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Niclosamide: A career builder

PART I: (Almost) Everything You Wanted To Know About Niclosamide But Were Too Afraid To Ask

PART II: Nanomedicines? A Carrier-Free Nanomedicine for Cancer and a Simple Buffered Solution to Prevent COVID19 and Other Respiratory Infections: They Just Need Testing

Invited Manuscript: J Controlled Release VSI in Honor of Prof. Park

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ABSTRACT

My contribution to honoring Professor Kinam Park celebrates and resonates with his scholarly career in drug delivery, his commitment to encouraging the next generation(s), and his efforts to keep us focused on clinically effective formulations. To do this I take as my example, *niclosamide*, a small molecule protonophore that, uniquely, can "target" all cell membranes, both plasma and organelle. As such, it acts upstream of many cell pathways and so has the potential to affect many of the essential events that a cell, and particularly a diseased cell or other entities like a virus, use to stay alive and prosper. Literature shows that it has so far been discovered to positively influence (at least): cancer, bacterial and viral infection, metabolic diseases such as Type II diabetes, NASH and NAFLD, artery constriction, endometriosis, neuropathic pain, rheumatoid arthritis, sclerodermatous graft-versus-host disease, systemic sclerosis, Parkinson's, and COPD. With such a fundamental action and broad-spectrum activity, I believe that *studying* niclosamide in all its manifestations, *discovering* if and to what extent it can contribute positively to disease control (and also where it can't), *formulating* it as effective therapeutics, and *testing* them in preclinical and clinical trials is a career builder for our next generation(s).

The article is divided into two parts: Part I introduces niclosamide and other proton shunts mainly in cancer and viral infections and reviews an exponentially growing literature with some concepts and physicochemical properties that lead to its proton shunt mechanism. Part II focuses on repurposing by reformulation of niclosamide. I give two examples of "*carrier-free formulations*", — one for cancer (as a prodrug therapeutic of niclosamide stearate for i.v. and other administration routes, exemplified by our recent work on Osteosarcoma in mice and canine patients), and the other as a niclosamide solution formulation (that could provide the basis for a preventative nasal spray and early treatment option for COVID19 and other respiratory virus infections).

My goal is to excite and enthuse, encourage, and motivate all involved in the drug development and testing process in academia, institutes, and industry, to learn more about this interesting molecule and others like it. To enable such endeavors, I give many *proposed ideas* throughout the document, that have been stimulated and inspired by gaps in the literature, urgent needs in disease, and new studies arising from our own work. The hope is that, by reading through this document and studying the suggested topics and references, the drug delivery and development community will continue our lineage and benefit from our legacy to achieve niclosamide's potential as an effective contributor to the treatment and control of many diseases and conditions.

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Contents

1. Introduction

Niclosamide and other proton shunts are a class of drugs called protonophores that can "target" all cell membranes, both plasma and organelles. Actually, they can't help it; rather than stay in the water, that's where they naturally go into all the cell membranes at a level of almost 10,000 times more than in the water. By entering the lipid bilayer membranes of mitochondria in particular, and also endosomes, lysozomes, and even Golgi, they act upstream of so many (maybe all) cell pathways. They therefore affect many of the essential events that a cell, and particularly a diseased cell or other entities like a virus, use to stay alive and prosper. As a person currently in the last throes of what they call "phased out retirement", (I'm almost as old as Kinam!), and, having stumbled on this molecule about 10 years ago, I am convinced that studying and understanding niclosamide in all its manifestations is a career builder for the next generation.

1.1. Overall goals

With this article I have two overall goals, and so divide it into two parts.

1.1.1. Goals in PART I

Part I is to simply introduce niclosamide and other proton shunts and review what is currently known with some concepts and physicochemical properties that lead to its proton shunt mechanism. I also include a review of its effects mainly in cancer and viral infections – a body of knowledge that is still in its early stages and is exponentially growing.

As a prototypical protonophore, niclosamide has the potential to affect many diseased states and conditions. Opportunities abound for confirmatory and new studies. In fact, even the mechanisms we think it affects are only just being unraveled with many twists and turns in the story; for example, see the papers that are now challenging Warburg [29]. Having a career-long interest in how to treat cancer and lately prevent COVID19, I focus mainly on niclosamide in anti-cancer and antiviral activity. As we know, most drugs target indiscriminately (chemotherapies) or discriminately (targeted therapies) the DNA, RNA, and proteins in a diseased cell; but they also spill over into healthy cells. In contrast, by entering the cell's membranes, protonophores like niclosamide influence pathways for survival, growth, and dissemination in cancer, or the host cell's own (healthy) pathways that a virus uses to promote its RNA entry, replication, and assembly or shuts down to evade being destroyed. While every cell might get niclosamide it's not as toxic to the healthy ones as it is to the cancer ones. As such, I believe that there is so much more to learn and evaluate about not only where and how niclosamide can function as a safe and effective therapeutic, but also where it can't.

1.1.2. Goals in PART II

In Part II, I want to give what could be a slightly new, even uniting, perspective on the "nanomedicine issue" in the context of repurposing by reformulation. Having written for Kinam's latest book, "*Biomaterials for Cancer Therapeutics. Evolution and Innovation*" [213] on "*Development of clinically effective formulations for anticancer applications: why it is so difficult*?" [180] my goal here is to stimulate more interest in the rationale, underlying science, and development of more clinically effective reformulations of existing drugs in general, and this class of compounds in particular, especially a consideration of "carrier-free" nanomedicines.

With the successes of sildenafil (Viagra) and metformin (to name but two) there has been a greater appreciation for "repurposing" of existing therapeutics for alternative disease indications. Invariably, this is simply off-label prescriptions of the existing dosage forms, usually as oral tablets. One of the main motivations is expeditious approval and saving costs. As Hernandez et al. say in their recent review [98], such repurposing, "*is an attractive approach that can save significant investments of time and money during drug development*".

I would agree, but there is also a lot to be gained from not just repurposing (as the existing tablets) but reformulating as more clinically effective formulations [180]. Recognizing that formulations really do determine the ultimate fate of a drug, their PK and biodistribution [142], and hence their arrival and efficacious concentration at the site (e.g., tumor interstitium), I also see a need for the correct "purposing" of existing drugs for existing applications that are not formulated optimally to have their potential efficacy. In fact, I would suggest that ALL DRUGS need a clinically effective formulation, if we have the will and the way to figure it out.

So, for me, it's not just repurposing pills for costs (and profits) but rethinking formulations of existing drugs to reach their full potential, for the patients. As I lay out here, I have focused mainly on cancer, and, in addition to a thermal sensitive formulation for doxorubicin, how to reformulate niclosamide as an acyl ester-prodrug therapeutic. There are many other approved anti-cancer drugs that can be so derivatized. Clearly, what we learn here could be applied to other diseases and conditions.

Thus, herein lies a very rich area of study, for new projects, grant proposals, and for subsequent papers on how to utilize this particular protonophore-class of drugs in a way that is somewhat different to the usual novel-drug discovery and testing pipeline. I see this as perhaps an underserved and underappreciated effort requiring a special team of researchers that engage in the repurposing-purposing-reformulation of

existing drugs, as well as new drugs coming down the pipeline with a more formulation-centric approach.

And, as I will expand on below, I suggest that we should not immediately go to "scaffold-nanomedicines" such as encapsulation in a liposome, micelle, or chitosan, or attached to a polymer or dendrimer, or dissolved in a lipid nanoparticle. Rather, I believe we should start with an exploration of effective drug delivery focusing first on the drug itself and pick the best drug for the job, not a prototypical drug that validates the scaffold-approach. That is, truly understand what the drug is and what it really needs by carrying out a careful preformulation drug characterization for each application, and then decide on an appropriate advanced formulation, that may indeed include a scaffold, but not necessarily. As we and others like Tonglei Li have done with their "carrier-free nanocrystals" [82], maybe we can utilize a pure prodrug core for i.v. administration in cancer [65,66,225] or a simple niclosamide solution as a nasal spray in respiratory viral infections [185,187].

In attempting to achieve these two goals, I hope to stimulate others to consider undertaking new research in the medicinal chemistry of niclosamide and similar proton shunts, for a range of targets on all aspects of cellular pathways, on preformulation drug characterizations that lead to the development of more clinically effective formulations of these molecules for cancer, viral infections and more, and to test them in preclinical and eventually clinical trials.

1.2. My audience

As Kinam knows so well, my audience for many of the papers that I write these days are for the people who actually do the work in the labs, —the grad students, post docs, and technicians. I therefore resonate with his plea [212] "*Instead of focusing on the senseless, pointless pursuit of new and innovative nanomedicine, i.e., more complex and fancier looking nanoparticles …. we could have trained young scientists to open their eyes to see, open their ears to listen, and open their heart to feel what is important*". They are the ones who carry out the experiments, the modeling, draft their theses and study reports, and generate most of the work that goes into the literature. However, in dedicating this "VSI in Honor of Prof. Park", I now want to focus on all researchers, new teams of researchers, including clinicians, and, with the new emphasis on Research and Knowledge Exchange, also the RKE officers, —everybody involved in the drug development and testing process in academia, institutes, and industry.

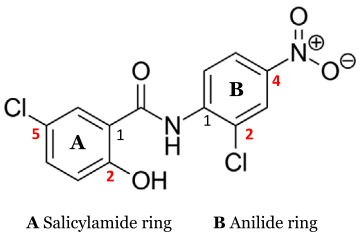
While I will include some reviews of the literature and some original scientific content, I see this as more of a document that recommends and directs your own reading, exploration, and grant proposals. I hope to excite and enthuse, encourage and motivate, all researchers to learn more about this interesting molecule and perhaps others like it, and their unique and ubiquitous membrane "target" that has influence on down-stream mechanisms. If done properly, niclosamide could be a major and effective contributor to the treatment or control of many diseases and conditions.

I know this is a long article, and it has an Appendix. I purposefully wrote it in the form of a story that one could read all the way through, like a novel, or, by consulting the table of contents, could dip in and out, like chips and salsa. Beyond the pedantic scientific details and potential research studies, this is a story. I have always felt that's what all research is, personal, (it's done by persons), a story of individual and collaborative endeavors, a timeline, a consequential arrow into the future. And it is not finished yet, it is waiting for you to write the next chapters. We recognize that you are our legacy, and can benefit from our lineage and so, in addition to honoring of Prof. Park, I also write it for you.

Organizational Note:

Peppered throughout the document are some consequential ideas of what I consider opportunities for collaboration or for anybody else to take on as their own. Each idea for a new study or proposal is highlighted in pink-orange as a "***Proposed Idea***" (numbered in sequence in each section). In light grey, I give a few tangential comments "***As an Aside***". There will also be a few footnotes for comment and information, and a few "***Bottom Line***s". I will also include quite a few "*quotes*" for titles and text from the author's themselves. Many times, the authors say things that are just worth reading rather than me doing a word rearrangement, and so I give them the credit.

There is also an *Appendix* of what I think could be additional and interesting information including: Structure Activity Relationships (SARs) for niclosamide and other proton shunts; drug development for new and reformulated drugs; some background on oxidative phosphorylation and beyond Warburg; a brief review of nanomedicine clinical trials and an interesting take on Kaplan Meier % survival plots, revealing the head-room for improvement in drug performance; and a brief comment on financial toxicity, where our new formulations and how we now bring them through could have a significant impact.



Properties: (Chem Axon, and (van Tonder, Maleka et al. 2004))

- Mwt = 327.12 g/mol
- Intrinsic solubility = 1.8 μM
- pKa (phenolic OH) = 6.89
- Polar Surface Area = 92.47 Å²
- Molar volume 202.5±3.0 cm³/mol
- Log D

pH	logD
1.50	3.81
5.00	3.80
6.50	3.66
7.40	3.19

Fig. 1. Niclosamide: 5-Chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide and comprises ***A)*** a Salicylamide ring and ***B)*** Anilide ring. Canonical SMILES: C1 = CC(=C(C=C1[N+](=O)[O-])Cl)NC(=O)C2 = C(C=CC(=C2)Cl)O.

PART I: (Almost) Everything You Wanted To Know About Niclosamide But Were Too Afraid To Ask

2. Introducing niclosamide

Niclosamide, 5-Chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, is a chlorinated salicylanilide. It was originally developed as an anti-helminthic [1] used for the treatment of gut parasitic infections [216], and was manufactured and marketed by Bayer [17] as Niclocide or Yomesan. Such infections infect very large numbers of humans and domestic and farm animals and cause a broad range of diseases [222].[1]

While many research articles start by saying, "*Niclosamide is an approved drug*", it is not, or at least not in the USA without going to the CDC to ask for it as a practicing MD [31]. It was voluntarily withdrawn from the market in the USA by Bayer in 1996 [68]. Since it is not currently "regulated" in the USA, any new reformulations have to start pretty much from scratch as a 505(b)(2) application. However, niclosamide is available in other world-wide jurisdictions as Yomesan, or other generics. (I got my Yomesan samples for my lab research from the internet, ordering from a Belgian Apotek, where it is legal, —4 tablets for €6). Why this is important is that being currently "not approved" has made our lives much more difficult in trying to develop, test, and move through (or even start) the approval process with the FDA for our simple niclosamide-solution-based preventative-nasal and early treatment-throat sprays [183,185,187,192] as well as new formulations for cancer [65,225], (as introduced and discuss later in Part II).

[1] For more information on this aspect, the World Health Organization (WHO) reports there are health issues not just with the worms but also their larvae and epilepsy, WHO [273]. Taeniasis/cysticercosis https://www.who.int/news-room/fact-sheets/detail/taeniasis-cysticercosis, World Health Organization.

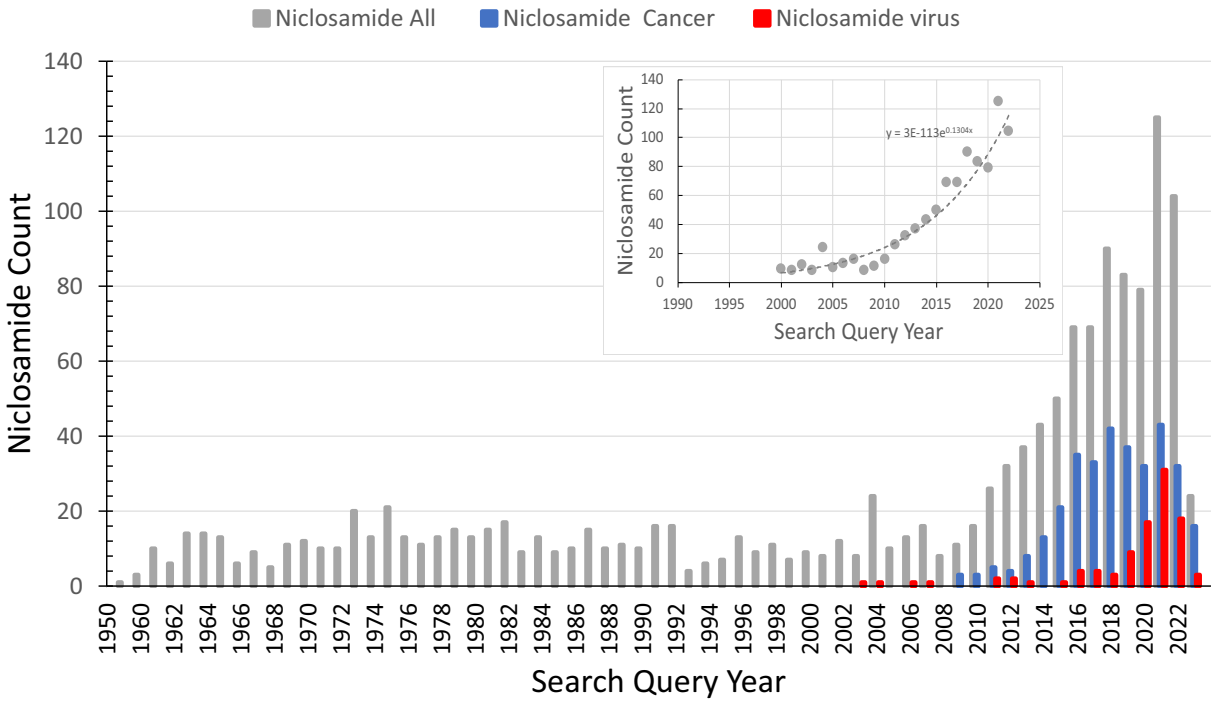


Fig. 2. Search Query: "*Niclosamide*" (***grey***) for all hits on PubMed from 1950 until the present (Feb 14th, 2023). Shown in ***blue*** are the papers for "*niclosamide*" AND "*cancer*", and in ***red***, the ones for "*niclosamide*" AND "*virus*". The inset shows the exponential rise in all publications since around 2000. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.1. Niclosamide the molecule

As shown in Fig. 1, niclosamide is a simple small molecule (327.1 g/mol) that comprises ***A)*** a Salicylamide ring and ***B)*** an Anilide ring (these distinctions will be important for later discussions of derivatives and Structure Activity Relationships (SARs).[2]

Its properties include a relatively low, 1–2 μM aqueous intrinsic solubility associated with its most stable monohydrate, —solubility (or, more accurately, amount dissolved in solution) depends on the niclosamide polymorph and the solution pH [185,261]. It also has a medium, positive LogD of ~4, typical of poorly absorbed BCS Class II drugs. Interestingly, it is a weak acid with a pKa calculated by Chem axon as 6.89 and quoted in many data bases as being between 5.6 and 7.2 [63,67,160,257]; I determined it to be 7.12 [185].

What is very curious though and turns out to be instrumental in its therapeutic activity, is that, at pHs above its pKa, its LogD hardly decreases; meaning, it stays relatively hydrophobic even though it is ostensibly negatively charged. Also important to its eventual fate and therapeutic target in the cell, niclosamide has a polar surface area of 92.47 $Å^2$ and a molar volume of 202.5 ± 3.0 cm^3/mol. These properties will be very important later as to how I (re)formulated niclosamide as a simple pH 8.3 buffered nasal spray solution [186,187] and its mechanism of action that stops viral infection in infected host epithelial cells and influences the workings of the mitochondrion and other organelles in cancer cells.

We will also see that its labile phenolic OH, at position 2 on the salicylamide ring, is important in our new prodrug therapeutic formulations [65,225]. Here, we esterified niclosamide with a long, C18-stearate, fatty acid-acyl chain for the expressed purpose of reducing its aqueous solubility and so controlling its nucleation, nano-precipitation, and ripening, thereby making highly insoluble prodrug nanoparticles that could be just the right size for i.v. infused anti-cancer applications [65,225]. For now, it is sufficient to note its chemical structure, comprising two characteristic aromatic rings, and the general nature of its properties.

Before we get into all of its potentially therapeutic effects, it is interesting to briefly review niclosamide's early days as an anti-helminthic and see what it has become by looking at its publication history.

2.2. Historical perspective: Niclosamide publication history 1950 - present

Shown in Fig. 2 is a search on PubMed for just the word, "*niclosamide*", carried out on February 14th, 2023 [223]. It yielded 1327 results. In the search, I grouped the papers into three main categories: parasitic (tapeworm), cancer, and virus.

2.2.1. Niclosamide(all) including for parasitic infections

The **grey bars** in Fig. 2 represent all the hits for "*niclosamide*". It was difficult to just separate out "*parasite*" because any search that includes "*parasite*", "*anti-helminthic*", or "*tapeworm*" generates all the papers that might have little to do with these conditions because everybody always says, "*The anti-helminthic drug niclosamide…*" etc. So, it was not really possible to obtain an isolated search just for its original application from this publication data base (without going through all 1327 results, which I did to some extent). Of these, a search for *niclosamide* and *bacteria* gave 138 hits with the majority coming after 2012.

Having said that, most of the grey bars in Fig. 2, especially the early papers, are on parasites. Niclosamide had humble beginnings stretching all the way back to 1950 in a paper by Holman et al., [101] entitled, "*Studies on experimental streptococcal mastitis. III. Observations on an alteration in mouse-lethal power of Streptococcus agalactiae, strain S 13 during growth in the udder of goats*". Its anti-helminthic days were launched in 1960 by a publication by Anna Teitze [255] entitled "*Short report on clinical experiences with Bayer 2353 in tapeworm infections in man*". This was rapidly followed by Knorr's "*Treatment of tapeworm with Yomesan in 36 patients*" [125] as Bayer introduced it for parasitic infections worldwide [17]. These and later papers through the 1970s and

[2] To reiterate, "SARs" here are "Structure Activity Relationships", —not to be confused with "Severe Acute Respiratory Syndrome".

early 2000s were focused mainly on its anti-helminthic applications as well as field-tests of its activity against other parasites and some evaluations of its environmental impacts.

There is one, 50-page, incredibly comprehensive study by Andrews et al. in 1982 [6] of Bayer's product, entitled, "*The Biology And Toxicology of Molluscicides, Bayluscide*". They give physicochemical properties, structure-activity relationships, analytical methods, and a host of data on efficacy and toxicity against all-and-sundry including: snails, worms, aquatic invertebrates (benthic and planktonic fauna), amphibia, aquatic and terrestrial plants, and in human and veterinary medicine. They include dosing, efficacy, and LD_{50} data on laboratory mice, rats, guinea-pigs, rabbits, cats, and dogs. This paper truly is "everything you wanted to know but were too afraid to ask" (up to 1982). Multiple papers eventually established niclosamide as an inhibitor of oxidative phosphorylation and therefore of cellular metabolic processes that, presumably, helped to stop the worm from holding on.

2.2.2. Niclosamide for cancer

As also seen in the graph (**blue bars**), interest started to pick up in niclosamide for cancer, with the first paper in 2009 by Wang et al. [265], in Taiwan, that showed niclosamide had some inhibitory effects on the Notch growth pathway, that could have implications in cancer and as a screening agent for Notch. They were motivated to do the study because, apparently, aberrant transduction of Notch signaling contributes to many diseases, especially cancers in humans, and the activated form of the Notch receptor was apparently extremely difficult to detect in normal cells. From their data they concluded that: "*modulation of Notch signaling after treatment with any of these three drugs (including niclosamide) may affect tumorigenesis of K562 cells*[3] *suggesting that these drugs may have therapeutic potential for those tumors associated with Notch signaling*".

Also in 2009, the Chen-Lyerly lab at Duke, who had been studying the Wnt pathway in colorectal cancer [41] published their first paper with niclosamide, "*The anti-helminthic niclosamide inhibits Wnt/Frizzled1 signaling*" [40]. Through 2010–2011, a series of papers then followed showing that niclosamide positively affected various cancers including, leukemia [111], as a STAT3 pathway inhibitor [227], and more from the Chen lab on colon cancer [42]. Here, a screen of over 1200 drug and drug-like compounds confirmed the identity of niclosamide as a Wnt pathway antagonist in colorectal cancer cells. Their studies later included APC (adenomatous polyposis coli) mutations [205] as more and more researchers and groups at Duke got in on the studies. How it all developed from there is obviously beyond what I could exhaustively review here (but see below ***4. Niclosamide in cancer***). The story though is readily seen by pulling up the search on PubMed [223] yourselves and scanning through the list in chronological order as more and more pathways are found to be affected. Papers on niclosamide and cancer peaked at around 40 per year through 2018–2021 including ours [225] on a new formulation of a niclosamide-stearate prodrug therapeutic (NSPT) entitled, *"Preclinical Testing of a Novel Niclosamide Stearate Prodrug Therapeutic (NSPT) shows efficacy against Osteosarcoma"* -preclinical studies of this NSPT in an Osteosarcoma mouse model of lung metastases, (as presented and discussed in more detail later, (***7. The Niclosamide Stearate Prodrug Therapeutic (NSPT) for cancer (Osteosarcoma)***).

2.2.3. Niclosamide as an anti-viral

Finally, (in the **red bars**) the other major area that caught researcher's attention was niclosamide's activity on bacteria and viruses, starting in 2003. This included niclosamide's activity on the original SARS-CoV virus in 2004 by Wu et al. [279] and a really interesting paper on its anti-viral (influenza) mechanisms by Jurgeit in 2012 [115]. As they report, *"In an image-based high-content infection screen of HeLa cells, we identified niclosamide from a library of 1200 known bioactive compounds as a potent, low micromolar inhibitor of HRV16 infection"*. These studies showed that niclosamide, at only micromolar concentration, when given as a prophylactic before vial genome release into the cell, inhibited infection of human rhinoviruses (HRV) and influenza virus in HeLa cells. Deviating from the usual, *"niclosamide is an uncoupler of oxidative phosphorylation"* they posited a new mechanism for niclosamide as a *"proton carrier that targets acidic endosomes"* with broad antiviral effects. Also, as discussed by Miyauchi [167], the fusion between viral and cellular membranes is the first critical step of the enveloped viral infection, and these pH-dependent viruses need a drop in pH for that RNA release from the endosome. (See also [195] in the special issue, *Virus Entry by Endocytosis*).

In the review by Xu [281], entitled *"Broad Spectrum Antiviral Agent Niclosamide and Its Therapeutic Potential"*, niclosamide, *"was found to be effective against various viral infections with nanomolar to micromolar potency such as SARS-CoV, MERS-CoV, ZIKV, HCV, and human adenovirus, indicating its potential as an antiviral agent."* The anti-viral activity of niclosamide though, really took off reaching 31 papers per year in 2021 when (as you might expect) researchers started screening thousands of compounds against SARS-CoV-2 and niclosamide was among them as it popped up, at or near the top of the list, in multiple cell screens.

In the early days of the pandemic (March 20th 2020) a paper by Jeon et al. [109] from the Institut Pasteur Korea, Seongnam, South Korea, came out. They screened a panel of 48 FDA-approved drugs in the very robust Vero6 cells against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This is the paper that my colleague, Christina Barkauskas in Pulmonary Medicine at Duke, saw and alerted me to it in March 2020 as a BioRxiv preprint [109] and that was later peer reviewed [110]. I immediately pivoted away from niclosamide for cancer and started my own explorations of niclosamide formulations for COVID. (see later for this story, ***8.2 Formulations: the early days (March 2020 - Oct 2020)***).

2.2.4. Niclosamide has broad spectrum activity

Niclosamide-the-molecule was actually brought to my attention earlier than these anti-virus studies back in ~2013 by the well-respected Duke Oncologist Kim Lyerly. As briefly mentioned above, in 2009 Lyerly along with Wei Chen and collaborators, were particularly interested in the Wnt pathway in cancer [40]. The Wnt pathway is a conserved pathway in metazoan animals [129], that regulates critical genes for normal embryo development. It is also important in adulthood for the maintenance of somatic stem and progenitor cells, and tissue regeneration following injury. Here, secreted glycoproteins bind to the seven-transmembrane-Frizzled receptors which leads to Frizzled receptor internalization and down-stream signaling that regulates the developmental and regenerative effects. However, while this is its normal biological function, the Wnt pathway was actually first discovered to be active and dysregulated in many cancers [203,288]. For example, in colorectal cancer (CRC), >80% of all sporadic and hereditary cancers show hyperactivation of the pathway due to mutations in that APC or the β-catenin gene. At the time Chen et al. were studying it [40], there were no drug candidates or even tool-compounds that modulated Wnt-mediated receptor trafficking, and subsequent Wnt signaling.

Then in 2015, in what was an incredibly comprehensive piece of medicinal chemistry, Mook et al. made a series of 35 different niclosamide derivatives (Niclosamide chemotypes) [169]. They were looking for structure–activity relationships and any derivatives with improved drug exposure for the Wnt/β-catenin pathway inhibition in both the anilide (25 derivatives) and salicylamide (10 derivatives) ring substitutions. Focusing on the most successful derivatives, they conducted a numerical assessment of the inhibition of Wnt/β-catenin transcription by their TopFlash assay. (See a review of their work in *Appendix A1. Lyerly*

[3] K562 is an ATCC cell line: lymphoblast cells isolated from the bone marrow of a 53-year-old chronic myelogenous leukemia patient, https://www.atcc.org/products/ccl-243.

Chen- Mook: Niclosamide inhibits the Wnt pathway). Especially for the medicinal chemists, it is a revealing study as to niclosamide's best structure for best action, and it has implications for future chemical modifications.

In 2018, Chen and Mook summarized all their remarkable findings, and had discovered more, when they wrote a comprehensive literature review on niclosamide, [44], "*Niclosamide: Beyond an anti-helminthic drug*". In it they reviewed what was known about this old drug that had been used for over 50 years since the 1960s [1] to treat gut parasites. In my 2013 meeting in his office, Dr. Lyerly had impressed upon me that Niclosamide was somehow something special, and the subsequent Chen and Mook review underlined why he was so enthusiastic. In it, they reported that niclosamide when tested, mainly in cells and a few preclinical animal cancer models, had efficacy against, (wait for it): cancer, bacterial and viral infection, metabolic diseases such as Type II diabetes, NASH and NAFLD, artery constriction, endometriosis, neuropathic pain, rheumatoid arthritis, sclerodermatous graft-versus-host disease, and systemic sclerosis. There have also been more recent reports of activity in Parkinson's [15] and COPD [25,165]. Specifics of activity in cancer will be saved until later (***4. Niclosamide in Cancer***), along with ***Proposed Ideas*** for potential future studies.

3. So what makes niclosamide so active and why is it so ubiquitous?

As we have seen above, niclosamide affects everything from worms to cancer, virus infection to Parkinson's. How can a single drug molecule that was just used for worms affect all these pathways? In order to start to answer this question (and pose more for your grant proposals) it is useful to put the "drugging" characteristics of niclosamide in perspective with all other drugs.

3.1. Almost all drug-targets are proteins, carbohydrates, DNA, and RNA

As I expect we all know, the process by which DNA is copied to RNA is called *transcription*, and that by which RNA is used to produce proteins is called *translation*. The central dogma of molecular biology, as posited by Crick himself, is that (biological) information flows between genes and proteins. Or, as Watson put it, DNA ➔ RNA ➔ proteins, [50].[4] Given that it is usually the DNA, RNA, or proteins that go wrong in disease, especially in cancer, this, in turn, means that pathways involving these molecules have been the main focus of pharmacology for generating therapeutic targets in the disease condition and the pharmaceuticals that are supposed to treat them. In general, as reviewed by Singh et al., [245], the Biochemical Classes of Drug Targets are spread over enzymes (47%), GPCRs (30%), Nuclear hormone receptors and transporters (8%), Ion channels (7%), Integrins (1%) and other receptors (4%), DNA (1%), and Miscellaneous (2%). As they say,

"*The list of potential drug targets encoded in a genome includes most natural choice of virulent genes and species-specific genes. Other options include targeting RNA, enzymes of the intermediary metabolism, systems for DNA replication, translation apparatus or repair and membrane proteins*".

However, plasma membranes and intracellular membranes as targets per se, are largely unrepresented, except for general anesthetics that dissolve in the lipid part. Again, see Singh [245] and references therein.

In cancer the main focus has been on polynucleic acids as the repository of genetic information, with DNA itself as a target for many chemotherapeutic drugs. These drugs have been shown to work in cancer cells through a variety of mechanisms including chemical modification and cross linking of DNA (e.g., cisplatin), cleavage of the DNA (e.g., bleomycin) or intercalation into DNA (doxorubicin) to name but three. And so, many current anti-cancer drugs either attempt to disrupt the synthesis and assembly of proteins or target host protein receptors and enzymes and mechanisms required by the cancer cell's replication cycle. Thus, as reviewed by Kumar et al., [135] classical chemotherapeutics directly target the DNA of the cell, while more contemporary anti-cancer drugs involve molecular-targeted therapy such as targeting the proteins possessing abnormal expression inside the cancer cells. In the case of *Lapatinib* (an interesting story, –see *The Discovery of Lapatinib* (GW572016) [234]), and other tyrosine kinase inhibitors, they block the phosphorylation of a pathway that leads to cancer cell proliferation [235]. However, because every cell has DNA, RNA and synthesizes and uses proteins, these strategies (when administered as oral pills) are often systemically toxic before they are efficacious and so are not optimally delivered to the tumor at all.

> ***As an Aside***: We did actually solve this problem of local anti-cancer chemo-drug delivery in Mark Dewhirst's Hyperthermia Program with a Low Temperature-Sensitive Liposome containing and rapidly releasing Doxorubicin (LTSL-Dox) at 41 °C–42 °C mild hyperthermic temperatures in tumors. As I reported in Kinam's [177,180] and Anya Hillary's [179] edited books, it is worth bringing to your attention how one might go about doing this (See ***Appendix A2 We actually did cure cancer with one of these highly toxic chemo drugs***, for a brief account of this including more background on the LTSL-Dox, drug release images by confocal video in a tumor showing that the Dox was rapidly released and filled the whole tumor, and a review of the clinical trials that attempted to test it).
> See also *Appendix A3 The Drug Development and Testing Process for New Drugs and A4 The Drug Development and Testing Process for Reappropriated Drugs,* where I introduce what I see as the drug development and testing process for new drugs, focused on Lapatinib, and then what I have experienced for *repurposing drugs* using Niclosamide as my prime example, and that can be adapted to all *repurposing by reformulation*. This fairly long discussion also has a Laboratory-to-Clinic Translational Research and Development scheme for what I call –Endogenous-Inspired Anti-Cancer Therapeutics. The drug development cycle starts with Failures (or suboptimal performance) in the Clinic; through Medicinal Chemistry Reappropriated or New Drugs; Chemical Analytics; Advanced Drug Delivery Formulation and Characterization; Cell Cytotoxicity, Pathways, and Efficacy; Preclinical testing; and on to the IND and Clinical Testing; returning to the clinic with hopefully a more clinically effective formulation.

3.2. Niclosamide is different

Niclosamide offers a very different mechanism. As introduced above (Fig. 1), and discussed next, niclosamide is a small hydrophobic molecule. As we will see, it is small enough, hydrophobic enough, and delocalizes its negative electron charge enough for it to enter the core of the very low dielectric constant (dc = 2) of cell membranes, as a lipophilic anion. Here, it acts as a proton shut, dissipating pH gradients across the plasma membrane, and a range of host cellular-organelle membranes, including mitochondria, endosomes, lysosomes, and even the Golgi. Before I discuss a few cellular examples of where niclosamide has its proton shunt activity, let's just look at the physico-chemical characteristics that favor this mechanism of action., i.e., what gives it its actual "drugging" characteristics?

[4] For a more nuanced discussion of these statements some history, and references therein, see Cob, Cobb, M. (2017). "60 years ago, Francis Crick changed the logic of biology." PLoS Biol **15**(9): e2003243.

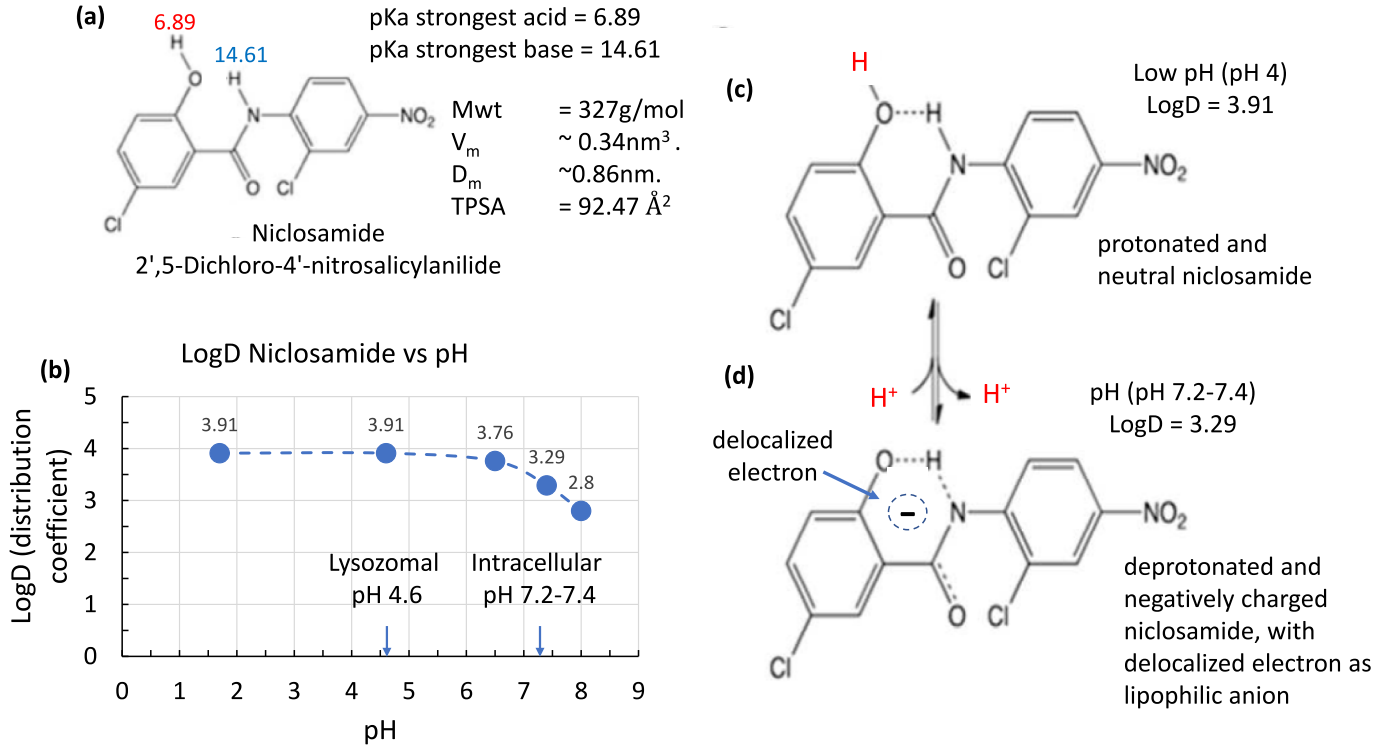


Fig. 3. Niclosamide and its pKa-enabling conversion from a neutral highly lipophilic molecule to a (still) highly lipophilic anion. ***(a)*** Niclosamide and physicochemical parameters; ***(b)*** Niclosamide LogD vs pH showing a LogD of 3.9 at the Lysozomal pH of 4.6 and a Log D of ~3.3 at the Intracellular pH of 7.2–7.4; ***(c)*** protonated-neutral niclosamide; ***(d)*** deprotonated and negatively charged niclosamide, with delocalized electron as a lipophilic anion (adapted from Terada [252] and Jurgeit [115].

3.3. The "drugging" characteristics of Niclosamide: Solubility, pKa, LogD and polar surface area

While, as above, niclosamide affects multiple cell pathways in multiple diseases and conditions, it appears that the main mechanism by which niclosamide can do this is to dissipate any proton gradient, across any lipid bilayer membrane, in any cell. Further discussion of where and how these might occur in the cell and their consequences will be presented later, see ***4. Niclosamide in cancer*** and ***5. Niclosamide in viral infection***. For now, especially for the medicinal chemistry, physical chemistry, and drug delivery readership, let's focus on the "drugging" characteristics of niclosamide that allow it to do all this.

So that we can talk with some knowledge (in our proposals) about the likely mechanisms of niclosamide in any future cell-organelle and -pathway studies on whatever normal and diseased cell states we are interested in, I put together a few adapted and original simple-minded illustrations. I want to provide an over-view and show how its intrinsic molecular properties (that we listed in Fig. 1) lead to niclosamide being able to target lipid bilayers, as opposed to binding to proteins and DNA/RNA directly, (which it may also do, see ***3.2 Proposed Idea***).

The principle molecular-level physico-chemical characteristics, (some calculated using Chemicalize [37]) that allow niclosamide to act as a proton shunt in lipid bilayer membranes is shown in Fig. 3.

These characteristics include the following. As above, it is a relatively small molecule with a molecular mass of only 327 g/mol, a molar volume (V_M) of 202.5 ± 3.0 cm^3/mol, and a molecular volume (V_m) ~ 0.34 nm^3. This represents a molecular diameter (D_m) of ~0.86 nm (Fig. 3a). It has a relatively low Topological Polar Surface Area (TPSA) of only 92.47 $Å^2$. Typically, molecules with a TPSA of <140 $Å^2$ [208] are >90% soluble in lipid membranes, and so niclosamide can readily partition into liquid-phase lipid bilayer membranes. The pKa of its strongest acid (the calculated value) is 6.89, so that, at physiological pH, it is ~50% negatively charged and ~ 50% is neutral [38].

It turns out though that, whether neutral or charged, it is still lipophilic. That is, its intrinsic logD (pH-dependent Octanol/water distribution coefficient) shown in Fig. 3 ***(b)*** is 3.91 [37]. More importantly, its LogD is still significantly hydrophobic and relatively unchanged in the physiologic range. Plotted as LogD vs pH in Fig. 3 ***(b)*** the neutral protonated form at pH 4.6 (lysozomal range in cancer cells [72] has a LogD of 3.91 and, at pH of 7.2–7.4 of the cancer cell cytoplasm, it is still 3.29.

With a pKa of ~7 then, this is right in the range of physiologic pH and, once partitioned into a lipid membrane, niclosamide allows proton-cycling between a neutral and an anionic form within and across the lipid bilayer membrane itself. Thus, when the protonated and neutral niclosamide (as depicted in Fig. 3 ***(c)***) converts to the deprotonated and negatively charged niclosamide, Fig. 3(d), it still remains as a lipophilic anion (adapted from Terada [252] and Jurgeit [115]). This is because, when it does deprotonate with increasing pH, the electron is delocalized across the molecule. It therefore seems to be the formation of this six-membered hydrophobic ring between the -NH in the aniline moiety and a phenolic -OH in the salicylic acid moiety by intramolecular hydrogen bonding that contributes to the high hydrophobicity and structural stability important for its intramembrane uncoupler activity.

3.1 Proposed Idea: (molecular simulations and lipid vesicle-liposome experiments) How is the electron shared within the molecule (electron density map?), and what exactly are the atomic and molecular level interactions between niclosamide and the lipid acyl chains? How do protons transition from their hydrated state in water across the lipid head groups and bind to niclosamide, to be transported down the concentration and dropped off on the other side? What is the permeation rate? Could this be simulated? Maybe start here, [231]. *This review paper explores the role that computational modeling, using the toolkit of molecular dynamics (MD) simulation, can play regarding drug*

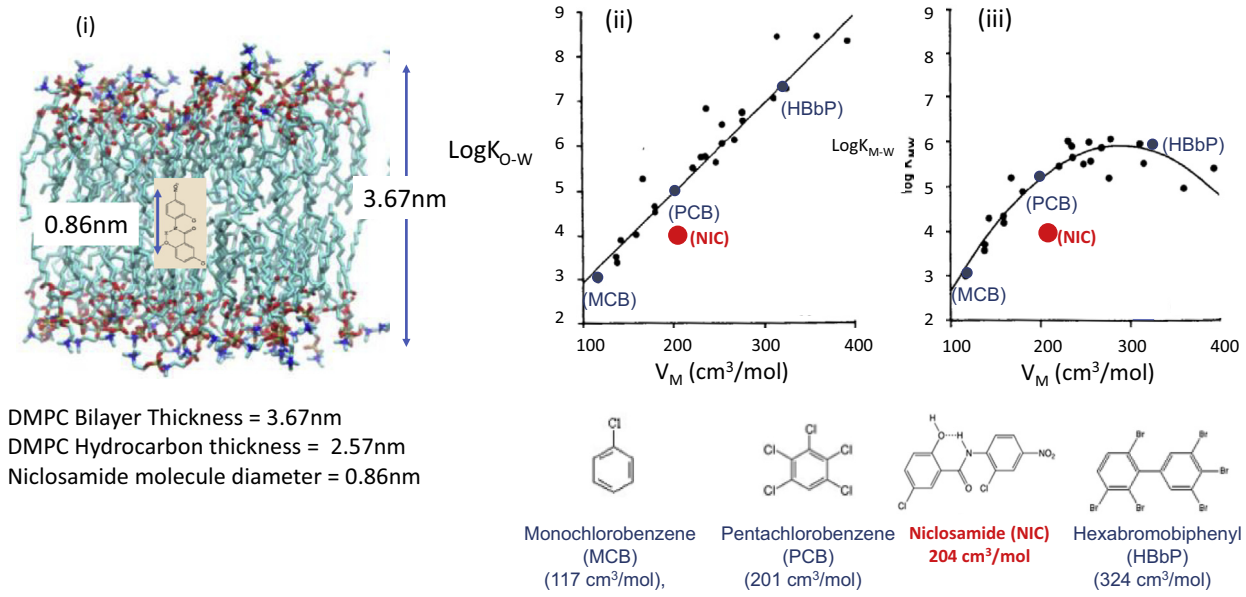


Fig. 4. Niclosamide partitioning into bilayers. (left) ***(i)*** Lipid bilayer system snapshot at the end of a 50 ns molecular dynamics simulation for DMPC at 30 °C, [295] with niclosamide drawn to scale. (right) Comparison between ***(ii)*** Octanol-water (LogK$_{O-W}$) and ***(iii)*** DMPC-membrane-water (LogK$_{M-W}$) partition coefficients as a function of the molar volume of the solute, (V$_M$ (cm^3/mol)) (Gobas, Jean M. Lahiitete et al. 1988).

design outside of this 'lock and key' paradigm, focusing on one fundamentally important aspect: the lipid membrane".
And also, here [262]: *"Experimental results on the crystal structure of the β-conformation showed that niclosamide exists in its planar conformation stabilized by the intramolecular hydrogen bond between the NH(11)–OH(20), which is in good agreement with both MD and DFT results".*
Could this be measured in reconstituted lipid vesicle-liposome experiments with niclosamide in their membranes and a pH gradient across the membrane and fluorescent pH sensitive dyes to monitor the kinetics and equilibrium as a function of lipid composition (e.g., SOPC with and without cholesterol), pH gradient, and niclosamide concentration? (the answer is probably "Yes").
Knowing this would help us understand how fast the pH gradients of internal organelles are dissipated and so how fast niclosamide might take to have its manifest physicochemical effects that then trigger the biochemical and cell-biological effects.

Thus, combining small size, low polar surface area, LogD, and the delocalized charge in the lipophilic anion means that weak acid molecules like niclosamide can readily partition into lipid bilayer membranes and shunt protons across them dissipating any gradients that happened to have been set up by experiment or already exist in nature (as in cancer, where, extracellular acidosis in solid tumors gives pH$_e$ values as low as 6.5 [52]).

As shown in Fig. 4, considering relative size, its diameter of ~0.86 nm compares well with the bilayer dimensions. Fig. 4 ***(i)*** is that of a dimyristoylphosphatidylcholine (DMPC) bilayer in its melted liquid phase at 30 °C [295], giving a total bilayer thickness = 3.67 nm, and hydrocarbon region thickness = 2.57 nm and so the small hydrophobic niclosamide readily fits inside the hydrocarbon region of the bilayer.

Considering a more biologically relevant lipid composition, as we showed back in 1990 in studies on the micro-mechano-chemical properties of giant vesicle bilayer membranes with and without cholesterol [193], the average biological membrane (diC12 to diC26 lipids with 0–6 double bonds per chain) is actually equivalent to that of Stearoyl Oleyl Phosphatidylcholine (SOPC) 18:0/18:1 C18 (di C 18 with one doble bond in one of the chains).

These stearoyl-chain lipids are clearly longer than the diC14 DMPC, and so have a thicker hydrocarbon region and overall bilayer thickness of 4.08 nm at 20 °C [134].

Also, while LogP is an indicator of hydrophobic-hydrophilic partitioning, for the biological milieu and especially lipid bilayer membranes, it is more appropriate to use the membrane- or bilayer-water partition coefficient, LogB, (or LogK$_{M-W}$).

In 1988, Gobas et al. [88] measured LogB for a series of 27 selected halogenated aromatic-hydrocarbons, from the simplest Monochlorobenzene (117 cm^3/mol), to the penta-substituted Pentachlorobenzene (201 cm^3/mol) to 2,2′,4,4′,6,6′-hexabromobiphenyl (324 cm^3/mol), structures. Thus, as shown in Fig. 4 ***(ii)***, while the octanol-water partition coefficient (LogK$_{O-W}$) increases linearly with molar volume (V$_M$), in contrast, in Fig. 4 ***(iii)***, the membrane-water partition coefficients, LogK$_{M-W}$, measured for DMPC bilayers, follow a more parabolic relationship with respect to the molar volume, V$_M$.

Their data show that the maximum ability to partition into bilayers, LogK$_{M-W}$, occurs for solutes with molar volumes of ~300 cm^3/mol, and then the ability goes down because of their increasing size, that limits their accommodation in the bilayer structure. Note that, as shown in Fig. 4***(ii)***, as the size and hydrophobicity of the molecular series increases so does the molecule's LogP, indicating that octanol does not present such a structured environment. The lesson here is that drug partitioning into cell membranes, and even drug absorption through gut epithelium, is not all about LogP, —molecular-size and polar surface area are also important [157].

Here then, a molar volume V$_M$ for niclosamide of 202.5 cm^3/mol, as shown in Fig. 4 ***(ii)*** and ***(iii)*** , means that, while the LogD value of 3.91 (red symbol) brings niclosamide a little lower on the LogK$_{M-W}$ axis than the series of halogenated aromatic-hydrocarbons, niclosamide is nevertheless on the rising slope of (LogK$_{M-W}$) vs molar volume (V$_M$) showing it is capable of fitting into the bilayer membrane. Thus, as above, when drawn to scale, in Fig. 4 ***(i)*** we see that, compared to the DMPC total bilayer thickness of 3.67 nm, and the DMPC hydrocarbon thickness = 2.57 nm, and a molecule diameter = 0.86 nm, niclosamide can easily fit into the bilayer (probably in parallel with the hydrocarbon chains) as schematically overlaid on this molecular dynamics simulation image of DMPC at 30 °C [295].

Next, consider the whole pH range, especially the physiologic range from cell lysozomes (pH 4 to 4.5) to the mitochondrial matrix

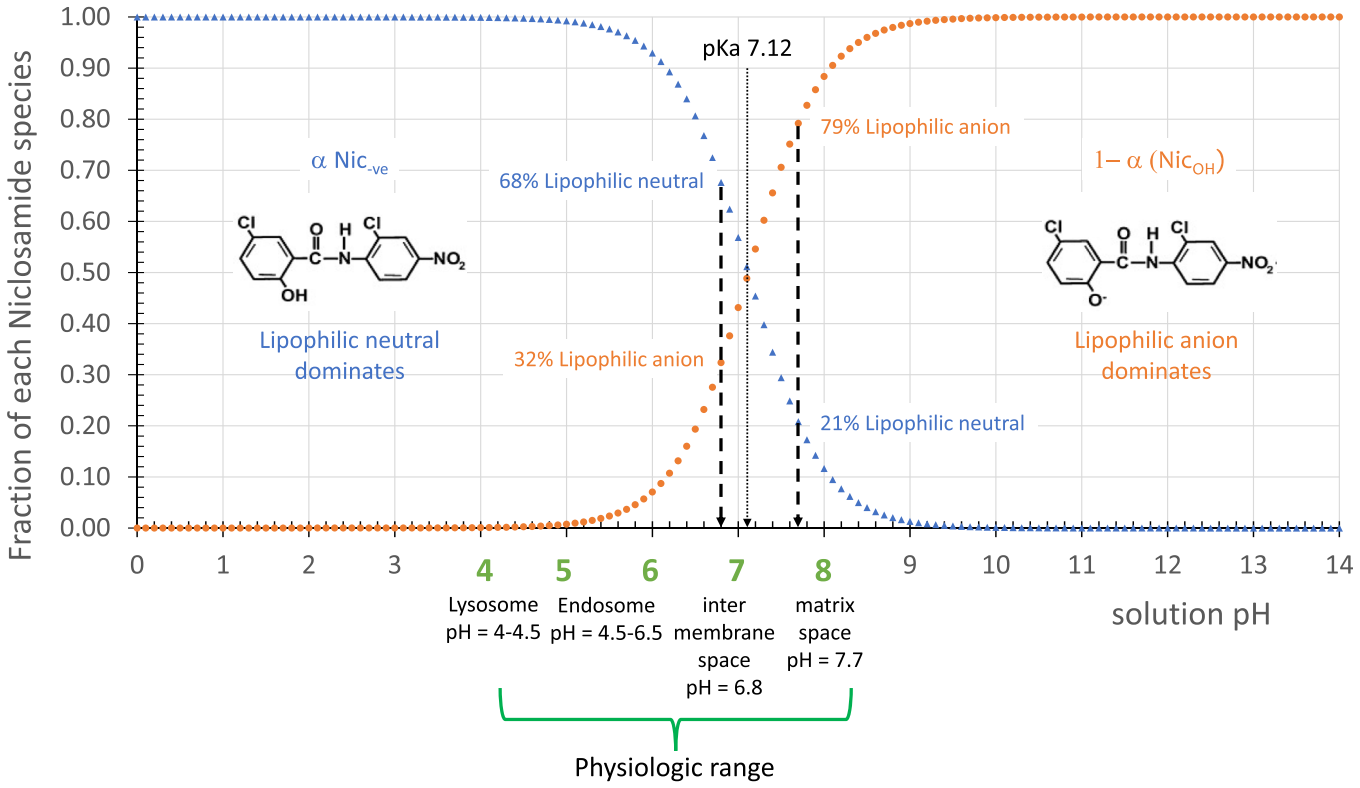


Fig. 5. Niclosamide pKa (Henderson–Hasselbalch), showing the fraction (and indicated %) of the Lipophilic neutral species (blue line), the Lipophilic anion species (orange line). The physiological range is also shown in green: lysosomes (pH 4–4.5), endosome (4.5–6.5), mitochondrial inner-membrane space (6.8), mitochondrial matrix (pH 7.7). Over this pH range, niclosamide's LogD only changes from ~3.91 to ~3.29 (Fig. 4b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

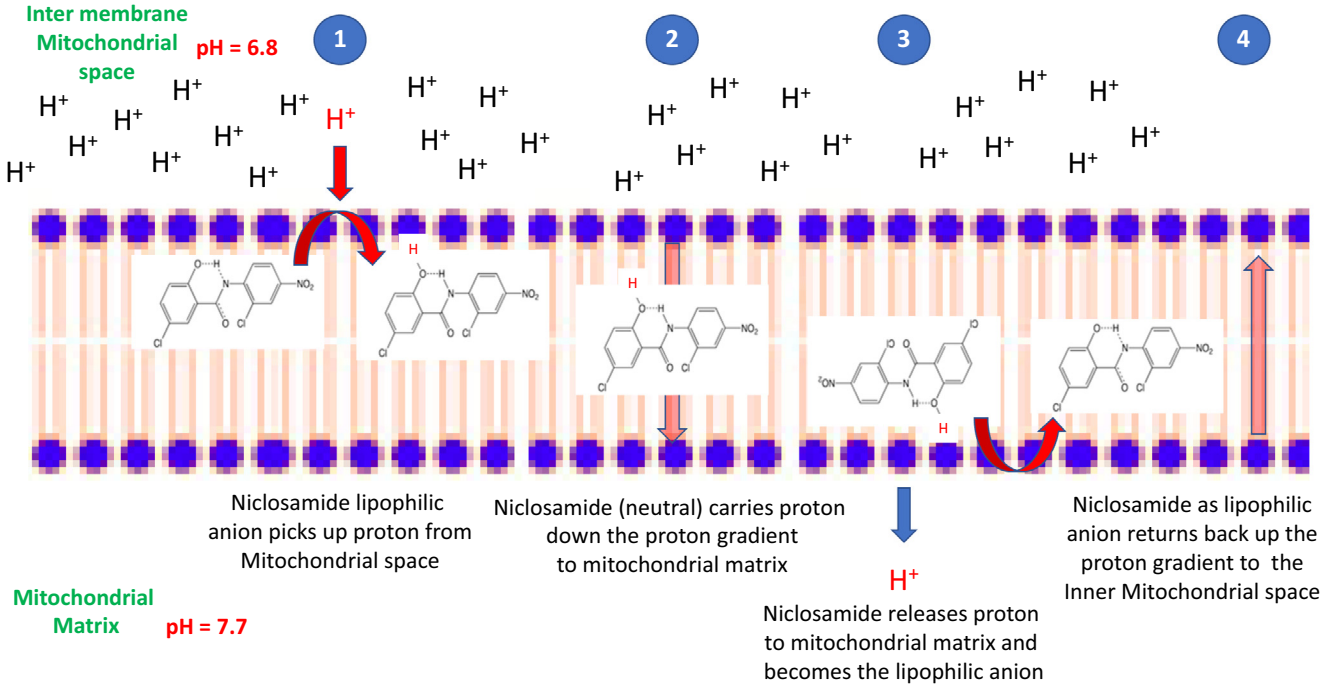


Fig. 6. Niclosamide acts as a proton shunt across the Inner Mitochondrial membrane. Shown schematically are the Inter membrane mitochondrial space (with its high H+ concentration) at pH 6.8; the Inner Mitochondrial membrane; and the Mitochondrial Matrix at pH 7.7. Red arrows depict the binding of H^+ ions at the inter-membrane space, their transport across the inner mitochondrial membrane, and their transfer to the mitochondrial matrix. The process is depicted as a series of events. ***1.*** Niclosamide lipophilic -anion picks up proton from Mitochondrial space; ***2.*** Niclosamide (neutral) carries proton down the proton gradient to mitochondrial matrix; ***3.*** Niclosamide releases proton to mitochondrial matrix and becomes the lipophilic anion; ***4.*** Niclosamide as lipophilic anion returns back up the proton gradient to the Inner Mitochondrial space. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(~pH 7.7). As shown in Fig. 5, using the value for the pKa of 7.12, that was inferred from the pH-dependence of niclosamide solubility vs pH measured earlier [185], we can plot the two-species distribution for niclosamide vs pH in the form of the familiar Henderson–Hasselbalch equation [217]. The important thing to note here is that, in the mitochondrial inner membrane space, where the pH = 6.8 and the niclosamide LogD is ~3.91, the amount of the proton-receiving lipophilic anion is ~68%, and the proton-carrying lipophilic neutral molecule is ~32%. However, in the mitochondrial matrix space, where the pH = 7.7, (i.e., a ΔpH of about 0.9) and the niclosamide LogD is ~3.29, the ratios are greater and more in favor of the proton carrier species, as ~83% lipophilic anion and ~17% lipophilic neutral. Unlike most weak acids, where the charged species has a much lower LogD, to reiterate, the delocalization of the negative charge of the anionic form maintains its lipophilicity and means it can readily partition into cell and organelle membranes. It is actually 1000 to 10,000 times more soluble in octanol, (i.e., lipids), than in water).

3.4. Niclosamide as a proton shunt across the inner mitochondrial membrane

As alluded to before, and discussed in more detail here, niclosamide's ability to collapse the outer mitochondrial membrane potential is one good example of how this proton shunt activity can affect a cell and then everything downstream from there. Basically, niclosamide dissipates the proton gradient and so reduces or inhibits ATP synthesis and available ATP for the cell**.** At the simple lipid bilayer membrane level, Fig. 6 schematically gives the steps in this H^+ transport.

Taking the steps in Fig. 6 in order, this is how I imagine niclosamide functions. A more detailed modeling may give a more sophisticated account (see ***3.1 Proposed Idea***), or may show something different:

1. The niclosamide lipophilic anion, that is partitioned into the inner mitochondrial membrane, picks up a proton from the relatively low proton-rich pH (pH 6.8) of the inter-membrane mitochondrial space.
2. The lipophilic, now neutralized, niclosamide carries the proton down the proton concentration gradient across the inner mitochondrial membrane to the other side, which is the mitochondrial matrix where the pH is relatively high (pH 7.7).
3. The neutral niclosamide releases the proton to mitochondrial matrix and becomes the lipophilic anion again.
4. Niclosamide as the lipophilic anion returns back up the proton gradient to the Inter Mitochondrial space, ready to transport another proton.

All these movements of niclosamide result in the dissipation of the pH gradient.

Further details of Oxidative Phosphorylation are given in *Appendix A5. So, what is Oxidative Phosphorylation again and how does niclosamide influence it?* including a fascinating review by Santo-Domingo and Demaurex [236], called "*The renaissance of mitochondrial pH*"and data by Alasadi et al., at Rutgers, in 2018 [4] who used the Seahorse oxygen consumption rate (OCR) assay, and confirmed that (in this case) Niclosamide ethanolamine (a slightly more soluble salt of niclosamide) uncoupled mitochondria at 2.0 μM.

As reviewed next under sub heading ***3.5***, Terada [252] concluded that these uncouplers show highly efficient membrane targeting action and become a "non-site-specific" type of bioactive compound. In studies by Park et al. [214], where they screened a chemical library in HeLa cells (derived from cervical cancer) they identified niclosamide as a potent inducer of mitochondrial fission, i.e., niclosamide treatment resulted in the disruption of the mitochondrial membrane potential, promoted mitochondrial fragmentation, and reduced cellular ATP levels. And so there seems to be a different way mitochondria functions in healthy and cancer cells, i.e., niclosamide can have a lesser or greater effect depending on the nature of the cell and its mitochondrial organelle.

Briefly, other data in the literature had this to offer:

- ATP produced by oxidative phosphorylation may still be necessary to initiate glycolysis through hexokinase II activation [233].
- Uncoupling oxidative phosphorylation has been associated with shifts in oxidation of pyruvate to the oxidation of glutamine and fatty acids [80].
- Other mitochondrial un-couplers, that also act by carrying protons across the mitochondrial membrane, can all induce cell cycle arrest and apoptosis. These molecules include *dinitrophenol* [91], *nemrosone* [211], and *carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP)* [92].
- Some uncouplers (*gallic acid derivatives* [108] with selective antitumoral activities) act not by proton shunting, but include targeting: *(a) decreased mitochondrial membrane potential (ΔΨm); (b) induce permeability transition pore opening; (c) promote the release of cytochrome c, apoptosis-inducing factor (AIF), some procaspases that will be further activated, and endonuclease G (Endo G); (d) upregulate the expression of Bcl-2-associated X protein (BAX) and caspase-4, caspase-9, and caspase-3 proteins, subsequently causing DNA damage and fragmentation.* Thus, other parts of the electron transport chain can also be targeted that consequently reduces ATP synthesis.

Having some idea now of its mechanism of action, it could still be interesting and informative to briefly review some aspects of the molecular structure-activity relationships (SARs) of this proton-shunt activity.

3.5. Brief historical perspective on structure-activity relationships (SARs)

While we are mostly concerned with niclosamide, it is also informative, to any budding medicinal chemists, to include literature on other salicylanilides and start to get a sense of what is involved chemically in terms of Structure-Activity Relationships (SARs) with niclosamide and also other proton shunts. These molecules are more or less effective than niclosamide and so it's good to see what chemistry is involved.

The detailed exploration of structure-activity relationships for Niclosamide's proton shunt activity started in 1967 at the Department of Entymology, University of California, Riverside by Williams and Metcalf [276]. It was already known that salicylanilides possessed fungicidal, bacteriostatic, cestocidal, and molluscicidal properties, and niclosamide had become the poster-child agent for schistosomiasis control.[5] Williams and Metcalf [276] reported a new series of 11 salicylanilide derivatives. While others had shown IC_{50}s to uncouple oxidative phosphorylation (OXPHOS) in rat-liver mitochondria of 0.1 mM for salicylanilide (the bare double ring structure with no electron-drawing groups) and 0.02 mM (20 μM) for N-salicyloyl anthranilate, Williams and Metcalf, for their derivatives, showed IC_{50}s for the inhibition of mitochondrial oxidative phosphorylation in housefly and rat-liver mitochondria at much lower concentrations of only 5 nM to 363 nM.

At that time, in 1967, these 11 salicylanilides appeared to be the most effective uncouplers of OXPHOS then recorded. They compared their salicylanilides with 2,4-dinitrophenol, a recognized anti-metabolic drug and one of the first anti-obesity therapies used in the 1930s. The effect of Williams and Metcalf 's salicylanilides on mitochondrial adenosine triphosphatase (ATPase[6]) was shown to be approximately 1000 to 10,000 times more effective than dinitrophenol.

[5] parasitic worms, https://www.cdc.gov/parasites/schistosomiasis/index.html.

[6] "**ATPases** are a group of enzymes that catalyze the hydrolysis of a phosphate bond in adenosine triphosphate (ATP) to form adenosine diphosphate (ADP). They harness the energy released from the breakdown of the phosphate bond and utilize it to perform other cellular reactions". https://www.tocris.com/pharmacology/atpases This was confusing to me because mitochondria are all about making ATP, then, I found the answer to the conundrum, they can also work in reverse! (good old Wikipedia). "ATPase (also called F_0F_1-ATP Synthase) is a charge-transferring complex that catalyzes ATP to perform ATP synthesis by moving ions through the membrane, — it's all in a name! and they are everywhere, transporting all sorts of things https://en.wikipedia.org/wiki/ATPase.

They defined the structure-activity relationships that achieved the best inhibition as:

- *presence of strong electron-withdrawing centers, (NO, CN, or CF_3), located within a certain spatial distance from a halogenated aryl ring; and*
- *the attachment of an additional bulky group such as naphthyl, biphenylyl or t-butyl*

Not suspecting the target could be a membrane, they suggested that "*inhibition results from preferential adsorption at an active site on the enzyme surface*". (See protein binding project below ***Proposed Idea 3.2***).

The mechanism of action for these uncoupling agents was somewhat resolved by Weinbach et al. in 1969 [269]. What was being realized at the time was that these reagents had little or no effect on the rate of mitochondrial respiration (citric acid cycle that makes NADH and $FADH_2$ –part of the electron transfer chain, see again, *Appendix A5. So, what is Oxidative Phosphorylation again and how does niclosamide influence it?*) but inhibited, or "uncoupled", the associated phosphorylation of ADP. Again, the chemical requirements seemed to be,

"*The possession of an ionizable group of low pK value (usually between 4 and 6) and lipid solubility*".

However, because the uncoupling agents all showed a propensity to bind to proteins in general, Weinbach et al. again proposed that,

"*the interaction of uncoupling reagents with mitochondrial proteins induces conformational changes that are the basis of the uncoupling phenomenon*".

Hiroshi Terada in 1990 [252] also confirmed that the effect of the uncoupling was to.

"*inhibit ATP synthesis without affecting the respiratory chain and ATP synthase (H+-ATPase)*".

And he added more physical requirements to the compounds:

- *an acid dissociable group,*
- *bulky hydrophobic moiety and*
- *strong electron-withdrawing group.*

He showed that, the hydrophobic salicylanilide "S-13" – (5-Chloro-3-tert-butyl-2′-chloro-4′-nitrosalicylanilide) a molecule very similar to niclosamide but with a tertiary butyl at the 3-position of the salicylamide ring, (making it more hydrophobic and a lower calculated solubility of 0.44 μM with a higher LogD at pH 7.4 of 5.2), was active *in vitro* at concentrations as low as 30 nM. It was Terada who proposed a different mechanism and suggested that these weakly acidic uncouplers produced uncoupling *by their protonophoric action* in the H^+-impermeable mitochondrial inner membrane (as illustrated schematically in Fig. 6).

"*Ah ha! so its direct target is not necessarily a protein at all, but the lipid bilayer of the inner mitochondrial membrane*", was, maybe, the collective cry.

Fonseca et al., [76] also carried out a structure-activity analysis of niclosamide in experiments in which the measured cytoplasmic pH was lowered from 7.04 to 6.61 in MCF7 cells. They showed that in these cancer cells, niclosamide at 10 μM dissipated protons (down their concentration gradient) from lysosomes to the cytosol, effectively lowering cytoplasmic pH and interfering with the pH-dependent Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling. This will be discussed further later in ***4. Niclosamide in Cancer***.

Two other more recent and very interesting papers reviewed structure-activity relationship, Mook and Chen at Duke [169] and Childress et al., [46] at Virginia Tech. Mook and Chen focused exclusively on niclosamide and published "*Structure–activity studies of Wnt/β-catenin inhibition in the Niclosamide chemotype: Identification of derivatives with improved drug exposure*" that has considerable structure-activity data for just niclosamide, in an *in vitro* assay and *in vivo* PK. This is definitely worth reading; its where Bob Mook made 35 different derivatives looking for enhanced activity in the Wnt pathway. This is the part of the story that got me started on niclosamide for cancer, and so I include a brief review of this work in the *Appendix A1. Lyerly-Chen-Mook: Niclosamide inhibits the Wnt pathway*, so that you can see some SARs specifically for niclosamide that may inform your own concepts of further drug development.

For other, more recent, protonophores Childress et al. [46] provide another comprehensive list of uncouplers in their mini-perspective, "*Small Molecule Mitochondrial Uncouplers and Their Therapeutic Potential*". It is also an excellent review (and references therein) of much of what we have been talking about regarding uncouplers of OXPHOS. It includes details of some molecular properties like pKa, and calculated LogP, minimum effective concentrations at inhibiting OXPHOS, toxicity, and comments about each drug, uses, successes and failures, and a section on synergistic drug delivery to increase uncoupling potential.

They list 21 prototypical uncouplers grouped into categories of: prototype uncouplers; repurposed FDA-approved drugs (including niclosamide as niclosamide ethanolamine); repurposed chemicals that were found to have uncoupling actions mostly by chance; chemical library screens for drug discovery that has led to the identification of numerous mitochondrial uncouplers, including small-molecule and natural-product library screens; and an interesting section on synergistic drug delivery to increase uncoupling potential. Also, they mention that, as an adjunct to chemotherapies, niclosamide has antineoplastic effects through direct STAT3 inhibition [141], where this inhibition of cell proliferation enhances the responsiveness of esophageal cancer cells to chemotherapeutic agents. (see some highlights of this paper in *Appendix A6. Medicinal Chemistry of Uncouplers*) ***[46]***).

Bottom Line: To reiterate, the reason for niclosamide's broad range of influence is its action at one of the highest levels in the cell; as an uncoupler of oxidative phosphorylation in mitochondria.

Thus, the inner mitochondrial membrane sets up a pH gradient across it and the H^+ ions pass back down their gradient a drive the ATP Synthase. Mitochondrial uncoupling is a process that uncouples nutrient oxidation in the mitochondrion, generated from the citric acid cycle, from ATP production. By virtue of the protonophore being in the membrane, Niclosamide and other proton shunts facilitate proton influx across the mitochondrial inner membrane without passing through ATP synthase and generating ATP, stimulating a futile cycle of acetyl-CoA oxidation without generating ATP. It is within this context of a mitochondrial membrane, that puts a lot of effort into creating and maintaining a proton gradient for the expressed purpose of synthesizing ATP, that niclosamide has at least one of its main, (upstream) actions for all downstream intracellular processes and pathways that use ATP. Niclosamide can also affect the pH balance in cancer cells.

Hence, a reduction or absence of ATP grinds many of the downstream processes to a halt. This also extends to its influence on viral infection at the single cell host level, as introduced and reviewed later, see ***5. Niclosamide in viral infection***.

The above literature generated two Proposed Ideas.

3.2 Proposed Idea (Pharmacology and cell and molecular biology): So, we know that niclosamide is a proton shunt in cell membranes, but, like many drugs, niclosamide also binds to albumin [154,285]. Q: Does niclosamide bind strongly enough to any other proteins to affect any intracellular pathways? (For a list of signaling pathways, we could start here, [51] "Pathways of Intracellular Signal Transduction" in "The Cell: A Molecular Approach").

3.3 Proposed Idea: (Cell Biology): Test other Mitochondrial uncouplers listed above along with niclosamide on any cell line both healthy and diseased and compare your favorite pathway metric/outcome.

Having reviewed the physicochemical features of niclosamide and other protonophores, some history and mechanisms, I want to focus now on two specific bodies of literature, namely, cancer and viruses. These are the two areas for which I have tried to generate formulations. They will constitute the latter PART II of this article on "nanomedicines" and the utility of pre-formulation drug characterization in the design and development of "carrier free" formulations, i.e., essentially pure drug or prodrugs as nanoparticles or simple buffered solutions.

4. Niclosamide in Cancer

First, what is the accumulated evidence for niclosamide's action in cancer? In fact, what is the current thinking about potential mechanisms, that seem to be largely associated with the altered metabolism and other downstream effects in cancer cells in general that niclosamide might affect? Again, this is a complicated story that I can only briefly review here, but it is one that is ripe for further investigation, of not just the effects of niclosamide on cells but also the pathways for cancer in the cancer cells themselves. It's a toss-up which to do first, cancer metabolism to lay foundation or niclosamide in cancer to get to the point. I decided to start with cancer metabolism and a perspective discussion of what seems to be the underlying target, the mitochondria, and how new evidence is moving us beyond Warburg. This is followed by a brief review of the evidence for niclosamide having an effect in cancer. As new students of niclosamide, combining the two opens up a wealth of opportunities for drugs like niclosamide that can target this organelle, especially in cancer, and including enhancing efficacy for other drugs and even radiation as we will see later.

4.1. Cancer metabolism: Beyond Warburg

Given what seems to be a very robust effect on mitochondria and that there does seem to be a difference between normal cells and cancer cells, it might be a good idea for us to start our exploration with some basics of cancer cell metabolism and see where niclosamide might have an effect.

As I mentioned earlier, recent papers are now challenging Warburg [29] (See *Appendix, A7. Warburg and Beyond: (Cassim* et al*)* for a more detailed review). According to Warburg, "aerobic glycolysis" (glycolysis that occurs in the presence of oxygen) is a series of reactions that extract energy from glucose (C_6-H_{12}-O_6) by (eventually) splitting it into two pyruvates 2(CH_3-CO-COO-). This makes 4 molecules of ATP per glucose, but it costs two ATPs to get them. And yes this is all done in the presence of oxygen and mitochondria that could do it much more efficiently making 32 ATPs per glucose. It turns out though, that tumors can be deficient in oxygen (they have to make their own feeding blood vessels and don't do a perfect job) and so this preserved and ancient glycolysis pathway is one solution.

As an Aside: This could be one great topic for us to propose for a "Reverse Engineering" exercise where we view cancer not from the host's, but from the cancer cell's point of view. We would start by asking "How did tumors solve the survive in low oxygen problem?" and "What is the low oxygen problem?" –a limited transport of oxygen by diffusion throughout the tumor tissue from the mal-formed feeding blood vessels and uptake by intervening cells. From what I can gather, because of their rush to grow and attract new blood vessels for food supply, they don't do a very good job of building that supply chain. The only process available for bringing in the oxygen is diffusion from those sporadic and far away blood vessels, and transport is physically limited by $x^2 = 2Dt$, plus, again, the intervening consumption. Thus, any cancer cell more than about 100 μm way from a blood-vessel is pretty much out of luck when it comes to getting oxygen; they are at the back of the queue, and this leads to some pretty radical strategies for gaining energy and food to build whole new cells with. To get started on the methodology of reverse engineering, a short section is included in the ***Appendix, A8 How to Reverse Engineer Anything***. And see my chapter "*Reverse engineering of the low temperature-sensitive liposome (LTSL) for treating cancer*" [178] and let me know if anyone is interested in going through this reverse engineering process for invention and innovation, in this case, "from the cancer cell's point of view".

But back to Warburg. The best review I found and can recommend you all read was "*Fundamentals of cancer metabolism*" (and all 200 references therein) by DeBerardinis and Chandel [54]. They give a very detailed and quite readable review of some of the recent papers supporting several fundamental principles in cancer metabolism. Here, I will obviously only be able to briefly highlight some important aspects that could be really interesting for further exploration in the context of niclosamide. Interestingly they also take on Warburg. While glucose was his main focus, it has now been recognized that it is all about supplying carbon (and H, N, O, P, etc.) to the cell via "*a finite set of pathways to support core functions like anabolism, catabolism, and redox balance*". This is done via glycolysis, fatty acid synthesis, glutaminolysis, serine metabolism, and mitochondrial metabolism. (Here, you really need to see their Fig. 1. "Signaling pathways that regulate cancer metabolism"). What caught my attention at a general level was: reprogrammed activities; stage of tumor; and bioenergetics, including some Proposed Ideas.

4.1.1. Reprogrammed activities improve cellular fitness

This reprogramming is all about providing a selective advantage during tumorigenesis. It either supports cell survival under stressful conditions or allows cells to grow and proliferate at pathologically elevated levels. In the review they say that "*there are many examples in which inhibition of an enhanced metabolic activity results in impaired growth of experimental tumors.*"

4.1 Proposed Idea (cell biology, mitochondria metabolism): Since niclosamide directly affects mitochondrial pH and membrane potential by its simple protonophore action, could niclosamide target any of these major activities that provide benefit to the malignant cells? While there is clearly a lot in the literature already (as reviewed next, *4.2 Niclosamide is a very "dirty" drug)*, has it been done and is completely understood on your particular cell line growing in your particular lab? If not, that proposal is ready to start.

4.1.2. Stage of tumor progression

They also make the excellent point that could generate lots of new experimentation for the tumor cell biologists when they say that success of metabolic therapy will depend on the stage of tumor progression in which each pathway provides its benefit to the cancer cell, requiring

biologically accurate models of tumor initiation and progression. So, add niclosamide to this equation, that could affect all down-stream pathways and I think we have a project or three.

4.2 Proposed Idea (Cell biology, cancer cell lines and development): If your lab has biologically accurate models of tumor initiation and progression, and I presume takes patient biopsies or has access to a biobank, how and to what extent does niclosamide act on any cancer cell line, especially patient derived, as a function of the stage of tumor progression?

4.1.3. Bioenergetics

I was most interested in the Bioenergetics section, where they start by pointing out that Warburg's hypothesis, ….

"… led to the widely held misconception that cancer cells rely on glycolysis as their major source of ATP".

It turns out that *"the great majority of tumor cells have the capacity to produce energy through glucose oxidation (that is, the process by which glucose-derived carbons enter the TCA cycle and are oxidized to CO_2, producing ATP through oxidative phosphorylation).*

And furthermore,

"Mitochondrial metabolism is necessary for cancer cell proliferation and tumorigenesis. Thus, despite their high glycolytic rates, most cancer cells generate the majority of ATP through mitochondrial function".

So, if we were under the impression (as I was) that cancer cells only use glycolysis and don't use OXPHOS, and therefore niclosamide would not have an effect in cancer, this statement opens up a whole new appreciation and lines of study. I can see the titles for at least a couple of new grant proposals.

4.3 Proposed idea *(cell biology, cancer metabolism)*
Proposal titles:

- *"How Does Niclosamide Affect The Functioning of Dysregulated Mitochondria In Cancer Just With a Simple Proton Shunt Activity; Or Is There More To It?"*
- *"Delineating All The Downstream Effects in All the Pathways that Stop Cancer in its Tracks".*

I was particularly intrigued by the discussion of how cancer cells, especially under hypoxic conditions, need glycerophosphate lipids and cholesterol for maximal cancer cell growth. Apparently, hypoxia suppresses *de novo* fatty acid synthesis from glucose, so they have to get it from the extracellular space to supply membrane biosynthesis. And so, to do this, they upregulate expression of the low-density lipoprotein receptor (LDLR). As we will see next, this was the endogenous-inspiration for our prodrug therapeutic nanoparticle design, as presented and discussed below in Part II, ***7. The Niclosamide Stearate Prodrug Therapeutic (NSPT) for cancer (Osteosarcoma)***. There is so much more that I cannot possibly do justice to, you just have to read it yourselves [54] and their other 2008 paper, "*Brick by brick: metabolism and tumor cell growth*" [55].

4.2. Niclosamide in cancer: a very "dirty" drug

4.2.1. Niclosamide affects multiple pathways

As we saw from the literature search in Fig. 1, niclosamide has recently attracted considerable interest as a novel antitumor agent [8,111,150,206,210]. In cell culture studies in a range of cancer cell lines, niclosamide has shown to be a very "dirty drug", —it inhibits (at least) 17 different pathways in cancer cells [145,209], including, Wnt/β-catenin, mTORC1, STAT3, NF-κB, aromatase, and Notch signaling. Especially in cancer there appears to be additional mechanisms associated with mitochondrial fragmentation that induce cancer cells to go into Apoptosis [147]. However, niclosamide is relatively non-toxic to healthy cells [206]. Niclosamide also induces cell cycle arrest at the G1 phase in head and neck squamous cell carcinoma through tumor suppressor mechanisms [93]. In recent studies, S phase was blocked after incubation with only micromolar niclosamide [94,147]. In breast and prostate cancer lines, we have also found that IC_{50} concentrations for cell viability are in the 100 s of nM to few μM to range and arrest the cell cycle in G_1/G_o, —there are no S phase cells [10,118]. In fact, *in vitro* studies with the NCI-60 human cancer cell lines indicate that niclosamide inhibits cell growth in all tested cancer cell lines [173].

4.2.2. Intracellular as well as plasma membrane gradients in cancer

Other intracellular pH gradients that are also affected by niclosamide include endosomes, lysozomes, and even the plasma membrane.

Endosomes: Niclosamide prevents normal endosome acidification. This will be quite important in the anti-viral effects of niclosamide, and so will be reviewed and discussed in that section. New data in cancer suggests that functionally competent endosomes are important in cancer malignancy as reviewed by Ko et al., [128] in their paper, "*Emerging links between endosomal pH and cancer*", and references therein. This was an unexpected and obviously fascinating find when the reference popped up in an inspired search for "endosomes" AND "cancer".[7]

The main point is that, since endosomes are where receptors (like the epidermal growth factor receptor (EGFR)) end up in their recycling after binding their ligand and initiating their downstream effects, "*endosomal trafficking could decide the fate of cancer cells by termination or prolongation of oncogenic signaling*". The experiments that showed this link between destroying pH and having a positive effect on cancer integrity are studies that used electroneutral Na^+/H^+ exchangers in order to effect dissipation of the endosome acidic pH. So, this is clearly where niclosamide could come in.

4.4 Proposed idea, (Cancer cell receptor-recycling biology): Take your well-characterized cancer cell receptor recycling model and determine if and to what extent niclosamide interferes with this process? How and to what extent does this limit the cancer cell's ability to do any of its downstream signaling using receptors that respond to ligands or are constitutively already turned on; or are they, with niclosamide present?

Lysozomes: Niclosamide also causes lysozome permeabilization and acidification of the cytosol. This also has an effect in preventing viral infection [115], where niclosamide was one of the top hits against HRV16 infection and again was shown to act as a proton carrier from acidic vesicular lumen to the pH neutral cytosol. This also has an effect in cancer. In cancer though, as mentioned above, in the Fonseca study [75], in MCF7 breast cancer cells, niclosamide dissipated protons (down their concentration gradient) from lysosomes to the cytosol, effectively lowering cytoplasmic pH thereby inhibiting the mTORC1[8] pathway. How niclosamide affects normal cells and how it affects cancer cells can be

[7] We are so lucky to have Google, "seek and ye shall find". I would have never found this in a conventional library. Google was founded (on September 4, 1998) and so here we are, 24.5 years, AG, (Anno Google).

[8] mTORC1– mammalian target of rapamycin complex 1 – a protein complex that functions as a nutrient/energy/redox sensor and controls protein synthesis.

two very different things requiring specialized expertise in the altered metabolism pathways. Because of the altered metabolism and, in particular, how the mitochondria (dys)functions in cancer, this is a rich source for more research projects.

In acute myelogenous leukemia, niclosamide causes mitochondrial damage, increases reactive oxygen species, and induces apoptosis through increased levels of cytochrome C [111]. Speaking of leukemia, I often wonder if we cannot get at the blood cancer cells more directly since prodrug nanoparticles do not need to extravasate, they just have to be at a level where collisional frequency and uptake by leukemic can be optimized. Details of the nanoparticles that could do this, and also release drug into the blood stream as long-circulating depots are given in Part II, ***7. The Niclosamide Stearate Prodrug Therapeutic (NSPT) for cancer (Osteosarcoma)*** but this could be worth mentioning here.

4.5 Proposed idea: (drug delivery, preclinical leukemic cancer models): Evaluate if and to what extent i.v., injection of leukemia-targeted particles of Niclosamide stearate that can be taken up by individual cells and/or provide a level of circulating niclosamide bound to albumin that the cancer cells take up as well. What is their effect on killing leukemic cells *in vitro* and *in vivo*?

Plasma membrane: Ko et al. [128] actually started their paper on endosomes by briefly reviewing the pH-status of normal versus cancer cells. While normal differentiated cells maintain an intracellular cytoplasmic pH of ~7.2 and the extracellular pH stays at ~7.4, cancer cells function, (perhaps surprisingly), in an alkaline cytoplasmic pH condition that is at a pH >7.4. However, their surrounding extracellular pH is actually more acidic at ~6.7–7.1 or even lower. Here, as you might imagine, given the Warburg effect, it's all about glycolysis and lactic acid, as explained by Romero-Garcia et al., [232] (and references therein) which is worth again hearing in their own words,

"*As a consequence of the Warburg effect, cancer cells secrete large amounts of lactate to the extracellular microenvironment, which in turn lowers extracellular pH to 6.0–6.5. Lactate contributes to acidosis, signals for angiogenesis, acts as a cancer cell metabolic fuel, and induces immunosuppression. Several reports demonstrate that acidosis leads to loss of the T-cell function of human and murine tumor-infiltrating lymphocytes; the T-cell function can be restored by buffering the pH at physiological values*".

As Ko also state, "*This reversal of the pH gradient between cytoplasmic and extracellular milieu promotes malignant phenotypes*",

Apparently, because of the high intracellular pH, cells start to: re-enter mitosis (grow); by-pass cell cycle checkpoints (could this be coupled with check point inhibitors?); promote proliferation; evade apoptosis (that requires a lower pH); destabilize their genome driving cancer evolution and drug resistance. What? This is all due to a cancer cell controlling its internal and external pH, but, wait, that's a pH gradient across the plasma membrane that niclosamide could dissipate! This is amazing, and I thought niclosamide could only affect mitochondria. Furthermore, extracellular pH "*promotes the expression of stem cell markers, angiogenic factors and hypoxia response factors enhancing tumor aggressiveness and angiogenic potential*".[9]

4.6 Proposed idea(s): (cell biology, endosome, and intracellular pH markers): Since niclosamide dissipates pH gradients across all membranes, as a matter of course, here is another mechanism to explore in each of your cancer cell lines and determine if and to what extent niclosamide dissipates the pH gradient across the plasma membrane, and if the dissipation of these intra and extracellular pHs in cancer cells correlates with control of cancer proliferation
Furthermore, does immunotherapy really work in an acidic extracellular environment?

4.2.3. Ameliorating the highly toxic chemotherapy and myelosuppression

One other area of interest in cancer drug treatment is when chemotherapy induces myelo-suppression, limiting dosing until the bone marrow has recovered. As mentioned later in Section ***7.1 Kaplan Meier and more***, one approach to help alleviate this drug-induced toxicity is to use Cell Cyclin Dependent Kinases (CDK) inhibitors to protect other dividing cells and put them into cell cycle arrest until the chemo has gone, and so can act to help reduce the toxicity of chemo [228], called "transient cell cycle inhibition" [60].

4.7 Proposed Idea (preclinical and clinical pharmacology): If CDK inhibitors can put cells to sleep, so can niclosamide. We know niclosamide stops cells in the S phase at only micromolar concentrations [94,147]. So, could niclosamide be used to do the same thing as a systemic protective, while niclosamide itself has its own anti-cancer activity? That is, due to the altered metabolism of cancer cells versus normal, as we have seen above, the cancer cells are much more sensitive to niclosamide and even kill themselves at concentrations that do not damage normal cells, it just puts them to sleep.

One drug that could use this niclosamide-mechanism is SN38 (irinotecan) that causes quite serious myelosuppression[10] and it is actually ameliorated by CDK inhibitors in the clinic. CDKIs like palbociclib also synergize with irinotecan to promote colorectal cancer cell death under hypoxia [290]. This obviously suggests a proposal or two since we are currently making SN38 prodrug nanoparticles ready for testing in a new R21 just submitted on preclinical colorectal cancer [194]. Hence the reason this could be interesting is that we know niclosamide also could act by inhibiting the cell cycle of normal cells without being lethal, yet still put cancer cells into apoptosis.

4.8 Proposed Idea: (preclinical toxicity):
1. Could a SN38 stearate prodrug therapeutic reduce the normal SN38 (irinotecan) toxicity while accumulating in the tumor interstitium?
2. Could niclosamide itself be both a transient cell cycle inhibitor for normal cells as well as an anti-cancer drug for cancer cells? Is the therapeutic index more about if, to what extent, and how healthy cells versus cancer cells respond to niclosamide in a

[9] These cancer cells really do take advantage of every opportunity…. and pH seems to be an under-appreciated aspect that influences so much both inside and outside the cells, including immunosuppression!

[10] Myelosuppression: A condition in which bone marrow activity is decreased, resulting in fewer red blood cells, white blood cells, and platelets. Myelosuppression is a side effect of some cancer treatments. When myelosuppression is severe, it is called myeloablation, https://www.cancer.gov/publications/dictionaries/cancer-terms/def/myelosuppression.

concentration dependent manner in these mechanisms? i.e., test the hypothesis: *niclosamide is not toxic unless the cell makes it toxic*.

4.2.4. Niclosamide can enhance other drugs, radiation, and immunotherapy

Two more recent reviews in 2022 that are worth reading have updated the data that niclosamide can enhance other drugs, radiation, and immunotherapy. Wang et al. [267] (and references therein) at the Ministry of Public Health Shenyang, China, systematically reviewed the pharmacological activities and therapeutic prospects of niclosamide in human disease in their paper *"Niclosamide as a Promising Therapeutic Player in Human Cancer and Other Diseases"*. Like Chen and Mook [44], they found that niclosamide, again, has influences that include metabolic diseases, immune system diseases, bacterial and viral infections, asthma, arterial constriction, myopia, and cancer. They offered therapeutic prospects of niclosamide and summarized the related molecular mechanisms and signaling pathways, indicating that niclosamide is a promising therapeutic player in various human diseases, including cancer.

The same group, Ren et al. [226] (and references therein) then focused specifically on niclosamide in combination with current anti-cancer therapies. They make the really rich and explorable-statement that, *"niclosamide can be used in combination with chemotherapeutic drugs, targeted drugs, radiotherapy, and immunotherapy to enhance the anti-tumor effect"*. They report an impressive list of co-activity for approved drugs for a range of cancers that include: human- breast, prostate, colon, ovarian, multiple myeloma, acute myelogenous leukemia, glioblastoma, adrenocortical carcinoma, osteosarcoma, head and neck, lung, and oral. They also say that,

"Niclosamide can inhibit the proliferation of cancer cells and have minimal effect on normal cells and has no obvious toxicity to nonmalignant tumor cancer cells and can inhibit the migration and invasion of cancer cells and the activity of cancer stem cells".

4.9 Proposed Idea (Preclinical and eventually clinical trials): If this is not a golden opportunity I don't know what is. As presented later, (***7. The Niclosamide Stearate Prodrug Therapeutic (NSPT) for cancer (Osteosarcoma)***) we already have a niclosamide formulation that has shown promise in a mouse model of metastatic Osteosarcoma [225], and in a feasibility clinical trial in canines. In addition to testing this monotherapy in many different tumors, this same formulation is just waiting to be developed and co-administered with any of the already approved drugs in a series of new preclinical and clinical trials. Test niclosamide as our Niclosamide Stearate Prodrug Therapeutic in combination with all current anti-cancer therapies as described by Ren et al., [226]. I'm happy to teach you all I know about how to make them from my lab to yours on zoom for cell and preclinical studies, and then we can arrange GMP manufacturing for any subsequent canine or human trials.

5. Niclosamide in viral infection

As in the brief history section, and reviewed by Xu [281], Niclosamide has very broad spectrum antiviral activity across many viral infections, and not just respiratory, –that will be the main focus for our eventual preventative niclosaspray nasal and early treatment throat sprays (See later ***8. Formulation of Niclosamide for COVID19 and Other Respiratory Infections***).

5.1. Respiratory viral pandemics – niclosamide could help prevent all of them

In their overview of "*Viral Pandemics in the Past Two Decades*", Bhadoria et al. in 2021, [20] state that, "*Major viral pandemics in the last 2 decades mostly involved respiratory viruses like corona, causing 3 major pandemics earlier: severe acute respiratory syndrome (SARS CoV-1), Middle eastern respiratory syndrome (MERS) and ongoing SARS CoV-2 (COVID -19), followed by influenza viruses causing Influenza A H1N1 pdm 2009 (swine flu), Ebola and Zika virus infections*".

And niclosamide has been shown to inhibit all of them [281]. And so, this is another must-read paper for any anti-viral researcher but also institutional research and innovation offices, RKE staff, tech transfer offices, funding agencies, and investors, to appreciate what niclosamide could do, ….. if you give it the chance.

The initial route of transmission appears to be via air (as aerosolized virus) [105] and viral load is highest in pharyngeal secretions early in the course of infection [278]. Thus, it is now well accepted that the initial infection site is mostly the nasaopharynx, and then the replicating viruses travel down into the throat in muccal secretions, often at night while the person is asleep. The problem here is that the viruses then head on down into the bronchi and end up in the lungs where they can have their most devastating damage. Searching on this, I was actually surprised to find that, as reported by Hönzke et al.[102] alveolar ACE2 expression in the lungs is scarce and it's the macrophages that do the damage, "*.. severe lung injury in COVID-19 probably results from a macrophage-triggered immune activation rather than direct viral damage of the alveolar compartment*".

Yes, you guessed it, the next search was for niclosamide and macrophages… and the first hit was, Sekulovski et al.'s, ***"Niclosamide suppresses macrophage-induced inflammation in endometriosis"*** [238]. So, if niclosamide can suppress macrophages in endometriosis, what about in the lungs and COVID19? Thus, niclosamide, if formulated appropriately and used for each of these locations as a nasal spray preventative, early treatment throat spray, and even nebulized lung treatment, could have a major impact at the population level. Having created a simple niclosamide aqueous solution at a high enough concentration to be effective but not too high that it is toxic or lethal to cells, our, again, nanomedicine-scaffold-free formulation, could be adapted to each of these applications, —prevent, treat, stop, and enable all the other viral specific vaccines and anti-virals to have an even greater impact.

5.1 Proposed Idea, (preclinical and clinical COVID19): In order to reach the lungs and alveoli, droplets or drug particles should be ideally between 0.5 μm to 5 μm. According to Kooji, et al., ultrasonic nebulizers, especially the surface acoustic wave nebulizer (SAWN)) [131] could make aqueous droplets in this range (~1.1 μm). Also, by McDermott and Oakley [161] Aeroneb Solo Mesh Nebulizers (Aerogen, Mountain View, California) can aerosolize a 3% sodium chloride solution, and gave ~5 μm droplets at the nebulizer. Here we would investigate solutions with and without niclosamide and determine surface tensions and droplet sizes (a lower surface tension of the niclosamide solutions would create smaller droplets). Hypothesis: treatment with nebulized niclosamide solution *pre and post infection will reduce inflammatory macrophage activity*. In a preclinical model of lung infected and immune-inflammatory lungs, pre-treat and post-treat with nebulized niclosamide solution as a function of time (viral life cycle) before and after inoculation and concentration of niclosamide. Monitor the development or reduction in inflammatory macrophages and determine if and to what extent there is lung damage compared to untreated controls. Explore other

micronized forms of niclosamide. I'd love to engage with anyone on this project.

Let's start, as always, with some literature.

5.2. Niclosamide has broad virostatic activity, but what is its mechanism? (— it's a host cell modulator)

Many current anti-virals either attempt to disrupt the synthesis and assembly of viral proteins (viral proteases, RNA-dependent RNA-polymerase, virus helicases, viral spike and structural proteins) or target host proteins and mechanisms required by the viral replication cycle (including, boosting interferon response, ACE2 receptors, cell surface and endosomal proteases, and clathrin mediated endocytosis) [139]. As discussed above, Niclosamide offers a different and potentially very effective way to combat viral infection because it enters cell membranes as a lipophilic anion where it acts as a proton shut, dissipating pH gradients across a range of host cellular-organelle membranes, including mitochondria, endosomes, and lysosomes, revamps virus-modified endoplasmic reticulum, and affects the viral assembly via the Golgi.

While vaccines and antibody treatments are certainly effective, they are designed to work only after infection has taken place, i.e., they do not prevent infection. They are also limited to a particular virus, a particular viral strain, a particular viral protein, and may eventually need to be boosted or redesigned for emerging viral variants. In contrast, Niclosamide is a host cell modulator and so is "specific" for every virus, virus strain, viral protein, and every virally infected or infectable cell. As postulated by Laise et al. [139], *"Targeting host cell mechanisms may have more universal and longer-term value because the same host factors may be required by multiple, potentially unrelated viral species and because host target proteins mutate far less rapidly than viral proteins, thereby limiting emergence of drug resistance"*. We concur.

The action of niclosamide can be more *virostatic* (than directly *antiviral*). That is, as a proton shunt it can dissipate the gradients, and, as we shall see, appears to affect any virus and any emerging new variant that uses the cell's own machinery to enter, replicate, and be assembled, but, once it washes out, then what? Are any of the effects permanent (toxic to the host cell) or permanently deleterious to the virus? As we will see, one could be, —up regulation of autophagy that the virus seems to shut down, but when reactivated by niclosamide could phagocytose the virus in the cytoplasm and destroy it.

Thus, niclosamide's mechanism of action is not *directly* on the viral RNA, or on blocking transcription/translation enzymes, or inhibiting the receptor-ligand interactions that first bind the virus to the cells. Rather, its targets are the intracellular membranes that the virus co-opts to otherwise facilitate these processes and where it dissipates their pH gradients reducing or eliminating function.

To reiterate, as reviewed briefly above, (2.2.3 Niclosamide as an antiviral) since the early 2000's niclosamide has shown broad spectrum *virostatic* activity against various viral infections with nanomolar to micromolar potency [86,109,126,279,281]. These have included SARS-CoV, MERS-CoV, ZIKV, HCV, and human adenovirus. Let's now look at niclosamide and SARS-Cov-2.

5.2.1. SARS-CoV-2

With respect to SARS-COV-2, in March 2020 Sangeun Jeon et al. [109] at the Korean Pasteur Institute measured a niclosamide IC_{50} for viral replication of ***0.28*** μM in Vero 6 cells. This was followed by a second screen (by Ko et al., [126]) in the more relevant, although still not necessarily ideal, Calu-3 cells (human lung epithelial isolated from human lung adenocarcinoma) of ***0.84*** μM. Jeon et al. [109] also showed a complete IC_{100} inhibitory activity for viral replication at the level of ***~1*** μM in Vero6 cells and an estimated ***~2*** μM in the Calu-3 cells [126]. As we saw above (***3.3. The "Drugging" Characteristics of Niclosamide***) this active concentration is right around the intrinsic solubility of niclosamide's monohydrate of 1–2 μM, and so this solubility-efficacy equivalence could, in principle, limit niclosamide's bioavailability and efficacy in solution. This would especially be the case if the niclosamide solution is in equilibrium with a niclosamide suspension of undissolved material or a drug delivery system where the niclosamide equilibrates out as this low solubility monohydrate polymorph [185,187] (see later, ***8. Formulation of Niclosamide for COVID19 and Other Respiratory Infections***)

These virus infection-inhibitory results were recently confirmed by Braga et al. [23] for SARS-CoV-2 also in Vero 6 cells with an IC_{50} for viral replication of ***0.34 μM***. Brunaugh et al. [24] also showed niclosamide inhibited SARS-CoV-2 by viral-Cytopathic Effect (CPE) to 100% at 0.063 mg/mL (which is ***0.193 μM*** niclosamide) and MERS at 0.125 μg/mL (***0.383 μM***). Similarly, Mostafa et al. [171], also in Vero E6 cells, showed that, while the IC_{50} for inhibiting SARS-CoV-2 (NRC-03-nhCoV) viral replication was actually even lower, at ***0.16*** μM niclosamide, the half maximal cytotoxic concentration for the host cells (CC_{50}) was ***204.6*** μM, representing a therapeutic (selectivity) index of 1279×. Mirabelli et al. [166] also measured an IC_{50} of ***0.14 μM*** for niclosamide in infected liver Huh-7 cells. Finally, Zhu et al. [293] showed that SARS-CoV-2 causes multinucleated syncytial cells arranged in a net-like structure observed in plaque regions. Here, the data from Braga et al. [23] showed that niclosamide protected against syncytia *in vitro*. Incidentally, Ko et al., [126] identified the protease inhibitor Nafamostat as a more potent antiviral drug candidate, with activity of only 2 nanomolar, and so this could be another drug that is prime for formulation, but toxicities include: agranulocytosis, hyperkalemia, and anaphylaxis, cardiac arrest in dialysis patients (dyspnea); LD50 i.v. in rats = 16.4 mg/kg.

5.2.2. Other viruses

For other viruses, data from Jung et al. [114] in ***Dengue viruses*** also confirm an EC_{50} for niclosamide against DENV-1, DENV-2, DENV-3, and DENV-4 infection in Huh-7[11] cells of ***1.45 μM***, ***0.38 μM***, ***0.37 μM*** and ***0.25 μM***, respectively, while niclosamide toxicity to the cells at CC_{50}s was again >10 μM. And for ***RSV***, Niyomdecha et al. [202] in HEp-2 cells, pretreatment with ***0.25 μM*** niclosamide concentration for 6 h presented the highest anti-RSV activity of 94%. Li et al., investigated ***Hepatitis E virus*** (HEV), [143], and determined using a viral replication-related luciferase activity an IC_{50} for inhibition of viral infection of ***0.96 mM***, and a complete inhibition at a slightly higher niclosamide concentration compared to the rest of ***~10 mM***. Li et al. [143] also extended the cell lines to human kidney 293 T, hepatic PLC, neuronal U87, reporting IC_{50}s of ***1.09 mM***, ***0.16 mM***, and ***0.16 mM***, respectively, and more complete inhibition at widely varying, ***~10 mM***, ***10–100 mM*** and ***10 mM*** (90%) at 48 h, respectively for the same three cell lines. Finally, Niyomdecha [201] had earlier evaluated niclosamide against HIV-1.

Unlike the other viruses mentioned above, ***HIV-1*** is a pH-independent virus and requires the mTORC1 pathway for viral replication. Niyomdecha et al. showed that in TZM-bl cells (a Human papillomavirus-related endocervical adenocarcinoma cell line that is highly sensitive to infection with diverse isolates of HIV-1) "*niclosamide effectively inhibited HIV-1 through mTORC1 inhibition without disruption at the early replication phase of reverse transcription and pro-viral transcription*". The cell line was highly sensitive in terms of cell viability measured by MTT assay to be ***0.851*** μM (not necessarily cell death). Efficacy though was ***0.119 mM*** by RT-qPCR. They conclude that the antiviral mechanism of niclosamide in HIV-1 is via the AMPK-mTORC1

[11] an immortal cell line composed of epithelial-like, tumorigenic cells.

Table 1
Summary of cells used, virus and MOI (#virus/#cells), viral inhibition assays, measured IC_{50} and IC_{100} (or as otherwise stated) and references with comments.

Cells	Virus (MOI)	Assay	IC50 (μM)	IC100 (μM)	Reference and Comments
Vero6	SARS-CoV-2, CoV/KOR/KCDC03/2020 (0.0125)	cytopathic effect (CPE)	0.28	~1–2	[110] (CC50 > 50 μM)
Calu3	SARS-CoV-2, βCoV/KOR/KCDC03/2020 (0.1)	immunofluorescence of SARS-CoV-2 N protein	0.84	~2	[127] (CC50 > 50 μM)
VeroFM	SARS-CoV-2 (0.0005)	plaque assay and RT-PCR at 24 h	0.13	1.24 (>99%)	[87] (also proved autophagy)
VeroE6 (also, in Calu-3)	SARS-CoV-2 (0.05)	plaque	0.35	5.0	[23] (TCID/50 used for screening)
Vero E6	SARS-CoV-2 (0.1)	Immunoblot Immunofluorescence PCR (RT-PCR)	- 1–3 -	1.56 3.12 3.12	[279] CC50 > 250 μM after 48 h
Vero E6	SARS-CoV-2, human/Korea/CNUHV03/2020 (unknown MOI)	Viral RNA, TaqMan real time fluorescent PCR, (RTqPCR)	0.092 (0.03 μg/mL)	0.193 (92.7%) (0.063 μg/mL)	[24] Niclosamide added 24 h post infection
Vero6	MERS, EMC2012 strain	Viral RNA (RTqPCR)	0.193 (0.063 μg/mL)	0.383 (0.125 mg/mL)	[24]
Vero E6	SARS-CoV-2 NRC-03-nhCoV (0.005–0.001)	Plaque Infectivity Assay	0.16	~5	[171] CC_{50} 204.6 μM Examined protein binding
Huh-7	SARS-CoV-2 (0.2)	TCID50 assay RT-qPCR anti396 nucleocapsid antibody	0.14		[166] (4 h pre-incubation with niclosamide 1 h incubation with virus) EC50 ~ 6 μM)
VeroE6, VeroE6 TMPRSS2, Caco-2 Human Airway Epithelial Cells	SARS-CoV-2 Wuhan D614 BatvPat D614G Alpha (B.1.1.7), Beta (B.1.351) and Delta (B.1.617.2) TMPRSS2 (0.001)	TCID50 assay RT-qPCR	0.13 0.06 (α) 0.08 (β) 0.07 (δ) 0.08	IC90 0.16 μM	[270] (used niclosamide ethanolamine)
VeroE6, VeroE6 TMPRSS2, Caco-2 Human Airway Epithelial Cells	SARS-CoV-2 Wuhan D614 BatvPat D614G Alpha (B.1.1.7), Beta (B.1.351) and Delta (B.1.617.2) TMPRSS2 (0.001)	TCID50 assay RT-qPCR	0.13 0.06 (a) 0.08 (b) 0.07 (d) 0.08	IC90 0.16 μM	[270] (used niclosamide ethanolamine)
VeroE6 (SNB19 glioblastoma)	SARS-CoV-2 USA_WA1/2020 (0.002) (Zika virus)	CPE activity focal forming units	0.112 with 30% efficacy (Zika 0.12)		[242] 57% cell viability at 30 μM (Also docking studies)
Huh-7	Dengue DENV-1 thru 4	cytopathic effect (CPE)	1.45, 0.38, 0.37 0.25 (Ave 0.61)		[114] CC50s >10 μM
Huh-7	Hepatitis E virus (HEV)	viral replication-related luciferase activity	0.96	~10	([143]) CC50 = 16.17 μM
human kidney 293 T hepatic PLC neuronal U87	Hepatitis E virus (HEV)	viral replication-related luciferase activity	 1.09 0.16 0.16	@48 h 10 10–100 10 (90%)	([143]) CC50s, 10.7 μM, 134.9 μM, 6.74 μM
HEp-2	RSV-A —B and -A (V102–7025) —B (V102–7031)	200 TCID/50		0.25 (94%)	[202] (6 h exposure) EC50 = 0.551 mM)
TZM-bl cells SupT1 cells	HIV-1	RT-qPCR	0.119 0.102		[201] CC50 0.851 μM in TZMbl cells and 0.962 μM in SupT1 cells

pathway, downregulating this mTORC1 pathway and the production of the HIV-1 p24 protein, suggesting that this could be a common therapeutic target for various viruses. (mTORC is also a target for niclosamide in cancer).

Table 1 gives a summary of the references in the text for the SARS-Cov-2 studies including details of the virus strain if available.

From Table 1 then it is clear that the cell lines that were chosen for testing were mainly the robust Vero —6, -FM, —EM, -TMPRSS2, Calu-3, and Huh-7, and so there is now a need to test niclosamide in the more relevant human Nasal and Bronchial epithelial cells. A recent study by Chui et al. [47] has developed an organoid culture system of the nasal epithelium from easily accessible nasal epithelial cells. Consecutively passaged for over 6 months Chui et al. "*established differentiation protocols to generate 3-dimensional differentiated nasal organoids and organoid monolayers of 2-dimensional format that faithfully simulate the nasal epithelium*" and "*when differentiated under a slightly acidic pH, the nasal organoid monolayers represented the optimal correlate of the native nasal epithelium for modeling the high infectivity of SARS-CoV-2, superior to all existing organoid models*", which sounds like a very appropriate model *in vitro* system to test niclosamide.

Also given for most of the studies in Table 1 is the Multiplicity of Infection (MOI) in parentheses where MOIs varied from 0.0005 to 0.2 viruses per cell with an average MOI of 0.0382 which is 200 to 5 cells per virus and an average of 26.2. These ratios would give niclosamide the best chance but are also at a level where some had

shown the virus did not compromise the cells, and so did not cause cell death.

Several different viral inhibition assays were used depending on if the study was a quick screen using the semi quantitative TCID/50 assay and then followed with the more accurate RT-qPCR, and some used Immunofluorescence. Most of the studies gave 50% inhibitory concentrations (IC_{50}s) and some showed the full curve and so the 100% inhibitory IC_{100} (or as otherwise stated) could be estimated from the graph.

Other studies that evaluated niclosamide in other viruses are given for completion, including, Dengue, Hepatitis, RSV and HIV, which showed similar IC_{50}s in the sub micromolar range. Basically, the average concentrations for complete inhibition of viral infection with niclosamide are all in the 1 μM range across all cells, all viruses, and all assays. Maybe this is not surprising given that niclosamide can partition equally into all membranes and dissipate all pH gradients, although some [171,242], did model niclosamide (and other drugs) to see if they were docking into various viral proteins.

One other review is really worth reading, that by Singh et al., where they review, similar to here, "*Niclosamide—A promising treatment for COVID-19*", but they also give some data on Pharmacokinetics of oral niclosamide. Mainly, though, they list many of the other potential applications (some I have reviewed here and some I haven't) including:

- *Niclosamide in chronic medical states*: metabolic syndrome, autoimmunity, pulmonary pathology, and mechanism of action and overall effect in 8 different types of cancer –colorectal, lung, breast, head and neck, ovarian, glioblastoma, hepatocellular and prostate cancers.
- *Niclosamide as an anti-bacterial agent*: gram-positive bacteria, (*Staphylococcus aureus* and methicillin-resistant *S. aureus*), gram-positive bacteria (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), *Clostridium difficile*, *Helicobacter pylori*, *Pseudomonas aeruginosa* quorum sensing, multidrug-resistant *Mycobacterium tuberculosis*.
- *Niclosamide as an antiviral agent*: including the many already cited and also focused on SARS-Cov-2. One thing caught my eye, Garrett et al. [84] demonstrated that the total lipid profile is amplified during SARS-CoV-2 infection in VeroE6 cells. Treatment with niclosamide led to a reduction in lipids available for virus production, (does niclosamide also shut down fatty acid synthase (FASN) and reduce the lipids available for making new coated viruses? Could it also reduce FASN in cancer?).
- *Clinical trials of niclosamide for COVID-19*: lists that twelve of these trials are in Phase 2/3 and investigate the efficacy of niclosamide across the full COVID-19 disease spectrum, as: Oral formulations, Intramuscular injections, and Inhalational and intranasal administration including the —PROTECT-V trial currently being run by UNION therapeutics, [258,259] including some of the authors on the Singh et al. paper that are responsible for the formulation and running that trial.

The publication references are also collected together below the table for convenience with a few qualifying comments. Hopefully this will get you started on any anti-viral studies, and, since most have not yet been done on primary cells (e.g., human nasal and bronchial epithelial cells), the field is wide open for testing the activity of niclosamide on viruses and cell lines.

Some of the studies we are considering in our own lab and invite you to join us and collaborate or just do them yourselves, include the following:

5.2 Proposed Idea (Cell biology, pathways, viruses, intracellular pH, ATP metabolism): How long does niclosamide take to start acting and producing measurable biochemical effects on the host epithelial cells, like reducing ATP, possibly glycolysis (Seahorse assays) and reducing pH-gradients and membrane potentials in the various organelles in any cell line? We would want to do cell studies with read outs at 15 mins, 30 mins, 1 h, 4 h. There would seem to be little point going beyond 4 h because a single drug dosing is unlikely to last that long bathing the nasal epithelial cells with the required niclosamide concentration. And then how long does it take for cells to return to baseline and re-establish those same pH gradients?

5.3 Proposed Idea (Cell biology, intracellular and organelle pH): For normal endosomes and lysozomes in the host cell that are already acidified, how rapidly and over what concentrations upon addition of niclosamide (time and concentration dependence) is the endosome and lysozome pH dissipated and what is the new acidified cytoplasmic pH? Also, what does this do to any pH-dependent processes that don't function when acidified?

5.4 Proposed Idea (Cell biology, viral infection, and replication cycle): Pre-infection, how much niclosamide in solution does it take (concentration dependence) and how long before inoculation (time dependence) to stop the initial endosomal viral RNA release and completely prevent infection? Here we would again want to conduct fairly rapid sampling in minutes to 1 h.

5.5 Proposed Idea (Cell biology, viral infection, and replication cycle): Post-infection, how can we utilize niclosamide in the viral lifecycle (characterize that first) to slow down or stop replication via reductions in ATP (—turn down the dimmer switch) that slow or stop replication and/or secretion? As discussed next (section 5.3) the kinetics here appear to be: Viral diffusion through mucin 20s – 30s; viral entry via endosome (8–12 min); Replication (24 h); Secretion (12h) and maybe up to 48–72 h. Again, these would be planned experiments for concentration- and time-dependences for niclosamide dosing on the cells generating a series of measures of viral replication titer effect vs time for each concentration.

5.6 Proposed Idea (Cell biology, Air/Liquid Interface (ALI) epithelial cell cultures): Submerged cells are good for initial screening and some data, but Air liquid interface cells (and the recently developed organoids [47]) would be as close as we can get to a more real nasal epithelium. All of the above experiments on these kinds of preparations can be done on these models of the nasal epithelium, including the kinetics and deposition of drug transport through the ALI cultures. I proposed this but was not funded a couple of years ago entitled, *"Rational Design of Nasal and Throat Sprays for Respiratory Viral Disease: Development of Five Potential*

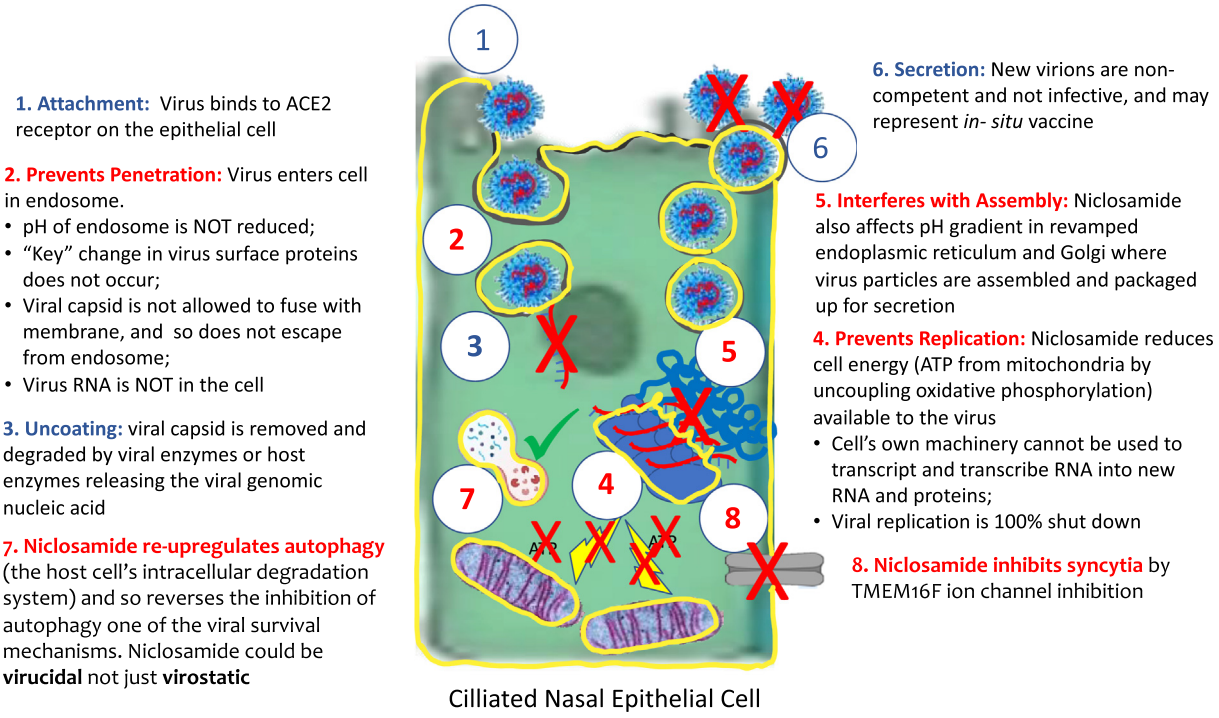


Fig. 7. Pathogenesis and kinetics of coronavirus infections and the influence of niclosamide at various stages. ***Blue*** numbers and text headings represent no niclosamide effect;. ***Red*** numbers and text headings represent known niclosamide effects on SARS-CoV-2 or other viruses. Schematic shows the stages in the infection process that are affected by pre-saturation with niclosamide of host cell membranes (shown in yellow for plasma membrane, endosome, mitochondria, and Golgi). ***1. Virus attachment and binding***; 2. ***Penetration*** via plasma membrane or endosome by spike protein catalyzed membrane fusion; ***3. Uncoating*** and RNA release from the capsid; ***4. Replication***, requiring ATP from mitochondria; and ***5. Assembly*** of viruses in the Golgi, and hence ***6. Secretion*** of non-competent virions; ***7. Autophagy*** can be re-upregulated; ***8. Inhibition of syncytia***. Via TMEM16F ion channel inhibition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Formulations of Niclosamide and Measurement of Drug Deposition and Activity in Respiratory Cells", if anybody (cell biology, drug delivery) want to collaborate and resubmit with me, please let me know.

5.3. Niclosamide inhibits (at least) 3 of the 6 stages of viral infection, re-upregulates autophagy and inhibits syncytia

Similar to cancer, where the cancer phenotype makes huge changes in the normal cell metabolism and other pathways, in an infected cell, it is the virus that changes the host cell's machinery and uses it to replicate and secrete new competent viruses. Looking deeper into the mechanisms of niclosamide activity, specifically with respect to SARS-CoV-2 viral infection, as reported by Goulding [89] because viruses are obligate intracellular pathogens, they cannot replicate without utilizing the machinery and metabolism of a host cell. As shown in Fig. 7, there are six basic stages that are essential for viral replication: *1. Attachment, 2. Penetration, 3. Uncoating, 4. Replication, 5. Assembly*, and *6. Release* of virions.

Here, I will attempt to bring together all the literature reviewed above under "***5. Niclosamide in viral infection***" and place it in the context of the viral sequence for infection. Shown in Fig. 7 then, is my simple-minded schematic of the known viral stages of infection. It shows where niclosamide, when sprayed prophylactically or subsequent to viral infection (in the first 24–48 h) as a simple solution, could have its anti-viral effects on the infection-sequence as reported in the various above literature on SARS-CoV-2 or other viruses. Referring to Fig. 7 and taking the stages in order, niclosamide can inhibit at least three of these (possibly four).

5.3.1. Attachment

As shown in Fig. 7 ①, the virus spike protein on the virus surface binds to the specific ACE 2 receptors on the host cell membrane. As early as May 2020, Lee et al., [140] reported that the "*ACE2 receptor protein robustly localizes within the motile cilia of airway epithelial cells, which likely represents the initial or early subcellular site of SARS-CoV-2 viral entry during host respiratory transmission*". Being outside the cell, we do not expect niclosamide to necessarily affect this stage unless it can prophylactically affect the recycling and expresson of the ACE2 receptors at the plasma membrane surfaces (project for someone?).

5.3.2. Penetration

Following attachment to the ACE2 receptor, the virus penetrates the cell plasma and/or endosome membranes, Fig. 7 ②. It is here that an, already-present prophylactic niclosamide, could have its first preventative effect. The binding of the virus to the ACE2 receptor induces internalization [220] and can lead to fusion of the viral spike protein with the plasma membrane or proceeds to internalization via the formation of an endosome, as in Fig. 7 ②. The direct plasma membrane fusion requires furin or a transmembrane protease (TMPRSS2), that cleaves off portions of the S-protein, and needs to be already present on the host cell. As described in several papers and especially one by Kreutzberg et al., [132] "*SARS-CoV-2 requires acidic pH to infect cells*", and the SARS-Cov-2 virus can enter the cytosol directly through the plasma membrane. Their experiments showed that the S-protein cleavage needs an extracellular pH in the pH 6.2–6.8 range, which, (fortunately for the virus) is within the slightly acidic natural physiological pH of the nasopharynx where the overall range in pH is 5.17–8.13 for anterior pH and 5.20–8.00 for posterior pH [268]; (and the saliva is ~6.7 [13]). Other viral infections such as the pH-dependent ASLV, have shown that a modest elevation of extracellular pH, or a raised pH in early endosomes, delayed the acid-induced fusion of endocytosed avian sarcoma and leukosis virus ASLV [58]. Thus, our spray of niclosamide

solution that is at pH 8.3 (see below, ***8.3 The development of a simple niclosamide solution-based formulation for a nasal preventative and an early treatment throat spray***), could, in and of itself, also act to stop any virus from fusing with the plasma membrane as it raises the local nasal pH.

Otherwise, receptor internalization with the virus bound to it leads to endosome formation by the formation of a clathrin-coated pit. Other mitochondrial uncouplers actually inhibit the endocytic process altogether. The thinking was that since uncoupling in mitochondria reduces available ATP, and since, clathrin mediated endocytosis uses ATP, that this was seen to be the reason for this endosome inhibition, (which it may still contribute to). However, Dejonghe et al. [56] have shown "*mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification*". If this holds for niclosamide and, as a prophylactic spray, niclosamide has already acidified the cytoplasm of the host epithelial cell by transporting H^+ ions out of the lysozome, then this clathrin building process may also not occur and even endocytosis itself is inhibited.

Under normal circumstances, when the virus enters the cell via endosome-early lysosomes, upon normal endosome acidification, cathepsins cleave the S-protein and allows exit from the endosome directly to the cytosol. As described by Fehr and Perlman for coronaviruses [71] and confirmed for SARS-CoV-2 [106,243], the lower pH is required to expose a fusion peptide that inserts into the membrane and allows for the mixing of viral and cellular membranes, resulting in fusion and ultimately release of the viral genome into the cytoplasm. However, this is where the drug properties of niclosamide really come to the fore. To reiterate, as a lipophilic anion, niclosamide can partition into the lipid membrane, and can shunt protons out of the endosome, and so reverses the usual acidification of endosomes that the virus needs for the conformational change required by the spike protein to fuse with the endosome membrane. Niclosamide has therefore been shown to prevent viral fusion, and the entry of viral RNA in cells infected with influenza [115], the *Dengue virus*, [114] and this has now been shown to be the case for SARS-CoV-2 [221]. Interestingly this acidification of endocytic vesicles, itself, occurs by the action of by an ATP-dependent proton pump [282] Thus, because of niclosamide's proton shunt effects across the endosome membrane and also its reductions in available ATP, the pH of the endosome is not reduced, the "key" change in the virus surface proteins does not occur, the viral capsid is not allowed to fuse with membrane, and so does not escape from endosome, and the viral RNA does not penetrate into the cell.

Furthermore, as above regarding overall signaling outputs, endosomes are integral not only to pathogen entry, they are also involved in the constitutive or regulated internalization of membrane-bound receptors and their ligands [162], that therefore (as specualted above) could also be inhibited, thereby reducing ACE2 at the nasal epithelial surface.

Summary of niclosamide and endocytosis.

In summary for this important penetration-entry process then, the series of niclosamide's proton shunting effects that provide multiple anti-viral, and virostatic mechanisms include:

1. The nasal mucosa can have a natural acidic pH but can be between ~6–8.3, (rhinitis apparently generates the higher pH range). So, in the normal nose at pH 6, the pH is already low enough for the virus to bind to the ACE2 and a TMPRSS coreceptor and enter the cell without going through clathrin mediated endocytosis. Spraying a nasal spray buffered at pH 8.3 (as the niclosamide spray is buffered) could cause some inhibition to all that even without niclosamide. So, raising the pH in the nasal epithelium prevents viral fusion with the plasma membrane, no spike protein change, no fusion, no RNA in the cell, no infection.

2. Niclosamide causes the dissipation of the proton gradient in the already acidified lysozomes, and so releases hydrogen ions into the cytoplasm and acidifies the cytoplasm to ~pH 6 or less. At this pH many of the cell processes grind to a halt, including the formation of the clathrin-coated pits that form the endosome in the first place. That is, even though the virus can bind to the ACE2 receptors on the ciliated cells of the nasal epithelium, it is not invited in: so, no clathrin mediated endocytosis, no virus in the cell, no infection.

3. Niclosamide at only micromolar concentrations or less, can prevent endosome acidification by dissipating any proton pumping into the endosome, as well as reducing available ATP for the pump, and stop endocytosis of the virus all together; so, no spike protein change, no membrane fusion, no RNA in the cell, no infection.

Clearly, opportunities abound to establish new mechanisms of these cellular processes *per se* by using niclosamide as an investigative modulator and establishing new mechanisms of its use as a therapeutic. Again, some experiments we are currently conducting that could be expanded into full blown proposals include:

5.7 Proposed Idea: (Cell biology, endosomes): While many of these experiments and data have been done on robust "VeroX" cells etc., they have not been done on primary nasal and bronchial epithelia or ALI cultures or organoids. Test in primary hNE and hBE cells niclosamide for the following:
1. Endocytosis: Use a fluorescent polymer (polyIC) and evaluate if and to what extent niclosamide can stop endocytosis by inhibiting the uptake of the inert marker PolyIC into endosomes ± niclosamide over a range of concentrations from 10 nM to 10 μM.
2. Endosome acidification: Use a pH-sensitive dye like Lysotracker and determine inhibition of endosome acidification ± niclosamide over the same range of concentrations from 10 nM to 10 μM.
3. Cargo and pH co-localization in endosomes: Colocalize the PolyIC and lysotracker with a pH sensitive dye like Lysotracker and determine inhibition of uptake and endosome acidification ± niclosamide over the same range of concentrations from 10 nM to 10 μM.
4. Cytoplasmic acidification: Independently evaluate the acidification of the whole cytoplasm ± niclosamide over the same range of concentrations from 10 nM to 10 μM.

5.8 Proposed idea: (Cell biology, endosome, viruses): This series of four experiments above could then be done in nasal and bronchial epithelial cells in a BSL3 with live viruses and couple this to viral entry, infection, and replication by adding in niclosamide pre incubation, and at various times post incubation that match or predate the stages of that life cycle, in those all-important primary nasal and bronchial epithelial cells.

5.3.3. Uncoating

In the absence of niclosamide, and if the above processes are not prevented, the next stage is stage 3 (Fig. 7 ③) uncoating of the entered capsid. That is, once out of the endosome, the viral capsid is removed and degraded by viral enzymes or host enzymes releasing the viral genomic nucleic acid. While we have no evidence (yet) that niclosamide can influence this process it would appear that in the presence of niclosamide, that has already reduced the ATP production and content in the cell, if these enzymes need phosphorylation there could be an inhibitory role. It appears that, as reported by Moreira et al. [170], in an incredibly detailed paper including amazing figures of how they see the processes evolving, they state that, "*ubiquitination and ubiquitin-like*

modification is usurped by many viruses to establish infection, and IAV uses ubiquitin-enhanced viral uncoating mechanisms". Histone deacetylase 6 also seems to be involved in the uncoating process. These are active ATP-dependent reactions and so, could niclosamide's presence also slow or inhibit these processes too?

5.3.4. Replication

Normally, if uncoating is successful what we think of as the main stage of infection is replication of the virus, the transcription and translation of its RNA Fig. 7 ④. As a very clearly, "non-molecular biologist" I'm not even going to try and attempt to describe any of this, and just refer interested parties to this recent review, by Emrani et al., [62], "*SARS-COV-2, infection, transmission, transcription, translation, proteins, and treatment: A review*".

Briefly then, "replication" is called the *Eclipse period* and is the time between viral entry and appearance of intracellular virions. As reported by Harcourt et al., this can take ~24 h [95] and it requires ATP from the mitochondria. As mentioned earlier, by dissipating the pH gradient across the mitochondrial inner membrane [252], niclosamide inhibits oxidative phosphorylation and so reduces available energy for a virus to replicate in the host cell [44]. There is a reduction in cell viability, but not necessarily death for normal cells, as Kim at al have recently shown, [123]. Thus, niclosamide, at again micromolar and sub micromolar concentrations, effectively slows down the metabolism of the infected cell, and the extent to which is does this can be titrated with niclosamide dose. The consequences of even partially reducing ATP in a potentially infectable or, indeed, infected host cell, is that the virus cannot use the cell's own machinery to replicate itself [86,109]. The virus is put in lockdown!

Interestingly, viruses may need more ATP than the cell normally produces. For *vaccinia virus (VV)* in HeLa cells and *tomato bushy stunt virus* (TBSV) in plants, virus production actually requires increased amounts of ATP [35,172]. In VV, two mitochondrial genes for proteins that are part of the electron transport chain that generates ATP, ND4 and CO II, were up-regulated after VV infection. As a result, ATP production was increased in the host cells after VV infection by ~60%. And so, if this holds for coronavirus (and other viruses), niclosamide could provide an even greater inhibitory effect.

5.9 Proposed idea: (Cell biology, virus replication, ATP): Test the hypothesis that, like *vaccinia virus*, under normal infection conditions in nasal and bronchial epithelial cells, SARS-CoV-2, and other respiratory viruses, like influenza and RSV, need more ATP than is usually generated. This could be done by running metabolic assays using seahorse assays (https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-analyzers) in the absence and presence of virus and then in the presence of niclosamide. This will again require experiments to be done in a BSL3 for SARS-CoV-2 but could be done at lower containment levels for influenza.

Specifically for SARS-CoV-2, as mentioned above, niclosamide shows prophylactic inhibition of SARS-CoV-2 replication [109], such that, when niclosamide was already present in Vero 6 cells, SARS-CoV-2 could not replicate. Of 47 antiviral drug candidates tested against SARS-CoV-2 by Jeon et al. [109], niclosamide completely inhibited viral replication at an IC_{100} of 1uM in Vero 6, and 2 μM to 3 μM in Calu-3 [126]), while host cell viability (and not necessarily cell death) extended to >100uM. Interestingly, in the Vero 6 cells, the IC_{50} for niclosamide of 0.28 μM was 30 to 40 times more effective than chloroquine, lopinavir, and remdesivir, with IC_{50} values of 9.12 μM, 7.28 μM, and 11.41 μM respectively [109].

What is also interesting about this replication process, is that viruses co-opt the normally pH-neutral, endoplasmic reticulum. For example, in the *dengue virus* (*DENV*) experiments of Mazeaud et al. [159] they evaluated the biogenesis of virus Replication Organelles (vROs). As they report, "*In infected cells, the DENV induces extensive morphological alterations of the endoplasmic reticulum (ER) to generate viral replication organelles (vRO), which include convoluted membranes (CM) and vesicle packets (VP) hosting viral RNA replication*".[12]

Being a multi-membrane structure, for the ER, and its subsequent remodeling by virus infection, it is certain that niclosamide will also partition into these membranes and, if the vROs have a functional pH gradient, it would dissipate that too. DENV replication requires VCP – ATPase valosin-containing protein. It has been shown in Huh-7 cells by Jung et al., [114], that, "*Neutralization of Acidic Intracellular Vesicles by Niclosamide Inhibits Multiple Steps of the Dengue Virus Life Cycle In Vitro*". Thus, assuming this is a transmembrane protein that, as usual, requires a H^+ gradient to run the ATP synthesis, it would be interesting to see if indeed this is the case for SARS-CoV-2 and other respiratory viruses as they remodel the ER in, especially, nasal and bronchial epithelial cells and if the pH gradient is set up for the ATP-ase to operate.

Thus, the virus commandeers the normal endoplasmic reticulum and converts it into a virus factory vesicle, requiring ATP, maybe more ATP than the cell normally makes. If the concentration of niclosamide and cell exposure time is sufficient for niclosamide to reduce the excess cell energy that is available to the virus and this process, then the cell's own machinery cannot be used to transcript and transcribe RNA into new RNA and proteins, and it would appear that viral replication is 100% shut down (again, we put the virus in lockdown).

5.10 Proposed idea: (Cell biology, virus replication, vROs): Evaluate the kinetics of the formation and existence of these *viral replication organelles (vROs)*, upon infection with SARS-CoV-2 in nasal and bronchial epithelial cells. Check their evolving pH as a function of time without and with a range of concentrations of niclosamide. Does this correlate with inhibition of viral infection by plaque or PCR assay?

5.3.5. Assembly

In Fig. 7 ⑤, all the parts made in the adapted *viral replication organelles* are then assembled in the Golgi. This, normal process of 12 h or so, seems to be disrupted by niclosamide (at least in Dengue virus [114]). The Golgi does have a pH gradient, that is also up for dissipation, and so Niclosamide also inhibits the manufacture of viral protein assembly in the pH-dependent activity of the Golgi [114]. Here, the presence of niclosamide prevents E glycoprotein conformational changes on the flavivirus virion surface resulting in the release of non-infectious immature virus particles with un-cleaved pr-peptide from host cells. Interestingly then, the cell secretes, non-competent virions, (Fig. 7 ⑥), which may, hypothetically, act as their own "vaccine".[13] It is therefore interesting and curious to think that even if the virions are made, and a late spray of niclosamide interrupts this process in the Golgi, that these "duds" may represent an *in-situ* vaccine. This hypothesis is still to be tested.

[12] Looks like we have to learn a whole new set of acronyms: vRO –viral replication organelles; CMs – convoluted membranes; VCP – ATPase valosin-containing protein; VP – vesicle packets; NS4B –an integral membrane protein.

[13] As suggested by my good friend and colleague Gary Fujji at Molecular Express Inc. CA http://www.molecularexpress.com/.

5.11 Proposed idea: (cell biology, virus infectivity, vaccines): If niclosamide does inhibit making fully functional virions, could they indeed be their own *in situ* "vaccine". Adding in niclosamide only at the point where the viruses are being assembled in the Golgi, test that the secreted virions are, in fact, plaque forming. If not, utilize an in vivo vaccine model to see if the non-infecting virions could generate antibodies

5.3.6. Secretion

The assembled, complete, and functional virions are prepared for secretion out of the cell. Thus, the time between viral entry and appearance of extracellular virions, the so called *Latent period* is, in total, about 36 h [95] but could be longer up to 48 h [293]. This secretion process itself, Fig. 7 ⑥ , could also be an active, ATP dependent process and so inhibited by niclosamide. The extent to which secreted viruses then move within the retrograde shifting mucus and reinfect new epithelial cells is also an area for future study. As reported by Li and Tang, [146], and as we all know from having a cold or flu, "*Respiratory viruses invade human airways and often induce abnormal mucin overproduction and airway mucus secretion, leading to airway obstruction and disease*". This mucus then moves all these secreted viruses down through the back of the throat and into the lungs. What does niclosamide do to this function of the goblet cells that make the mucin and ciliated cells that move it? In Air Liquid Interface cultures, we have seen ciliated motion slowdown in the presence of micromolar concentrations niclosamide (Zach Kelleher, personal communication).

5.12 Proposed idea: (cell biology, virus infectivity, ALI cultures): In air liquid Interface cultures, cells are made to produce mucin. Even in the absence of virus, what concentration and how long does it take for niclosamide to reduce the normal mucin production, and does it slow down the ciliated motion that sweeps it retrograde? In the presence of virus, is this mucin production increased by the goblet cells, and again, what is the concentration of niclosamide and time dependence of inhibiting this process?

As is clear now from the above, it would be an interesting series of projects working with a viral life cycle in one or more cell lines and adding in niclosamide at various stages to not only delineate the kinetics of viral entry, replication, and assembly but also to show that niclosamide is an effective anti-viral if it does indeed target autophagy, as described next.

5.3.7. Other intracellular targets: Autophagy

As depicted in Fig. 7 ⑦, while it appears that this and other viruses shut down autophagy so they themselves do not get "self-eaten" by the cell, along with all the other accumulated detritus in the cell, niclosamide re-upregulates autophagy (the host cell's intracellular degradation system) and so reverses the inhibition of autophagy that is one of the viral survival mechanisms. Thus, associated with its ability to dissipate other pH gradients in the endosomes and lysozomes and acidify the cytoplasm, niclosamide is also an autophagy-inducing compound [86], which adds to its potential as a treatment against SARS-CoV-2. Niclosamide will clearly partition into the lipid bilayers of these double-membrane autophagous-vesicles. Autophagosomes are essential for, and the maintaining of, cellular health. They are (in my mind) the recycling plant of the cell for old protein aggregates and damaged organelles and such, transporting them to the lysozome for breakdown and reuse or secretion. They also entrap and deliver viruses to the lysosome for degradation. This is obviously important in viral infection because, as reviewed by Choi et al., [48],

"*autophagy controls viral infections at multiple levels by causing the destruction of viruses, regulating inflammatory responses, and promoting antigen presentation. Moreover, viruses manipulate autophagy for their immune evasion, replication, and release from infected cells*".

Yang and Shen therefore proposed that targeting the autophagy (and endocytosis) could lead to therapeutic strategies in COVID-19, using lysosomotropic agents such as Chloroquine and inhibitors for clathrin-mediated endocytosis such as chlorpromazine. We can add niclosamide to this list with a few papers appearing that have screened for and identified niclosamide as an anti-viral therapeutic [86,87,113]. Thus, while viruses have acquired the potent ability to hijack and subvert autophagy for their benefit, it seems that the presence of niclosamide re-upregulates this viral clearing process and so could actually be not just a virostatic but a virus destroying anti-viral. That is, we might say that many of the niclosamide-inhibitory systems are *virostatic*, i.e., when niclosamide is washed out, the pH gradients may return, and its business as usual. However, by re-upregulating the autophagosome system and actually destroying the virus in its own recycling vesicles, niclosamide could be truly *virucidal*.

5.13 Proposed idea: (Cell biology, autophagy): Any labs that are expert at imaging and following autophagosomes could investigate and confirm this viral-down-regulation and niclosamide's re-upregulation of autophagosomes in hNE and hBE cells and see if it correlates with assays for viral number and replication.

5.3.8. Other intercellular targets: syncytia

Finally, as depicted in Fig. 7 ⑧, there is evidence that niclosamide inhibits syncytia by TMEM16F ion channel inhibition in SARS-CoV-2 as well as RSV as reported and reviewed earlier, by Braga et al. [23] and Niyomdecha et al., [202]. One of the more devastating effects of several viruses including, the human immunodeficiency virus, SARS-CoV-2, herpes simplex virus and, of course, Respiratory Syncytia Virus (RSV) in the lungs is to cause lung cells to fuse together into what are called *Syncytia*. In RSV this syncytia formation causes extensive alveolar damage and a reduction in the number of lymphocytes, that normally help protect the body from infection. Syncytia formation during RSV infection was also found to facilitate virus spreading. Here, two recent papers from 2021 are worth reading, again, by Braga et al. and [23] "*Drugs that inhibit TMEM16 proteins block SARS-CoV-2 spike-induced syncytia*" and by Niyomdecha et al., [202], "*Repurposing of antiparasitic niclosamide to inhibit respiratory syncytial virus (RSV) replication*". The Braga study used two high-content microscopy-based screens with >3000 approved drugs in two drug libraries, (Prestwick Chemical Library and The Spectrum Collection, MicroSource (MS) Discovery) to search for inhibitors of spike-driven syncytia. Niclosamide was the top hit in the Prestwick and the second in the MS Discovery library. Narrowing the screento 83 drugs, they identified niclosamide as a drug that could protect cells against virus replication and associated cytopathicity and suppressed the activity of TMEM16F – a calcium-activated ion channel and scramblase, that is responsible for exposure of phosphatidylserine on the cell surface. Niclosamide was active at inhibiting viral infection with IC_{50} value of only 0.34 μM in Vero6 cells. Preincubation exposure of Calu-3 cells with 2.5 μM niclosamide for 2 h and then infected with SARS-CoV-2 for 1 h, reduced the viral titer for this short drug exposure time. Also,the cells that were infected in the presence of niclosamide were no longer syncytial. As they conclude, like many screens for specific pathways that niclosamide affects, "*These findings suggest a*

potential mechanism for COVID-19 disease pathogenesis and support the repurposing of niclosamide for therapy".

In the Niyomdecha et al., [202] study, in bronchial and epithelial cell lines, a 6 h pretreatment of human epithelial type 2 (HEp-2) and human bronchial epithelial cell lines gave the highest anti-RSV activity of 94% with an EC_{50} of 0.022 μM (i.e., only 22 nanomolar). A 48 h cytotoxicity assay by MTT gave a CC_{50} of 0.551 μM, and so the cell-based therapeutic index (TI) (CC_{50}/EC_{50}) was 25. Niclosamide efficiently blocked infection of laboratory strains and clinical isolates of both RSV-A and RSV-B in a bronchial epithelial cell line. Interestingly, in contrast to other studies, Niyomdecha et al. showed that RSV inhibition by niclosamide was mTORC1 independent. Their data indicated that, "*niclosamide hindered RSV infection via proapoptotic activity by a reduction of AKT prosurvival protein, activation of cleaved caspase-3 and PARP (poly ADP-ribose polymerase), and an early apoptosis induction*". And so here are more opportunities to further tie down these mechanisms. Also, another area for future study is establishing the real value of a 48 h cytotoxicity assay (as opposed to an actual cell death assay like Lactate DeHydrogenase (LDH)) and whether cells can actually recover once the niclosamide is taken off, i.e., are cells just metabolically compromised (asleep) but the niclosamide exposure is not lethal?

A 48 h cytotoxicity assay is actually somewhat irrelevant for dosing a solution of niclosamide intranasally, where, as discussed below (8.5.1 Nasal spray, niclosamide's presence in the mucosa, and initial virus infection), niclosamide may pass through the nasal epithelium in a matter of minutes. Having said that, a 20 μM niclosamide solution concentration may not actually decay down to below therapeutic levels (1 μM) in several hrs. Thus, experiments here would evaluate dose and time dependent assays carried out on the time scale of an anticipated treatment modality.

5.14 Proposed idea: (Cell biology, cell viability vs cell lethality): While a 48 h cytoxicity assay is maybe considered standard, and traditional, it is actually not consistent with the 6 h exposure used for the syncytia assay. The question then becomes, "is niclosamide compromising cells, in a metabolic sense, at even shorter times where it is active in inhibiting syncytia?" And "at what level of concentration and over what time period of exposure does niclosamide become lethal and not just reduce viability?" Also, over what period of exposure and concentration of niclosamide can cells actually recover once the niclosamide is taken off – were they just "asleep" and not dead, so challenging the notion of "cell viability" using MTT or Cell titer glo assays, as a measure of incapacitated cells.

Bottom Line: Collectively, all these above literature studies support the potential application of niclosamide as a preventative and early treatment nasal spray, (and even nubilized lung solution), with both *virostatic* as well as anti-viral, *virucidal*, mechanisms against many viral infections. These could include the SARS-COV-2, and could extend to its more contagious variants [32], and also the perennial influenza and many others [281]. They highlight a series of unique mechanisms of action of the drug, i.e., niclosamide can partition into lipid bilayer membranes as a lipophilic anion. Thus, all of niclosamide's multiple different pathways in cancer [209] and now infected host cells [86,109,126,279,281] are based on *lipophilic proton shunt activity* that dissipate pH gradients in endosomes and lysozomes, the mitochondrial inner membrane, viral replication organelles, and in the Golgi, autophagosomes and syncytia. Importantly, these actions inhibit viral entry, viral replication, and even the mis-assembly of non-infective virions. They also clear intracellular viruses and inhibit multinucleated formations in the lungs. Niclosamide has therefore emerged as a very interesting and potentially successful ubiquitous anti-viral that could be used as a preventative and early treatment option in conjunction with all other vaccines and treatments of COVID19 and other viral infections.

As above, there is therefore a huge opportunity now to repeat all of these studies and more in the more relevant human respiratory nasal, bronchial, and lung epithelial cells that are the main site for cell entry replication, assembly, and secretion of new virions. If possible, these could be extended to fresh patient derived cells. This niclosamide solution is just waiting to be tested in preclinical animal studies for all respiratory viral infections. There is likely to be no systemic toxicity because the doses, that are administered locally, are in micrograms. And so, if successful, a collective effort (when the formulation is fully optimized as a human nasal spray, see below), could move this preventative and early treatment on to human testing and be readily available to a suffering world, perhaps at cost or reduced reinvested profit.

PART II: Nanomedicines? A Carrier-Free Nanomedicine for Cancer and a Simple Solution to Prevent COVID19 and Other Respiratory Infections: They Just Need Testing.

6. Nanomedicines

As we all know, the headline that precipitated Kinam's "*The beginning of the end for nanomedicine*" [212] was the article in Science, May 2019 [239], "*National Cancer Institute Will Stop Funding Nanotechnology Centers*." Their reasons were that this shift marked nanotechnology's 'natural transition' from an emerging field requiring dedicated support *to a more mature enterprise* able to compete head-to-head with other types of cancer research. OK, money is tight, and we want to move on. But, have "nanomedicines" really reached maturity? As Kinam posits, "*It is inconceivable to expect that such pancreatic cancer and 100 other forms of cancers can be cured by simply placing anticancer agents in nanoparticles*". I would agree, and would suggest, that, as shown in my examples below, "*it depends on the nanoparticle, the drug (or prodrug), the delivery route, and the disease*". "Horses for courses", as they say – would you put a carthorse in a steeple chase?

In this Part II, I will try to give some ideas of how we can, as Kinam suggests, "*examine the sources of difficulty in clinical translation and move forward*". And, as in the title of this Part II "*They Just Need Testing*" is again my invitation to collaborate. Again, I will also try and give some indications as to new studies where this could happen and/or where you can write your own proposals to address various aspects of niclosamide in cancer, especially from a formulations point of view.

6.1. Nanomedicines: but is it really the end?

Given that many medicines for cancer are not optimally effective, and this whole field of "nanomedicines" arose to meet this challenge, why do nanomedicines for cancer fail so miserably?[14] According to the review by Wilhelm et al. [275] "… *after surveying the literature from the past 10 years, only 0.7% (median) of the administered nanoparticle dose is found to be delivered to a solid tumour*". Invariably, they are talking about i.v. injections or infusions of a nanomedicine-scaffolded

[14] Apart from the difficulties in crossing the "valley of death" from academia to corporations via university licensing and ventures (see again Needham, D. (2016). Bringing Research to Clinical Application: Lessons from Thermodox - A Thermal Sensitive Liposome for Treatment of Cancer. Drug Delivery and Targeting: Fundamentals, Applications and Future Directions. A. HILLERY, S. J and K. PARK. Boca Raton, CRC Press**:** 523–583, Needham, D. (2020a). Development of clinically effective formulations for anticancer applications: why it is so difficult? Biomaterials for Cancer Therapeutics, Evolution and Innovation. K. Park. Cambridge, UK, Woodhead Publishing.), there is also the study site, study coordinator/investigator, and the effects on participating patients, as reviewed by Fogel in, Fogel, D. B. [74]. "*Factors associated with clinical trials that fail and opportunities for improving the likelihood of success: A review*." Contemp Clin Trials Commun **11**: 156–164.

drug-laden nanoparticle. And so here, it's all about, can the nanomedicine be injected or infused i.v., survive long enough in the blood stream, is small enough to access the perivascular space of the tumor, and can carry and deliver enough effective dose of drug, in relation to its volume-occupying inert scaffold, to be effective?

I am actually not that concerned about the low delivery percentage compared to the initially delivered dose if the effective dose is actually delivered, and so potency of the drug is paramount here. And it's not always about drug-laden particles that have to get to the tumor. There is perhaps a different role for nanomedicines to play, as depots of prodrug that release parent drug, depending now on toxicity of the chosen drug that, as free drug and its half-life, but when released from a longer-circulating depot could provide greater efficacy.

Here then, we have started to investigate a new prodrug approach, making a carrier-free pure prodrug-cored "nanomedicine" as described in more detail later (*7.5 "Make the drug look like the cancer's food" –they all have to eat*). The hypothesis here is that, like the LDLs that cancer's feed on, our highly insoluble pure prodrug-cored nanoparticles are expected to extravasate at least into the tumor-interstitiums perivascular space. And so, we are also likely to be limited by that low tumor-accumulation percentage. However, one of the interesting mechanisms that we have discovered by using pure prodrug particles of Niclosamide Stearate (Niclosamide Stearate Prodrug Therapeutic (NSPT)), in mice [225] and recent canine studies (unpublished data) is that they could act as a depot that releases the drug by enzymolysis of the prodrug in circulation. Thus, if a drug (like niclosamide) is active with an IC_{50} of 1 μM and, by i.v. injection, we can achieve 300 μM of the niclosamide stearate prodrug therapeutic in plasma, with a half-life of 5–7 h, that releases ~100 μM niclosamide with a similar circulation half-life, then that 100 μM of the drug (probably bound to albumin which is also the cancer's food) bathes the tumor, and we might have efficacy at 100 times the IC_{50}.[15]

As discussed later, in any event, and all events, key determinants are the choice of drug, the choice of delivery system (which could be the drug particle itself, as Tonglei Li et al. have also shown [81,82,100,152,289,292]), and the extent of tumor-exposure. Perhaps these are not necessarily achieved by a drug-encapsulating-scaffolded-particle-nanomedicine, that is too big to extravasate into the tumor interstitium and might not efficiently release its drug.

As I wrote in Kinam's edited book [213], in my chapter [180], in the section 22.10 "*What is nanomedicine? And why?*", I tried to understand where it all went wrong. I started by looking up other people's definition of what they thought nanomedicine was. According to Nature portfolio, (https://www.nature.com/subjects/nanomedicine) its,"*a branch of medicine that applies the knowledge and tools of nanotechnology to the prevention and treatment of disease*". At NIH and their 2005 "Nanomedicine Initiative" [196] with a national network of eight Nanomedicine Development Centers, they said their goals were, "*(1) understand how the biological machinery inside living cells is built and operates at the nanoscale. (2) Use this information to re-engineer these structures, develop new technologies that could be applied to treating diseases, and/or leverage the new knowledge to focus work directly on translational studies to treat a disease or repair damaged tissue.*"

So, they were really looking for researchers to develop and test a series of new micro- and nano-based techniques and approaches, diagnostic and otherwise, to examine the nano-scale at which actual medicines act and perhaps quantify the fundamental parameters in cellular- and molecular-mechanisms.[16] Thus, according to the NIH original definition it was not just all about "*nanoparticles*" for medicine, or what has now become synonymous as the much-maligned term, "*nanomedicines*".

In that chapter in Kinam's edited book [180], I also asked the question (I have yet to receive an answer), "*So why is everybody making 'nanoparticles' containing, encapsulating, or loading drugs in various and myriad LPDMC*[17]*-matrix materials in the first place? Is it because we can? Or is it because we have to?*"

6.1.1. Clinical trials for "nanoX"

To begin to answer this question, I would encourage us to start by reading what Volkmar Wessing et al. had to say in their 2014 and 2015 two-paper series "*Nanopharmaceuticals (part 1): products on the market*" [272] and "*Nanopharmaceuticals (part 2): products in the pipeline*" [271]. It is quite telling that, in 2015, Wessing et al. report that,

"*… after an extensive search for information through clinical trials, we found only two clinical trials with materials that show unique nano-based properties, i.e., properties that are displayed neither on the atomic nor on the bulk material level*".

So, I looked some things up on the clincaltrials.gov web site.

> ***As an Aside:*** You should try it if you have a few days to spare. No, seriously especially for the young and up-and-coming researchers who want to make a difference. Track some of these stories yourselves and see how it all unfolds from the initial grants on NIH's grants web sites, to published manuscripts, to successful (or not) clinical trials, and even what happens after that when it is launched onto the public, including adoption, perhaps new or enhanced toxicities, and the all-important net profits.

From my search, at clinicaltrials.gov, on Tuesday, February 28th, 2023, there were ***95,296*** studies found for *cancer*. Of these, it seems that people are still considering liposomes, with ***1681*** studies (1.76%) found for *liposome | cancer*. So, there is still some significant activity for the quintessential liposome nanomedicine that started it all. And now, it looks like liposomes can deliver RNA and successfully make a vaccine, which really is a new step forward given all the anti-cancer failures, (ask my good friend Pieter Cullis, — *UBC's Pieter Cullis is gaining wider recognition for the discovery of a 'delivery system' used in mRNA technology* [14]). There were ***352*** studies for *nanoparticle | cancer*, including some for therapy and some for diagnostics. There were only ***5*** studies found for: *polymeric nanoparticles | cancer*, and it seems people are avoiding using the term "*nanomedicine*", as there were only 3 studies for *nanomedicine | cancer*.[18] Here is what I found with some personal comments for perspective.

1. Nab-paclitaxel (Abraxane) for breast cancer

The first one was on Nab-paclitaxel (Abraxane) for breast cancer, that was looking for data on Nab-paclitaxel-derived grade III neuropathy in 2013, now published [49]. Their introductory claim was that,

"Nanomedicines are currently being developed in the treatment of cancer due to their pharmacological advantages over traditional formulations; they provide a shorter infusion time and lower risks of hypersensitivity reactions associated with commonly used solvents"…..

Despite that seemingly wild claim "pharmacological advantages over traditional formulations", the results showed that, regardless of the dose, "*nab-paclitaxel did not differ from (solvent-based) sb-*

[15] I know C_{max} isn't everything, exposure time and AUC will come into play, hence the emphasis on long circulation.

[16] I actually attended that meeting with a proposal on cell mechanochemistry.

[17] *LPDMC* –Liposome, Polymer, Dendrimer, Micelle, Chitosan…. and Solid Lipid Nanoparticles.. etc.

[18] https://clinicaltrials.gov/ct2/results?cond=Cancer&term=nanomedicine+&cntry=&state=&city=&dist=.

paclitaxel in terms of neurotoxicity as evaluated with the Total Neurotoxicity Score (TNS)" So, here is a billion-dollar nanomedicine formulation that in efficacy and toxicity is not much better than Taxol, —the original emulsion formulation (also a $1Bn drug) where the cremophor is itself toxic. (See also my "Abraxane discussion" in the Kinam-book chapter [180]).

2. Hypersensitivity reactions to Doxil

As for reduced hypersensitivity reactions, the second one was a 2022 study that was trying to evaluate biomarkers for the hypersensitivity reactions fourteen patients had on Doxil, following a recently published paper, [294]. What? So, these are post-approval studies (maybe 27 years later) where there are still safety issues for Doxil in advanced breast cancer and other solid tumor therapy? I thought approval meant it was safe. Doxil has only 57% disease control rate and induces severe hypersensitivity infusion reaction (HSR) in up to 25% of patients, leading to allergic shock even presyncope[19] or threat to life. So, they were looking for biomarkers and mechanism of Doxil (also called Pegylated Liposomal Doxorubicin, —PLD) -induced HSR in advanced breast cancer. So here, although approved for systemic therapy in 1995 [69], clinical research is still trying to evaluate Doxil's toxicities.

And despite all this effort and hype around liposomes per se, Dou et al. [61] concluded in their review of thermal sensitive liposomes, that:

"*A recent meta-analysis found no significant difference in clinical anticancer efficacy between liposomal and conventional chemotherapeutics in terms of objective response rate, overall survival (OS), and progression-free survival (PFS)*". The only thing a traditional liposomal drug has achieved is improvement in the toxicity profile of conventional chemotherapeutic agents leading to better patient compliance and quality of life. Reduced toxicity is not better efficacy.

As an Aside: While its long-term circulation is >24 h, —an amazing achievement for any nanomedicine, it eventually generates systemic toxicity in addition to the usual doxorubicin chemo events, in the hands and feet where it extravasates in the apparently compromised vascular beds in these well used body parts. Regarding its less-than-optimal therapy, Doxorubicin was so well encapsulated in this very strong and tight hydrogenated soy lecithin-cholesterol liposome (we measured the tensile strength and elastic modulus of these membranes [193], and they are on the level of polyethylene) that it was not sufficiently bioavailable. That's probably why the Doxil nanomedicine reduced the solution infusion toxicity of doxorubicin (e.g., cardiac), it couldn't get out. But again, reduced toxicity does not necessarily lead to increased efficacy.
And what is worse, if any of it was taken up intracellularly, the intact Doxil is endocytosed and ends up as most such materials in the lysozome. As shown by the ten Hagan lab, [240], the lysozomal enzymes degraded the phospholipids and then released the doxorubicin, but (because of it being a weak base cation with a pKa of 8.3), it could not go back up the pH gradient of 4.5 to 7.4 …. "*the released doxorubicin was still sequestered in the lysosome*". Doxil was actually approved on reduced toxicity, not increased efficacy. So here is a good example of a choice of nanomedicine scaffold that solved one problem, reduced toxicity, but compromised another, bioavailability, and its ultimate goal of efficacy. This whole "nanomedicine" thing is not easy!

3. Image-guided targeted doxorubicin delivery

The third nanomedicine trial is in the Netherlands at UMC Utrecht. It's an ongoing, recruiting trial started in 2018, entitled, *Image-guided Targeted Doxorubicin Delivery With Hyperthermia to Optimize Loco-regional Control in Breast Cancer (i-GO)*. And guess what? They are studying how best to use my invention! [174,175] the Low Temperature-Sensitive Liposomal-Doxorubicin (abbreviated here as LTLD). The brief summary, that I might as well quote, is:

"In this phase I feasibility study, the investigators evaluate the combination of lyso-thermosensitive liposomal doxorubicin (LTLD, ThermoDox) with local hyperthermia and cyclophosphamide (C), for the local treatment of the primary breast tumour in patients with metastatic breast cancer".

When heated to 40–43 degrees Celsius (°C), LTLD releases a very high concentration of doxorubicin locally within seconds. Hyperthermia of the primary tumor *will be induced by Magnetic Resonance guided High Intensity Focused Ultrasound (MR-HIFU) on a dedicated Sonalleve MR-HIFU breast system.*

The investigators hypothesize that by substituting doxorubicin (A) in the AC-chemotherapy regimen for the combination of LTLD and MR-HIFU induced hyperthermia, optimal local tumor *control can be achieved without compromising systemic toxicity or efficacy. This will be the first study to evaluate LTLD with MR-HIFU hyperthermia in breast cancer patients.*

(NOTE: Additional searches for "nano" brought up trials for: nanocomposites (0); inorganic nanoparticles (4); DNA nanostructures (0); polymeric nanoparticles antibiotics (3); polymeric nanoparticles cancer (5); and carbon nanotubes (8), see ***Appendix A9 "Nano" in clinical trials***)

As an Aside: And also, while on the subject of clinical trials, and how difficult it is to move from bench to bedside, I was alerted to a very interesting group, —The Global Coalition for Adaptive Research (GCAR) https://www.gcaresearch.org/ see *Appendix A10. 3* Financial Toxicity for what others are doing to help alleviate costs at least the clinical testing phase.

6.2. Nanomedicines: or is it just a bump in the road?

So, as above, and as you could see yourselves by searching for "nanomedicine" on PubMed, (43,957 results) or on Google (About 40,400,000 results in 0.45 s), there are definitely some "nanoX" successes, but an awful lot of failures. And by "failures" I mean that, as Kinam rightly points out [212], "*they have all produced numerous research articles, all ending with the same lofty conclusion that nanomedicine has great potential*", yet few have made any impact on patient survival. How do we solve the underlying reason for this? i.e., beyond the very involved and expensive process of drug development and testing that the majority of us really can't do, even if we had the time and the funds necessary. I think there is a potential solution: more effective and focused preformulation drug characterization, before putting the drug in some "nanomedicine scaffold".

First, let's just focus on the "nanomedicines" themselves and how they come about. As Wessing says, "*The term "nano" became tantamount to "cutting-edge" and was quickly embraced by the pharmaceutical science community. Colloidal drug delivery systems reemerged as nanodrug delivery systems; colloidal gold became a suspension of nano gold particles*".

I take this to mean, we already had a good idea what was going on at the colloid and surface level, but then, "Colloid and Surface Science" per se [254][20] appears to have largely dropped out of the curriculum

[19] Presyncope: the feeling that you are about to faint. Someone with pre-syncope may be lightheaded (dizzy) or nauseated, have a visual "grey out" or trouble hearing, have palpitations, or feel weak or suddenly sweaty. https://www.hopkinsmedicine.org/health/conditions-and-diseases/syncope-fainting.

[20] The history of Surface and colloid science goes back to at least Sir Eric Rideal's founding of the Colloid and Surface Chemistry Group (CSCG) in 1958, and before that with the Society of Chemical Industry, (SCI), that have now merged to become the Joint Colloids Group (JCG)in 2002.

Fig. 8. Photographic image of the author at his solvent exchange apparatus making nanoparticles, taken by a camera placed on top of the DLS, just six feet away.

(especially in the USA) and students, and especially pharmaceutical students, are being trained less and less in such fundamentals. Instead, I agree with Wessing and feel that we have *embraced nanoX as "cutting-edge"*, without really understanding what constituted a "cutting edge blade".

As an aside: I have been in the Dynamic Light Scattering (DLS) instrument room in a Pharmacy School and got the chance to have conversations with some of the graduate students as they brought their samples in for size and zeta potential measurements. It sems that the fundamentals really have been lost, hence my attempts in recent papers to reintroduce such ideas. One issue is that in many institutions, the DLS instrument room is often across the building or across the campus from everybody's labs where they make their nanoparticles. Why this is important is that they can't possibly do any kinetic studies, and they may even think it's not important.

As shown in Fig. 8, in my lab, my DLS is within 6 ft of my solvent-exchange nanoprecipitation-making apparatus and so I can get kinetic data in particle size as soon as I can get a sample into the DLS and the instrument can make a measurement (usually within 30s). As a result I have discovered some critical kinetic phenomena.

I realize that DLS instruments are expensive and not everybody could have one in their lab, but still, ruling out some of the most important effects associated with kinetics of nucleation, primary condensation, Ostwald ripening, Smoluchowski collisional aggregation, and colloid stability, puts any formulation work at a disadvantage, scientifically. Maybe institutions could have a nanoparticle making station at least next door.

As my ex-advisor, friend, and colleague Evan Evans used to say, "*There are only two things you need to know in any scientific experiment (or theory): where is it going? How long does it take to get there?*" i.e., equilibrium thermodynamics and kinetics. And so, by administratively placing instruments "conveniently" in shared facilities, institutions may well have eliminated the possibility of any student or post doc doing the kinetics of their study , (unless the kinetics are on a longer time scale than their walking-pace to the DLS room).

6.2.1. The disconnect between scaffold-technology and the drug

Apart from this lack of measurement proximity, that ignores important fundamental kinetic data and so also stifles invention and innovation, I think one major underlying issue with many nanomedicines and their papers in the literature is the disconnect between the scaffold technology, which in many cases is well worked out and could certainly have an impact, and the drug, that is often somewhat randomly chosen to prove the scaffold's worth.

Do correct me if I am wrong, but, it seems that, in general, nanomedicine labs are mostly focused on their own scaffold technology than a particular drug for a particular effective administration route for a particular disease condition. For proof of principle, a prototypical drug is usually chosen that could potentially treat a prototypical disease or condition, without truly analyzing what the disease really needs and what the properties of the drug really are, and whether a scaffold for that prototypical application is even needed in the first place.

As we can all appreciate, if the drug is better understood from a preformulation drug characterization standpoint, and decisions can be made as to if a scaffold is need at all, and if it is, the drug is better matched to a particular nanomedicine scaffold, and the nanomedicine is well matched to the pharmaco-kinetics *in vivo*, the disease cell target, and mechanism of action, and the drug is ultimately bioavailable, … *then* …. we could be closer to more clinically effective formulations.

For example, see again *Appendix A2. We actually did cure cancer with one of these highly toxic chemo drugs*, the "nanomedicine", the Low Temperature Sensitive Liposome containing Doxorubicin (LTSL-Dox). Given the, at least preclinical [130,155,189,191,218,219,283] and some clinical success of Thermodox [22,96], I would suggest that when administered under the most optimal conditions (—warm the whole tumor first to 41 °C -42 °C, administer Thermodox, maintain whole tumor temperature at 41 °C - 42 °C for 30 min to 1 h, kill tumor), that this is a good example of a more appropriately designed and more clinically effective

nanomedicine utilizing a most effective heat-triggered drug release scaffold (permeabilized lipid bilayer) at the exact tumor site [178,179].

Bottom Line: So, that's my brief take on nanomedicines: Rather than develop a certain kind of hammer (nanomedicine scaffold) and go looking for a nail (drug, disease, and administration route) I would encourage all drug delivery nanomedicine researchers and developers to first identify a particular drug and a disease to be treated. Then, carry out an exhaustive preformulation drug characterization for that particular drug (that may well represent a whole class of drugs but really, they are all different). Then establish, does the drug itself make a useful nanoparticle (microcrystal or even just solution) that would solve the drug delivery problem? If not let's then choose a scaffold that really suits the drug and the application. Liposomes were very effective at loading and encapsulating doxorubicin, but the drug didn't get out fast enough or in enough quantities to be optimally efficacious. However, by creating a Low-Temperature-Sensitive Liposome that could respond to clinical hyperthermia, drug was released in the blood stream of the tumor in 2 seconds and (when finally tested for approval) could be much more efficacious.

Let me finish this tome by giving two new examples where we have used niclosamide in two different formulations – one as a prodrug "nanomedicine" to treat cancer and the other , a solution-suspension "micromedicine" to potentially prevent SARS-Cov-2 and other respiratory infections. Here, I am putting my money where my mouth is. By evaluating the properties and behavior of the drug and prodrug first, it turned out that we could make clinically effective formulations that were "carrier free" and "scaffold-free". Briefly, then, here are those stories.

7. The Niclosamide stearate prodrug therapeutic (NSPT) for Cancer (osteosarcoma)

Inspired by the fact that cancers feed on LDLs and VLDLs, for the past few years we have been evaluating a new prodrug formulation of niclosamide as a Niclosamide Stearate Prodrug Therapeutic (NSPT). The why, how, and what did it do are the subject of this section where we first evaluated the concept in a mouse model of metastatic osteosarcoma, and then in a feasibility clinical trial in canines with Osteosarcoma.

7.1. Osteosarcoma: Nothing has changed in over 40 years

Focusing now on Osteosarcoma and our own reformulations of niclosamide as a prodrug therapeutic, let's start with a review of how Osteosarcoma is treated today, including the options that the, mostly, children and adolescent patients are faced with. As researchers, with our heads buried in our lab work, we do not often look up to see what is really going on in the clinical efforts we might be trying to impact with drug formulation. Here's one example that hopefully is worth stating, informative, and motivating.

7.1.1. Osteosarcoma, a painful bone cancer affecting mostly children and adolescents

Osteosarcoma (primary malignant bone tumor) is a painful bone cancer that sadly, and predominantly, affects young adults and children [78]. While rare, (0.2 to 3 new cases/million per year in Europe [256]), it remains a lethal diagnosis for some due to pulmonary metastasis and there are few options for the patients and their metastatic disease. Of the total cases, as reported by Marko et al. [156], incidence of metastasis at osteosarcoma diagnosis when averaged as global pooled data, is 18% (95% CI: 15%, 20%). However, depending on the Human Developmental Index (HDI): it is 15% for a country with a very high HDI; 20% for high HDI; and 31% for medium/low HDI. Apart from there being a "*biological baseline for metastatic OS at diagnosis*", their conclusions suggest that bringing these numbers down is in the hands of the system:

"*In countries with medium / low HDI, where there are more barriers to accessing healthcare, the higher prevalence of metastasis may result from treatment delay or an artificial prevalence inflation due to patients with less severe symptoms not presenting to clinic. Additional research in countries with medium / low HDI may reveal that earlier detection and treatment could improve patient outcomes in those countries*".

After my Mum had her radical mastectomy in 1972 that took away her breast and all the lymph nodes up under her arm, I thought that was bad enough, but even successful treatments of OS are so debilitating and disfiguring and chemo is sickening. Sadly, "*there have been no significant improvements in clinical outcomes for osteosarcoma in the last four decades*" [85]. And that's how papers, including our own [225], usually start. So, let's try and fix it, sooner rather than later!

Again, while we, in the labs, rarely interface with the realities of treatments, I thought I would at least spell out this debilitating disease and the limited options and their consequences for the mainly adolescent suffers. I thought this short section was worth including rather than leave it out or again hide it in an Appendix. I wanted to include this, since scientific reporting, and as above, is usually an emotionless description of the statistics and options and Kaplan Meier "survival" curves. But research is driven by emotions, a need that grabs you, that makes you get up every day to find the answer using your developed scholarly skills. If you are interested in a career in cancer drug delivery, the following might just help to motivate it.

7.1.2. Imagine that you have osteosarcoma, what are they able to do about it?

Adapted and summarized from cancer.org [27], these are the situations a young child or adolescent patient can find themselves in and the current options for treatment including the toxicity it can cause in their bodies.

As bone cancer, this means it can occur in all parts of the kid's body.

7.1.2.1. Surgery. *Tumors in the Arms and Legs* are treated with surgery:

- Limb-salvage (limb-sparing) surgery: removing the cancer and some surrounding normal tissue but leaving the limb basically intact; or
- Amputation: removing the cancer and all or part of an arm or leg [78].

However, OS can occur in other bones such as:

Tumors in the pelvic (hip) bones, that can often be hard to remove completely with surgery.

Tumors in the lower jawbone, where the entire lower half of the jaw may be removed and later replaced with bones from other parts of the body.

Tumors in the spine or the skull, where it may not be possible to remove all of the tumor safely. Cancers in these bones may require a combination of treatments such as chemotherapy, surgery, and radiation.

Tumors in the Joints, where surgery may not succeed in reconstructing the joint, and so the bones will be fused together, (e.g., in the spine, shoulder, or hip) and results in significant loss of motion.

As mentioned, Osteosarcoma most often spreads metastatically to the lungs (15% to 31% depending on where you live) but also to the kidneys, liver, or brain. Patients who have tumors in both lungs and respond well to chemotherapy can have surgery on one or both lungs at a time. Removing tumors from both lungs at the same time may be another option. However, as you might expect, some lung metastases may not be able to be removed because they are too big, too disseminated, or are too close to important structures in the chest (such as large blood vessels).

7.1.2.2. Chemotherapy. By the time they come in for surgery, they could already have micro-metastases in their lungs. And so a treatment plan [27] starts with ~10 weeks neoadjuvant chemotherapy that could maybe, shrink the bone tumor, and reduce surgical invasion. After

surgery, patients will suffer adjuvant chemotherapy, given for up to a year along with suffering all the toxic side effects. These systemic therapy regimens typically utilize Methotrexate, Adriamycin, and a Platinum agent (MAP), and, of course, they have significant toxicity [7]. Of the patients who survive osteosarcoma (via neoadjuvant chemo, surgery, adjuvant chemo), their life expectancy and quality of life is often reduced due to the toxicity from systemic therapy. Despite its toxicities, the MAP regimen remains the preferred option for osteosarcoma chemotherapy [286] including neutropenia, thrombocytopenia, febrile neutropenia, cardiac toxicity, anemia, hypophosphatemia, mucositis, and infection. For patients with distant metastatic disease, the outlook is even more grim: only 26% of patients survive beyond 5 years after first diagnosis. One of the markers for more aggressive disease was given in a 2103 paper by Zhu et al., they found that, SOX9[21] is upregulated in aggressive osteosarcoma tissues indicating that SOX9 may participate in the osteosarcoma progression.

Other data in adolescents though by Gao et al., [83] does show significant limb salvage. While the median overall survival time was 8.6 years and cumulative three-year and five-year survival rates which were 64.6% and 52.6%, respectively, out of the 94 patients studied, "*81 cases spared the limb (86.2%), 9 cases underwent amputation (9.6%), and 4 cases were nonoperational (4.2%). Of the 94 cases, bone metastasis occurred in only 3 cases (3.2%), whereas lung metastasis occurred in 14 cases (14.9%)*".

However, for the 69 patients who were <18 yrs. old, 28 (40.6%) died; encouragingly, the numbers were better for the 25 kids ≥18 yrs. old, where only 9 (36.0%) died. I encourage you to go to this and other papers in the literature and also check out ***Appendix, A10. Kaplan Meier and more*** and do that simple spread sheet for a US-SS Actuary compounded calculation and plot that % survival for an adolescent of 18 yrs. Then overlay it on Gao et al.'s, [83] Fig. 1 Kaplan-Meier analysis of the cumulative three-year and five-year survival rates. Out to 15 years from 18 yrs. old, the US-SS probability of surviving 1 yr. is 99.0% for healthy males and 99.2% for healthy females, and so a compounded 15 yr survival is 97.7% for healthy males and 99.0% for healthy females. For both, this represents a normal life expectancy and the chance of another 58.97 yrs. and 63.89 of productive years of study, career, and family. But not if they have Osteosarcoma.

And, again, it is not like the chemotherapy doesn't have serious side effects. As we all know, the general side effects of chemo include: Nausea and vomiting, loss of appetite, diarrhea, hair loss, and mouth sores. But, chemo can also damage the bone marrow, where new blood cells are made leading to low red and white blood cell and platelet counts, which in turn, can result in an increased chance of infection; bruising after minor cuts or injuries; fatigue or shortness of breath, and, "*Some chemo drugs can affect your (child's) ability to have children (fertility) later in life*", requiring options such as sperm banking or egg preservation.

Even worse, the high doses of the particular chemotherapeutics that children are expected to be able to survive include, again, summarized from cancer.org [27]:

- *Ifosfamide* and *cyclophosphamide* can damage the lining of their bladder, and so give them blood in the urine.
- *Cisplatin* and *carboplatin* can cause them nerve damage (leading to numbness, tingling, or pain in the hands and feet); affect their hearing, (high-pitched sounds); and damage their Kidneys.
- *Etoposide* can also cause nerve damage and in rare cases can even increase their risk of later developing acute myeloid leukemia (AML).
- *High-dose methotrexate* can damage the white matter in their brain as well as affecting their liver and kidneys.
- *Doxorubicin (Adriamycin)*, perhaps worst of all, infused doxorubicin solution can damage their heart muscle in a dose dependent way such that there is a maximum lifetime dose of systemic doxorubicin of 550 mg/m^2 (for 21-day cycles), and in people already at high risk of cardiotoxicity, the maximum lifetime cumulative dose of doxorubicin should not exceed 400 mg/m^2.

Thus, given the extensive toxicity of chemotherapy agents, there is an urgent, and currently unmet clinical need to identify new therapeutics with lower systemic-toxicity and greater efficacy to treat OS with better outcomes and less treatment-derived morbidity.

As an Aside: I did want to bring to your attention something that struck me the other day. That is, the way we traditionally represent clinical data as % survival versus time after starting patients on the experimental drug –the common Kaplan Meier (KM) plot, that shows the drug compared to placebo or other drug data. As mentioned above and presented in the ***Appendix, A10. Kaplan Meier and more***, the two cancers I chose were prostate and triple negative breast cancers to exemplify this notion. It struck me that we never include what the % survival is for the general population i.e., for people who do not have the cancer. or are cured So, what would this look like? … I went to the US-SS Actuary table for life-expectancy and the chances of surviving 1 additional year for each age of life and compounded the calculation to get, as an example, 5 years survival for a 70-year-old man who does not have prostate cancer or a 50-year-old woman who does not have triple negative breast cancer. This simple exercise shows us where just where we are in terms of the success or failures of treatments and how far we still have to go to achieve cures. It also turns out that niclosamide can treat these two cancers and enhance the efficacy of current drugs (Ren, [267]). Thus, a lot of what I researched and wrote in this ***A10 Appendix*** section is clinical trial data asking where is the room for improvement?
I think you will find the presentation of data and analysis in ***A10 Appendix*** interesting, with short sections on: How far could we still go in Prostate Cancer?; Niclosamide has activity in Prostate Cancer; How far could we still go in Breast Cancer?; Niclosamide has activity in TNBC; and Financial Toxicity. There is also a personal story about a person I know, who was diagnosed with lung cancer 9 months prior and is now faced with hospice care. She recently wrote a paragraph to me, where she encapsulated the problems with today's cancer treatment options, their toxicities, and their failures. As you might expect, all the Kaplan Meier (KM) plots, treatment options, and toxicities seen here for Prostate and Breast Cancer could be listed for all cancers. It doesn't get any easier.

7.1.2.3. Inter- and intra- tumoral genomic heterogeneity. Like all tumors it seems, developing new therapeutics against OS is complicated by substantial inter- and intra- tumoral genomic heterogeneity and a complex micro-environment [116]. Somatic mutations can be found in several genes that are differentially expressed in tumors that responded poorly to the MAP-neoadjuvant chemotherapy [21]. These include genes (% expression in parentheses where known) that encode: the bone differentiation regulator RUNX2, (55%); the cell cycle regulator, CDC5L; the TP53 transcriptional inactivator, MDM2, (90%); the DNA helicase, RECQL4; the cyclin-dependent kinase gene, CDK4; retinoblastoma 1, RB1 (40%); the tumor suppressor, PTEN, (44%); vascular endothelial

[21] SOX9 is a developmental transcription factor, Sex determining region Y (SRY)-related high mobility group (HMG)-box 9 (SOX9) plays a vital role in the regulation of sex determination, cartilage development, intestinal differentiation, and adult progenitor cell pool maintenance. (As mentioned, later niclosamide is also active in inhibiting the NOTCH signaling pathway including SOX9 Esmail, M. M., N. M. Saeed, H. E. Michel, et al. [64]. "The ameliorative effect of niclosamide on bile duct ligation induced liver fibrosis via suppression of NOTCH and Wnt pathways." Toxicology Letters **347**: 23–35).

growth factor A, (VEGFA, 60%), and bone development pathways involving the Wnt/ß-catenin pathway that ultimately control key developmental gene expressions, the cell-fate receptor NOTCH1, and the Hedgehog (Hh) signaling pathway can also be dysregulated [116].

7.2. Niclosamide in osteosarcoma (OS)

As we might expect by now, niclosamide also inhibits multiple pathways that promote survival and growth that are known to be dysregulated in OS, including, again, the Wnt/ß-catenin, Akt/mTOR/PI3K, JAK/STAT, NOTCH, and NF-κB pathways (Chen, [9,265], Li, [3,144,250]). Niclosamide also inhibits cell cycle progression [147,148], and one of its main mechanisms of cell kill seems to be induction of apoptosis [147,246,284]. And, in a recent paper by Esmail et al. [64], the effects of niclosamide were expanded in an *in vivo* model of cholestatic liver fibrosis (CLF) where they include the usual pathways and also mention SOX9. As they report: "*Niclosamide (5 and 10 mg/kg) significantly reduced liver enzymes levels, oxidative stress, inflammation and phosphorylated signal transducer and activator of transcription3 (p-STAT3). Niclosamide (5 and 10 mg/kg) also significantly reduced NOTCH pathway (Jagged1, NOTCH2, NOTCH3, HES1, SOX9), Wnt pathway (Wnt5B, and Wnt10A), and fibrosis (transforming growth factor-beta1 (TGF-β1), alpha smooth muscle actin (α-SMA) and collagen deposition with more prominent effect of the higher dose 10 mg/kg. So, this study presents niclosamide as a promising antifibrotic agent in CLF through inhibition of NOTCH and Wnt pathways*".

We have focused on Osteosarcoma (OS), as our first cancer target, mainly because Dr. Will Eward, Surgical Oncologist at Duke is involved in OS treatment, and was the only person who initially believed in our formulations. He recruited two medical students (David Kerr and Giresh Reddy) onto the studies and they did extremely well, obtaining some very impressive initial results in mice [120,121,225] that motivated a subsequent feasibility study in 10 canine patients led by Steve Suter at NC State Veterinary College [65]. Thus, here again, as a stand-alone drug, niclosamide inhibits multiple pathways that otherwise promote survival and growth that are known to be dysregulated in OS. But, as usual, there are formulation issues with repurposing the oral tablets and so a more clinically-effective formulation had to be found.

7.2.1. Niclosamide (as oral tablets) suffers from formulation and administration-route issues

What if we wanted to do a clinical trial with niclosamide, we would naturally think of reappropriation as the lowest hanging fruit and start with the oral tablets. Unfortunately, when taken orally as Yomesan tablets, the 2 g of Niclosamide is so poorly absorbed it is essentially non-toxic and so not efficacious; LD_{50}s are in the 1 g/kg range, i.e., a 70 kg person would have to take 70 g of Yomesan (140 × 500 mg tablets) and would still only have a 50/50 chance of getting extremely bad diarrhea.

Niclosamide, as Bayer's Yomesan, has actually already been trialed in cancer and showed little to no efficacy at large oral dosing. Since its oral formulation is so clinically "safe", it is perhaps not surprising that, in a 2018 human prostate-cancer clinical trial [237], the short-lived plasma concentrations of niclosamide that were achieved at the maximum tolerated oral-dosing (500 mg given three-times-daily for four weeks) were only in the range, 35.7–82 ng/mL (0.1–0.25 μM). These values were only ~0.014% of the ingested dose,[22] —and you thought i.v. nanoparticle dosing was poor. They were so low as to be below the therapeutic threshold of 0.5 μM *in vitro* for colony formation, as measured for LNCaP prostate cancer cells [149]. Clinically, there were no PSA declines in any enrolled subject and the Data Safety Monitoring Board closed the study for futility.

Thus, although a drug with significant potential, new approaches have been necessary to repurpose and reformulate the active niclosamide drug molecule for a range of cancers, including Osteosarcoma. An effective mechanism of delivery for this drug (and similar agents) would mark an important step in providing new treatment options for patients. So, how would we reformulate niclosamide? Rhetorically, does nature itself give us any clues for how to deliver relatively insoluble molecules to tumors? The answer is "Yes".

7.3. LDLs are the cancer's food!

Lipoprotein particles (High-, Low-, Very Low-, and Intermediate-density and Chylomicrons) are a family of normal blood constituent nanoparticles serving as a means of delivering extremely insoluble molecules —triglycerides, cholesterol, and fatty acids, (water solubilities, S_w ~ nano to pico molar), to tissues in the body. As shown for breast cancer cells they need substantial amounts of cholesterol, triglycerides and lipids, obtained chiefly from LDLs, to establish new membranes [57], as they switch from that energy-based metabolism to that growing, spreading, metastasizing, anabolic metabolism [54].

As above, *(7.1.1.3)*, from genomic analyses all cancers have an extensive degree of inter- and intra-patient heterogeneity [224]; they have so many biological features that it makes it difficult to target just one aspect that will be sufficiently therapeutic. Thus, we asked, can we find a common ubiquitous feature that circumvents cancer heterogeneity [248] and that could enhance drug-uptake for more effective treatments? They all have to eat!

For growth, aggressive phenotype transformation [287], and metastatic spread, cancers feed on Low-Density (LDL) and Very Low Density (VLDL) lipoproteins from the patient's blood stream [73,151,158]. Lipoprotein uptake promotes proliferation and invasion in breast and other cancers [34,57,229,230]; and an abundance of LDL-Receptors is a prognostic indicator of metastatic potential [79].

Concerning brain cancer, as is well known, Glioblastoma (GBM) is the most lethal malignant tumor in the central nervous system, with a median survival of only 14 months. Here, again, niclosamide has activity [45,274]. In a collaboration with Ruman Rahman at the CBTRC we just published a new paper [2] suggesting, "*the LDLR pathway as a ubiquitous metabolic vulnerability in high grade gliomas across all ages, amenable to future consideration of LDL-mediated nanoparticle/drug delivery to potentially circumvent tumour heterogeneity*". Thus, our own analysis tested the hypothesis that brain tumors over expressed LDLs. It had already been observed that brain cancer cell lines like SF-539, U-87 MG, and U-343 MG (from the tissue bank of the Brain Tumor Research Center UCSF) were particularly rich in the LDL receptor-related protein (LRP) [153]. In fact, the LDL receptor (LDLR) is highly expressed in the BBB and glioma cells, whereas normal brain tissues and neurons have relatively low LDLR levels. In a recent review Pawar et al, [215] discussed the LDLR and the types of NPs that have been used to target the brain via this receptor. What's more, brain tumors orchestrate vascular niches that maintain the cancer stem cells pool and the cancer cells in the perivascular niche [26] were actually the most aggressive, and so could be accessible to nanoparticles from the blood stream if the blood brain barrier (BBB) was compromised in brain tumors or if, as seems to be the case, LDLR-targeted nanoparticles could be taken up by caveolae-dependent transcytosis [168,291].

In our new histology study [2], we wanted to see for ourselves across a range of biopsy samples from intra- and inter tumor regions of high-grade gliomas (HGG) in 36 adult and 133 paediatric patients to confirm LDLR as a therapeutic target. We found that widespread LDLR expression in adult and paediatric cohorts, localized preferentially within perivascular niches, but with significant intra-tumor variation observed between the core and either rim or invasive regions of adult HGG. As an

[22] 1500 mg ingested and 2.5 L of blood plasma is equivalent to 600 mg/L; 82 ng/mL = 82μg/L; and so, the % of the ingested dose in plasma was (82μg/L)/600 mg/L)x100% = 0.014%.

LDLR pathway across all ages, this now motivates new drug delivery efforts to try and target these cells using both nanoparticles in the blood stream and also intra-tumoral deposition right after surgical removal that potentially circumvents tumor heterogeneity. So, we have a new study to devise, and carry out.

7.1 Proposed Idea (Clinical Brain Cancer): While this could be done in an animal model, they are notoriously unreliable when it comes to representing the blood brain barrier in humans and especially for implanted human tumors with mouse blood vessels. They may be OK for drug validation but (in my experience) not necessarily for proving nanoparticle accumulation and actual drug delivery per se. So, could the clinicians get together and devise a clinical trial to see if injected LDLs did accumulate in brain cancer patients? Maybe in real time, or after surgical removal of the tumor tissue by immunohistochemistry? It is apparently done for plaque identification, as in the paper by Luliano et al., [107] where, autologous native [^{125}I]-labeled LDL or [^{125}I]-labeled human serum albumin were injected 24 to 72 h before endarterectomy, showing that they circulated long enough to rapidly accumulate in human atherosclerotic plaque. If this is done in brain cancer patients we would know what size nanoparticle they could be permeable to, or not, or taken up by receptor mediated transcytosis and so motivate a LDLR-ligand peptide targeted prodrug nanoparticle, as I proposed some years ago in an (unpublished, available on request) white paper [176], and has recently been shown to be feasible by newly-graduated George Bebawy at the University of Nottingham, School of Pharmacy, [19].

Thus, what is required to be explored, for all cancers, is a drug delivery system that can provide both tumor accumulation as well as perhaps long-systemic circulation to expose circulating cancer cells. As mentioned earlier and discovered in PK analysis when we tested this kind of prodrug nanoparticle, they may also act as a depot and release drug from the prodrug nanoparticles [225].

7.4. "Make the drug look like the cancer's food": They all have to eat

As above then, one common feature of all cancers is "cellular nutrition", — *they all have to eat.* The strategy for our particular nanomedicine approach therefore is to *"make the drug look like the cancer's food"*. The challenge was to provide a long-circulating particle of pure drug or, in this case a prodrug, of the same basic diameter (20 nm – 50 nm) so that, by definition, it could passively extravasate into the tumor interstitium like the LDLs and VLDLs can presumably do. As subsequent experimental data would show, this pure-prodrug nanoparticle might also act as a long circulating drug-releasing depot. That is, while direct tumor uptake was the initial motivation, PK data in mice [225], and now confirmed in canines Manuscripts in preparation), (see later Fig. 11A) suggests that such long-circulating prodrug particles are ~25% enzymolysed in the blood stream. Why this is important is because the prodrug nanoparticle concept could provide a niclosamide drug depot at safe and efficacious levels that releases niclosamide, and that would bind to albumin, that is also the cancer's food [103]. Curiously, low plasma albumin levels in patients have been associated with an increased risk of cancer [164] perhaps indicating that tumors are removing albumin from a patient's plasma.

Thus, we had to choose the right drugs and/or prodrugs that would first make the nanoparticles, and so this involved a bit of physical, colloid, and surface chemistry and what it takes for a compound dissolved in a water-miscible organic solvent to be precipitated into an aqueous antisolvent, a technique we explored for making the LDL-sized pure drug nanoparticles [190].

7.4.1. Our prodrug "Bricks to Rocks" technology (B2RT)

Since their "food" comes in the form of LDL and VLDL particles [73,151,158] that are ~20 nm–80 nm in diameter, by definition, they are expected to passively extravasate into the tumor interstitium and then bind to the LDL receptors on tumor cells via their own Apolipoprotein ligand. Our scientific strategy then was to make prodrug nanoparticles that matched the LDL/VLDL in terms of size but not necessarily LDLR-targeted, just passively-accumulated in the tumor interstitium and passivley taken up by cancer cells. Initially, even though niclosamide was a notoriously low solubility drug, we found that it was actually not insoluble enough to make stable nanoparticles of pure material. We therefore followed nature's designs (cholesterol being made into and transported as cholesteryl oleate) and had the idea to convert the low solubility drugs, commonly referred to as 'bricks", to even less water-soluble prodrugs ("rocks"). Hence our *"Bricks to Rocks Technology"* (B2RT). Thus, the reason we make the drug less water soluble is because lower (nanomolar to picomolar) solubility allows us to make similarly sized (25 nm – 50 nm) precipitated nanoparticle as pure-prodrug nucleates, stabilized by a protective lipid monolayer [190] suitable for i.v. injection.

Following initial experiments on triolein (a triglyceride, the main component in olive oil[23]) the first prodrug formulation we made was from niclosamide stearate. Basically we esterified niclosamide with stearic acid, as described in the patent [43] to make niclosamide stearate that reduced the solubility of this, now, prodrug acyl-ester into the pico molar range, and enabled the right-sized and stable nanoprecipitation. The idea then is for the prodrug nanoparticle formulation to be eventually enzymolysed *in vivo* to release the parent drug and so increase drug delivery of niclosamide.

The traditional pharmaceutical approach is to make low-solubility drugs more water soluble for oral -tablet -administration and absorption. However, by modifying the compounds to be even less water soluble, as shown schematically in Fig. 9, we make the drug almost insoluble (0.16 picomolar by chemaxon with a LogP of 10.6), so that our solvent exchange technique [190] can nucleate a pure, prodrug core. Thus, our first example is the Niclosamide Stearate Prodrug Therapeutic (NSPT).

This pure prodrug core is simultaneously coated by stabilizing surfactants or lipids that can be coprecipitated to coat the core and stabilize the nanoparticles against aggregation and degradation [99]. Such "carrier-free" nanoparticles allow for delivery of the highest amounts of prodrug per particle.

Simple geometric calculations, as represented schematically in Fig. 9, show that, at these very small diameters, of 30 nm total, a 16.8 nm prodrug core, and a 22.8 nm lipid coated core, that the prodrug makes up 37% of the volume and the lipid monolayer is 67% of the main particle (excluding the PEG). Given this surface-to-volume ratio scaling, if larger particles like 45 nm diameter can still extravasate, then the numbers become, a 37.8 nm main particle, and a 31.8 niclosamide stearate prodrug core with again a 3 nm lipid monolayer, giving 59.5% for the prodrug core and 41.5% for the lipid monolayer.

Thus, here is a carrier-free nanomedicine formulation, no polymer, no chitosan, no mesoporous silica etc., no scaffold, where the highly insoluble prodrug itself makes the particle. The only other component is the lipid monolayer for steric and chemical stability.

[23] As a way to teach this technique we can make 20 nm nanoparticles with just olive oil dissolved in ethanol and solvent-exchanged into water, Walke, P. [263]. Physico-Chemical Parameters of Nanoparticles that Govern Prodrug Design and Application in Anticancer Nanomedicine. PhD, Unnversity of Southern Denmark (SDU), Bebawy, G. [19]. Novel orlistat LDL-like nanoparticles as potential anti-cancer medicine, PhD Thesis. PhD, Nottingham University.

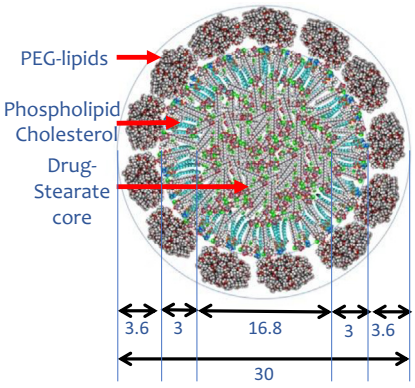


Fig. 9. Typical Drug-Stearate Prodrug Therapeutic nanoparticle (dimensions are in nm), e.g., Niclosamide Stearate Prodrug Therapeutic (NSPT).

Now, imagine what the drug-volume % might be for a drug in a scaffolded particle like a polymer or solid lipid nanoparticle that is also stabilized by a lipid or surfactant. Or, as some researchers have proposed and done, tried to load drugs like paclitaxel into an actual LDL particles [34,57,77,97,122,136,137,138,197–199,244]. The problem is that, at these 30 nm – 40 nm scales, the scaffolding material takes up a large fraction of the volume reducing the "payload". What is worse, for loading the LDL itself, as estimated by Teerlink et al., [251], it is already full of 1500 molecules of solid phase cholesteryl ester, 115 molecules of cholesterol and 200 molecules of left over triglyceride, and so there is no room for the drug, if the drug will even dissolve-partition into this semi-solid core.

In Fig. 9, the core is schematically represented in an amorphous state. By definition, when a nucleate forms, it is likely in an amorphous solid, even liquid state (depending on its melting point, Niclosamide Stearate is ~85 °C) but can soon solidify into a crystalline structure. All this also needs to be further investigated should anyone like to collaborate on this and other drugs, prodrugs, and compounds (see below, ***7.2 Proposed Idea***.)

In terms of innovation we already have an issued patent on the esterification concept applied to niclosamide (Chen, Mook et al. 2016), and unique compositions of matter for stabilizing formulations in the prodrug nanoparticle platform. We are ready to work with any other research groups on preclinical testing with, for example, *in vitro* Patient Derived Organoids (PDOs) and *in vivo* Patient Derived Xenografts (PDXs) as here for colorectal cancer, (PDX screening technology [280].

Thus, these pure-prodrug formulations provide nanoparticles for difficult-to-formulate, low water solubility compounds. What is required is a drug with either a hydroxyl or acid group that can be esterified by a long chain, (e.g., stearoyl) fatty acid or alcohol, respectively, by standard esterification chemistry. Such drugs are fairly common. There are at least 50 drugs from the 119 FDA-approved oncology drug library used by Hsu [5] in high-throughput drug screens of patient's colorectal tumors that are amenable to the esterification chemistry. As an example of the kinds of proposals that this technology can generate for already approved drugs that need reformulation here is a Specific Aim we wrote in a recent NIH R21 proposal. In addition to niclosamide we suggested five approved cancer drugs that are suitable for forming the prodrug therapeutic nanoparticles.

7.2 Proposed Idea: (medicinal chemistry, physical pharmaceutics): Acylate six prototypical parent drugs to prodrug stearate esters (Capecitabine, Doxorubicin, Gemcitabine, Methotrexate, Paclitaxel and SN38) suitable for forming the prodrug therapeutic nanoparticles. That is, convert this series of poorly soluble drugs ("bricks") into even less soluble prodrugs ("rocks") for the express purpose of making LDL-sized nanoparticles consistent with classic nucleation theory [190].
Each prodrug will then be formed into pure prodrug nanoparticles by the rapid solvent exchange technique and characterized, as done previously, and stabilized by lipids or surfactants [190,264]. This would include: Particle size immediately after making and over time (hrs-days and after Lyophilization) measured by Dynamic Light Scattering and optical and electron microscopies; Chemical stability against hydrolysis in buffer and enzymolysis in mouse and human plasma using UV/Vis and HPLC-LCMS; Mpts and Fpts associated with the esterified prodrug by Differential Scanning Calorimetry (DSC). Initial tests of lyophilization and reconstitution will lay the groundwork for manufacturing and storage-transport.

7.3 Proposed Idea (physical pharmaceutics): There could be other drugs that do not need to be esterified because they are already sufficiently insoluble to make the required sized nanoparticles. For example, here we already tried Cysmethynil [266,277] and also Orlistat [18,117] and they form beautiful pure drug-cored nanoparticles, the latter has activity in inhibiting Fatty Acid Synthesis in cancer cells [19,117,119,133].

7.5. Cell studies with NSPTs show viability IC_{50}s of ~ 1uM and complete induction of apoptosis at 20 μM

In the study by Reddy et al., [225], NSPTs inhibited cell viability, proliferation, and the amount of intracellular ATP present in human and canine osteosarcoma cells *in vitro*. All four canine and four human osteosarcoma patient derived cell lines showed growth inhibition IC_{50} values in a does dependent manner. While all cells were slightly more sensitive to niclosamide from DMSO (IC_{50}: 0.57 μM), when compared with NSPTs (IC_{50}: 1.22 μM), on average, there were no statistically significant differences between inhibition of human and canine osteosarcoma cells by niclosamide and NSPTs.

NSPTs also showed a marked dose-dependent inhibition in cell proliferation in both cell lines above 5 μM. Importantly, as cell proliferation was inhibited (at 4.9 μM), with increasing NSPT addition, the level of measured apoptosis rose. The EC_{50}s for apoptosis in human 43B and canine D418 lines were ~10 μM Niclosamide Stearate, and complete Apoptosis occurred at ~20 μM. (The mean across all 8 cell lines for the EC_{50} was 20.9 μM). Thus, as with many earlier studies, now shown in both human and canine patient-derived OS cells, niclosamide (as NSPTs) not only reduces cell ATP and reduces cell proliferation, it also induces cell kill by apoptosis.

7.6. Preclinical and (canine) clinical studies

The most convincing data we have so far for the B2RT is making and testing our Niclosamide Stearate Prodrug Therapeutic (NSPT) in an *in vivo* mouse model of Osteosarcoma [225]. Here the NSPTs prevented lung metastasis in the mice, and also in a Canine Feasibility Study [65] made at Duke (Spasojević, DCI PK/PD core), that enabled survivors.

7.6.1. Osteosarcoma mouse model

As reported by Reddy et al. [225] and shown in Fig. 10, intravenous administration of 50 mg/kg NSPTs as 45 ± 5 nm *Z*-average diameter

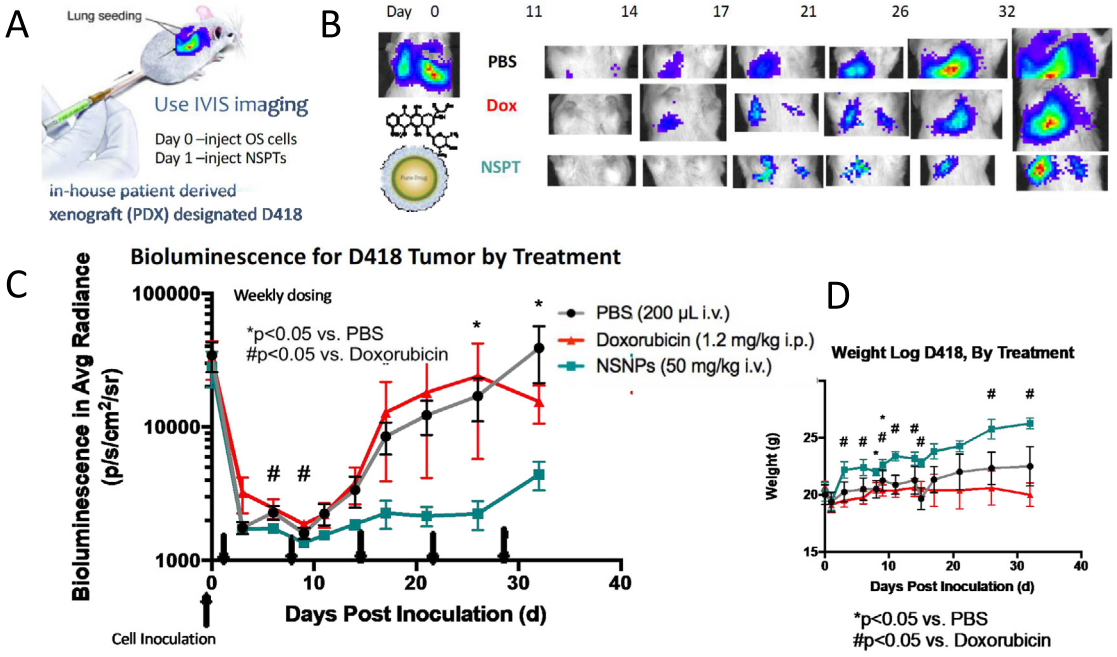


Fig. 10. Testing NSPTs in a Lung metastases model of Osteosarcoma. ***A)*** the "1 day chase" experiment where NSPTs 50 mg/kg were injected 1 day after luminescent zs green OS cells were allowed to colonize the lung. ***B)*** ZS green cells are imaged by IVIS imaging and then weekly for 4 weeks showing lung tumors for PBS, Dox i.p. and NSPTs i.v. ***C)*** Lung Bioluminescence for PBS control, Doxorubicin i.p. and NSPTs i.v. vs days post inoculation of ZS green cells, for weekly dosing of NSPT. ***D)*** mouse weight by treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nanoparticles, with only weekly dosing, actually prevented metastatic disease from occurring for a 4-week study.

Tail vein injection (Fig. 10 A) of zs-green OS cells inoculated on Day 0 were imaged by an In Vivo IMaging System (IVIS). This was followed on Day 1, (hence, called the "Day-1 chase" experiment) by tail vein injection of 50mgs/kg NSPTs (i.e., ~1 mg/mouse). The bioluminescence recorded in the IVIS Spectrum images in Fig. 10 B were converted to a calibrated bioluminescence in Fig. 10 C and plotted versus time (in Days) after initial inoculation of the zs green cells.

The data shows an expected initial drop off in bioluminescence from an initial 30,000 units of radiance (p/s/cm^2/sr)[24] to ~2000 units over the first few days as cells were lost from circulation. PBS controls showed a colonization of the lung starting at day 9–10, and so did i.p. doxorubicin, with bioluminescent lung tumor growth increasing to high levels over the next 25 days reaching levels comparable to the original ~30,000 bioluminescence units.

In contrast, for the NSPT cohort dosed on Days 1, 8, 15, 22 and 28, metastatic lung colonization remained at the low ~2000-unit level and was prevented out to 4 weeks, when the study was stopped. As also shown in Fig. 10 D, whereas mice on PBS and Dox showed constant or slightly reduced weight loss, mice on NSPTs actually gained weight while the mets were prevented from growing. The MTD was in fact not reached, even at 50 mg/kg.

7.4 Proposed idea (preclinical metastatic cancer models: In the mouse study we only did the "Day 1 chase" experiment, administering the initial dose of NSPTs 1 day after inoculation with the zs green cancer cells that rapidly lodge the lung and eventually within a ~ 9 days colonized to give new metastases. Could we allow say 15 days for these new metastases to get established and then deliver the NSPT dosing? Would a "Day-15 chase" experiment show that NSPTs act on the established tumors and not just on what might have been circulating and/or lodged cancer cells? Can we effectively treat established lung metastases?

7.6.2. Canine feasibility study

Based on these promising preclinical mouse experiments the NSPTs were then tested in a canine feasibility standard-of-care (SoC) study at NC State Veterinary College[65] (manuscripts in preparation). Briefly, 10 canine patients were enrolled with histologically confirmed OS and clear thoracic radiographs and the affected limb was subsequently amputated. Patients were given SoC carboxyplatin, @300 mg/m^2 q 3wks (days 1, 21, 42 and 63) for a total of four doses. Then, starting 1 week after carboxyplatin, 4 doses of NSPT were given i.v. weekly over 30–60 min @ 10 mg/kg, (Days 70, 77, 84, 91). The PK for Niclosamide Stearate (as NSPTs) for 4 canine patients showed that,at the end of the 1 h infusion , the blood concentrations were 227.2 NS and 70.6 µM Nic, giving a total equivalent niclosamide of almost 300 µM, They all decayed in an exponential fashion with an average terminal half-life = 4.57 h for NS and 3.23 h for Nic. The average Area Under the Curve (AUC) = 211.6 h × µg/mL for NS and AUC = 27.5 h × µg/mL for Nic which was well above the 1.27 µM cell-effective concentrations for OS cells in culture. The half-life for a bolus of free Nic is usually only 30 min [169].

It therefore appears that some niclosamide (~25% of the NSPTs) is released by enzymolysis from the long-circulating depot in the blood stream. This is supported by kinetics of *in vitro* enzymolysis in mice and dog plasma), which could represent an intersting new drug delivery mechanism of drug release from a long-ciculating prodrug depot. As a result, a significant concentration of niclosamide is now maintained at a circulation concentration that is well above the in vitro cell IC_{50} of only 1.27 µM.

In terms of efficacy while four patients failed on, or just after, SoC carboxyplatin, 6 patients, that did receive NSPTs, survived past the

[24] p/s/cm^2/sr, or photons/s/cm^2/sr is the number of photons per second that leave a square centimetre of tissue and radiate into a solid angle of one steradian (sr).

Disease-Free Interval of 257 days and five survived past the normal 321 Days. As of 10/24/2022 three patents (43%) were still cancer free after 1404 days ±81 days, i.e., ~ 4 years after starting the study.

Thus, it was this enhanced PK for the NSPTs passively targeting the metastases, and the seeming release of parent niclosamide from this long-circulating depot that may have been responsible for the observed cures. This technology is now ready for more advanced clinical trials in canines, if not in humans, starting with dose escalation Phase I and Phase II efficacy.

7.5 Proposed Idea (preclinical metastatic cancer models in canines): In the canine study we only treated 7 dogs with the NSPTs. This is now ready for a full-blown canine Phase I/II clinical study to establish a dose escalation MTD, and then efficacy studies at that MTD with all the outcomes tracked. We might start by adopting "Adaptive clinical trial design". As discussed by van Norman [260], in adaptive clinical trial design we *"learn from accumulating data in the trial and apply what is learned as quickly as possible in a prospectively specified way during the trial itself to hone flexible aspects of the study while it is still ongoing"*. Benefits include: reduced costs of phase II testing, earlier determination of futility, prediction of phase III success, reducing overall phase II and III trial sizes, and shortening overall drug development time. It would appear that this is the basis for a whole series of clinical trials in canine patients, if not in humans.

There is also an idea from ***Appendix 10.1.1*** regarding enhancing prostate cancer treatments that use enzalutamide with niclosamide.

7.6 Proposed idea (preclinical and clinical pharmacology in prostate cancer): Test the conclusions by Liu, using our i.v.-injectable NSPTs that, *"niclosamide is a promising inhibitor of androgen receptor variant to treat, either alone or in combination with current anti-androgen therapies, advanced prostate cancer patients, especially those resistant to enzalutamide"*. One of the main issues seems to be that inhibition of the androgen receptor (AR) by second-generation anti-androgens for metastatic castration resistant prostate cancer (mCRPC) inevitably leads to the development of resistance. Niclosamide targets the cell membranes so there is less, or even no, expectation of resistance. Could this eventually be a basis for a clinical trial?

Similarly, from *Appendix 10.2.1* for breast cancer, a series of studies show that niclosamide can have positive effects alone and in combination with other drugs including cisplatin and radiation, (see A10.2.1 Niclosamide has activity in TNBC).

7.7 Proposed Idea (preclinical and clinical pharmacology in breast cancer): Given the above positive activity in TNBC, conduct in vivo preclinical studies in a TNBC mouse model to test our NSPTs without and with cisplatin. If positive results are obtained with NSPT alone, then we could eventually introduce a treatment that uses less toxic chemotherapy and a more-gentle, yet still active, niclosamide on the cancer. Testing along with other Standard-of-Care drugs, as suggested by Ren et al., (Ren, [267]), would also be interesting and prudent, since, in general, *"drug resistance and recurrence are the main challenges of cancer treatment, but combination therapy for different pathways may be able to meet these challenges"*.

Appendix A10.3. Financial Toxicity also has some brief comments on this very important toxicity of today's, especially, anti-cancer medications. It also has a personal story of a friend of a friend who got diagnosed with cancer a few months ago. In "***An Aside***" paragraph, she encapsulated the problems with today's cancer treatment options, their toxicities, and their failures. Surely, we can do better than this.

8. Formulation of Niclosamide for COVID19 and other respiratory infections

This last section is on how we have developed niclosamide as the basis for a potential preventative nasal spray and early treatment throat spray for COVID19 and other respiratory infections [185,187]. Others in this area have taken to using their patented cyclodextrin [247] and thin film freezing technologies [24] to create more complex formulations. In contrast, our preformulation drug characterization [185] revealed a much simpler, less toxic, and potentially more effective approach [183,188]. That is, by understanding its solubility-pK_a-pH-polymorph relationships coupled to its IC_{100} for inhibition of infection in various cell lines, we created a simple niclosamide solution, perhaps supplemented with additional undissolved but dissolvable niclosamide-microparticle depot.

8.1. Motivation to create a nasal and throat spray comes from etiology of SARS-COV-2

For SARS-CoV-2, the initial route of transmission appears to be via air as aerosolized virus. While early infection targets the nasal-mucosa, advanced infection traverse to the lungs and can also go systemic, as well as some apparently in the brain. Long-COVID can ensue where, in 2022, "*more than 40% of adults in the United States reported having COVID-19 in the past, and nearly one in five of those (19%) are currently still having symptoms of "long COVID*" [33]. Given its presence in the nose and close to the nasal bulb, I also suspect this proximity may be promoting infection in the brain [249] and so a nasal spray that stops the viral infection in the nose or can safely follow it to the brain, could be a real boon here.

A new study by Hou et al. [105] showed that, for the angiotensin-converting enzyme 2 (ACE2) receptor the virus binds to, the highest expression is in the nose with decreasing expression throughout the lower respiratory tract. Thus, nasal surfaces appear to be the dominant initial site for SARS-CoV-2 respiratory tract infection. It is in this context that an effective nasal and throat spray, especially prophylactically, and also in early stages of the disease (for throat and bronchi), would be crucial in preventing or reducing initial viral load in the nasal and buccal-bronchial epithelia. In fact, data from Wölfel et al. [278] revealed that viral load is highest in pharyngeal secretions early in the course of infection. As time goes on, the viral load in these secretions goes down and viral load in lower respiratory tract secretions rises. There is anecdotal clinical evidence that oropharyngeal secretions are aspirated into the lung at night during sleep, leading to the devastating COVID-19 pneumonia [28]. Here, it should also be noted that while the lungs also contain levels of ACE2, the stimulation of alveolar macrophages can drive the cytokine storm and contribute to these most devastating effects [296,297]. We therefore propose that the use of a niclosamide spray by individuals both prior to entering environments of suspected virus exposure (including contacting infected individuals or groups with/without early symptoms), and if the individual becomes initially infected, will lessen SARS-CoV-2 titer in upper respiratory secretions, thereby reducing the risk of infection in the lower respiratory tract. With vaccines available, that actually do not prevent initial infection,

such preventative and early treatment sprays can only enhance all mitigation efforts.

8.2. Formulations: The early days (March 2020 - Oct 2020)

In March 2020 Jeon et al. at the Korean Pasteur Institute in a BioRxiv preprint [109] (later peer reviewed [110]) showed that niclosamide had infection-inhibiting activity on SARS-CoV-2. Having explored the potential for niclosamide as a new niclosamide stearate prodrug therapeutic (NSPT), I therefore pivoted away from niclosamide for cancer to formulations of niclosamide for this emerging respiratory infection. Through 2020 I designed and carried out a large series of experiments that would lead to a new niclosamide-based solution formulation for COVID19 [185,187] and other respiratory infections.

Over the next seven months, in an attempt to expedite some practical and effective formulations and what I (naively) thought would be more readily approved and taken up for development, I added niclosamide to everything, starting with commercial mouthwash, nasal sprays, eye drops, and contact lens salt solutions, that I bought from the grocery store. I made some interesting observations about niclosamide, and its solubility, precipitation, and morphology, and its interaction with various surfactants and preservatives. These experiments and results were dutifully submitted as invention disclosures to Duke and recorded in a series of provisional patent applications [181]. However, none of the commercial companies we approached (P&G, J&J, and Merck), were in a position to work with us, and Duke's Office of Licensing and Ventures (OLV) were unfortunately unsuccessful in finding a partner or funding, although they only focused on corporate and venture capital, and were told, words to the effect that, "no point investing in this, it will be over in a year" . The Duke OLV eventually assigned it back to me in February 2021, and so this preventative nasal spray and early treatment throat spray is ready for further development.

As an Aside: It is interesting to note that, when I started thinking about and then developing the niclosamide nasal and throat spray solutions (March 20th 2020), there were just 17,743 cases in the USA 238,584 worldwide [207]. I soldiered on, working on my own, days and nights and weekends, in my COVID19-separated lab. Here in Fig. 11 is the progression of the pandemic through 2020 and the dates of my submitted provisionals [181].

Imagine, as I watched, frustratedly, the nightly news as more and more people were catching this virus and how the pandemic was taking more lives, especially the elderly.[25] I had what could potentially be a preventative and early treatment option but couldn't get it through the system. I still often wonder if this could have made a difference if anybody had listened.

I even emailed all the experts appearing on the nightly news, Fauci, Kessler, Osterholm, Slavitt, Zha, and Hotez. Subject: ***A new formulation of niclosamide for your consideration***. The only one I heard back from, with an immediate email response, was Dr. Fauci. I was so excited to see Fauci, Anthony (NIH/NIAID) <afauci@niaid.nih.gov>, but, understandably, since he was serving the country during a pandemic, it was just an out of office reply, saying,

My work with the Coronavirus Task Force and the large volume of incoming emails precludes me or my staff from answering each individual message. I would encourage you to visit www.coronavirus.gov for the latest information and guidance related to COVID-19. Thank you, and best regards.
Anthony S. Fauci, M.D.
I will nevertheless treasure this email forever, ☺.

Looking back in the literature, as reviewed by Xu et al. [281], and then searching for the more granular details, (as reviewed above ***5. Niclosamide in viral Infection***), there was overwhelming evidence for niclosamide having activity in the original SARS-CoV and other viral infections, including influenza [115]. This new data from Jeon et al. showed that out of, again, a large screen of approved drugs, niclosamide, when dosed pre-inoculation in Vero6 cells, exhibited very potent antiviral activity against SARS-CoV-2 with an $IC_{50} = 0.28$ μM. It completely inhibited SARS-CoV-2 infection in the Vero6 cells at only 1 μM, yet did not reduce host cell viability until >250 μM. Others had shown that there were no deleterious effects of Niclosamide on Vero6 cell viability up to 250 μM for a 48 h incubation [279], giving a therapeutic window of 250 times. By comparison, (as mentioned above 5.3.4. Replication), chloroquine, lopinavir, and remdesivir showed much lower potency than niclosamide, with IC_{50} values of 9.12 μM, 7.28 μM, and 11.41 μM, respectively. Niclosamide was 40 times more potent than remdesivir and 32 times more potent than Chloroquine, with probably much fewer side effects. Niclosamide had also been shown to limit viral replication in already infected cells [86].

While others were focused on vaccines and antivirals that could limit systemic infection, but would not stop initial infectivity, my initial idea then was to go for the site of infection and early stages by looking to develop a nasal spray and early treatment throat spray. In that series of provisional patent applications [181], as shown in, Fig. 12, I discovered how niclosamide formed a range of microcrystalline morphologies that were everything from micro-blocks in P&G's Crest Scope mouthwash, to star-burst-like crystals in Benzalkonium Chloride (BAC) preservative, to wheatsheaves and spiky-balls in saline PEG40- stearate solution, to long hairy-like fibers in Equate's mouth rinse. This was the same niclosamide precipitated into each one? How is that possible?

Later, by reductive reconstitution of each component I found out, that the range of surfactant and preservative excipients present in the various commercial solutions were crystal habit modifiers that controlled polymorphs and morphology.

I also discovered a lot about niclosamide solubility and the amount of niclosamide that could actually be in aqueous solution (and therefore 100% bioavailable from the spray) due to those basic solubility-pK_a-pH-polymorph relationships.

By October 2020, and based on its physico chemical properties, I finally came up with a simple, "carrier free" pH-buffered niclosamide solution. The 1 L bottle I made by ethanol injection-rapid solvent exchange into pH 9 buffer is still sat, stably, on my shelf at the same 85 μM concentration that I made it at, 2.5 years later. This solution, when optimized for nasal and oral delivery, could potentially become a very effective preventative nasal spray and early treatment throat spray [185,187]. We are currently testing niclosamide in the more appropriate nasal and bronchial epithelial cells [192] as discussed in more detail below (8.4.6 Ongoing cell studies).

By November of that year, I had a provisional patent application submitted (just in case) [183] with my ex-graduate student, Jeff Mills now partner at the patent law firm Medler Ferro Woodhouse & Mills PLLC, in Mclean VA. And, by December 2021, working with Tonglei Li, the Editor-in Chief at Pharmaceutical Research, my first single authored experimental paper was published [184,185].

During that first year of the emerging pandemic, other people had also recognized niclosamide's potential including Union Therapeutics

[25] Our almost 89-year-old mother had already just passed away in December 2019, and I shuddered to think, after surviving breast cancer twice in her lifetime, how it could have all ended for her (and the other people in her care home) if she had been involved in that horrendous phase of the pandemic, when the UK government were happy to let herd immunity take its course, but only succeeded in killing off a large segment of the aged population.

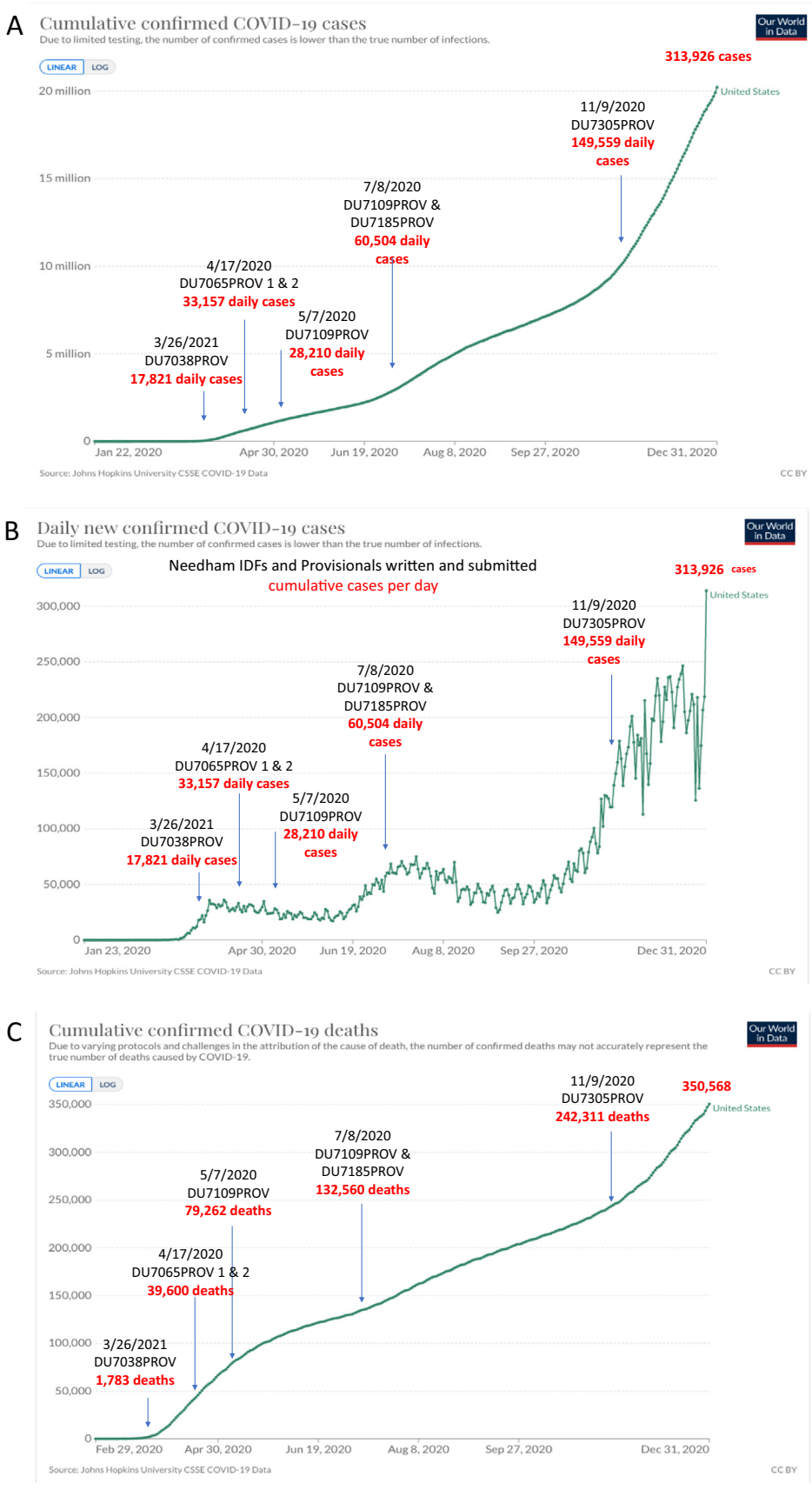


Fig. 11. Comparison between the progression of COVID19 as ***A)*** daily cases USA; ***B)*** cumulative cases USA; ***C)*** Total deaths Worldwide and all my lab work generating the series provisional patent applications, March – November 2020.

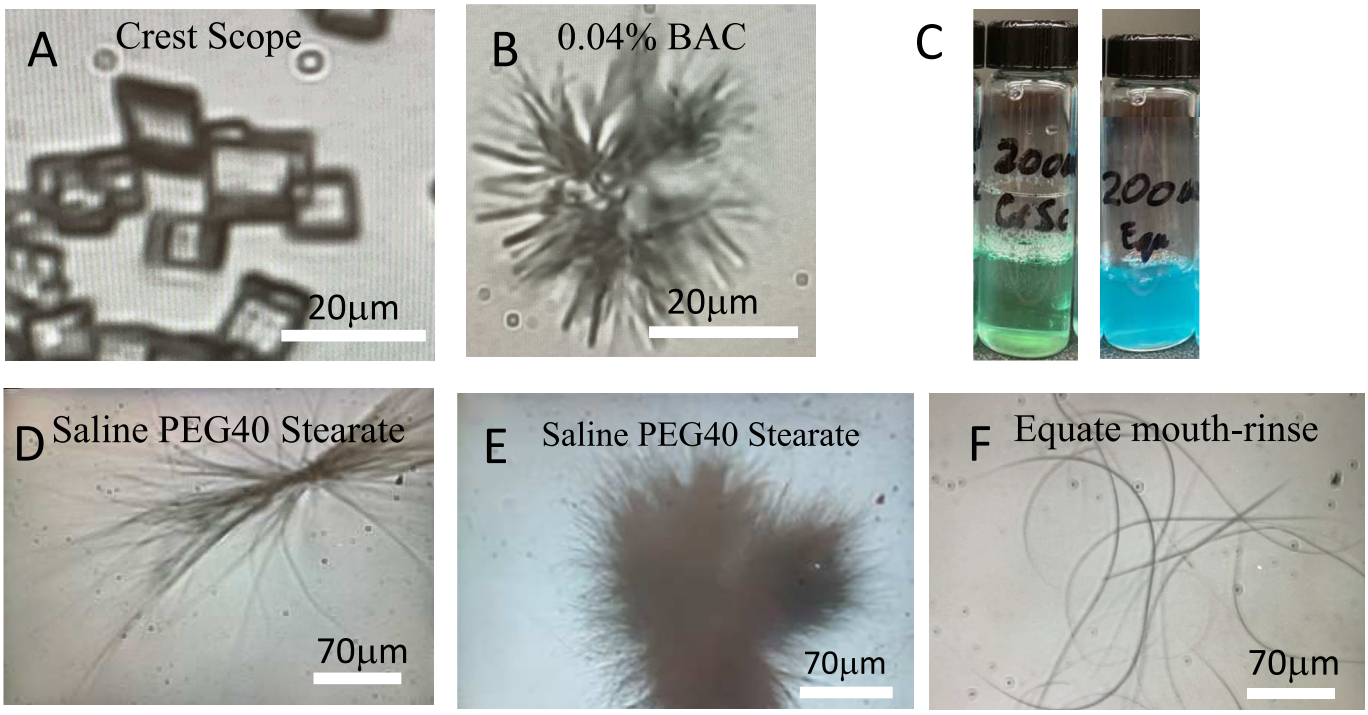


Fig. 12. Crystal morphologies of niclosamide precipitated into various commercial solutions: ***A)*** Crest Scope: block-like crystals; ***B)*** 0.04% BAC preservative: star-burst-like crystals; ***D)*** and ***E)*** 100uM PEG40 Stearate in Saline: wheatsheaves and spikey balls; ***F)*** Equate mouth-rinse solution: very long hairy fibers; ***C)*** The suspensions in Crest Scope (left) and Equate mouth rinse (right).

in Denmark who were starting to develop a formulation and test it in phase I trials [11]. This formulation comprised a 25 mM niclosamide (as niclosamide ethanolamine) solubilized in hydroxypropyl-beta cyclodextrin (HPβCD) and is now in Phase II trials in Cambridge and India [258,259]. Also, the Smyth group at the University of Austin had preclinical data showing that TFF Pharma's proprietary inhalable powders technology (0.7% micronized niclosamide in 99.3% spray-dried lysozyme) had some efficacy in a mouse model for SARS-Cov-2 as well as MERS infection [24]. This niclosamide inhalant formulation is also now in clinical trials dosed at 20 mM niclosamide [253].

While the Union cyclodextrin and TFF spray dried formulations may well eventually show promise in their clinical trials, the bioavailable aqueous concentrations will still be determined by the niclosamide polymorph and solution pH. That is, given an IC_{100} to completely stop infection of only 1 μM, are these "advanced" formulations at 25 mM and 20 mM really necessary, safe, and clinically optimal? –especially when one, the UN9011-cyclodextrin was tested intranasally and as an inhalant in phase I trials [11] uses the more toxic niclosamide ethanolamine. When purchased from any chemical company [30,39] niclosamide ethanolamine comes with a hazard warning as a skin, eye, and respiratory irritant and the New Jersey Department of Health and Senior Services is concerned about its toxicity [59]. Thus the ethanolamine component may be causing the extensive bronchial irritation found in the Phase 1 trial [11], by taking the UNI901 formulation, where two subjects actually discovered they were asthmatic, "*two receiving active were likely associated with undiagnosed asthma*".

It seemed to me that the researchers in these companies were again going with what they knew and their patent-protected technology, cyclodextrin, and thin-film-freezing spray-dried-protein (which is fine). And again, given the 1uM activity of niclosamide in stopping all viral infection in cells, 25 mM and 20 mM concentrations (including asthma-inducing niclosamide ethanolamine) seemed like over kill. So, as with our nanomedicine discussion earlier, they were creating nano- and micro-medicine formulations because they could, and maybe without really understanding the drug first. Basically, everyone knew that niclosamide was highly insoluble so "*let's formulate it in our cyclodextrins and spray dried powders*". But was it? –especially in relation to its already measured *in vitro* efficacy? (and my newly discovered increased solubility as a function of pH)

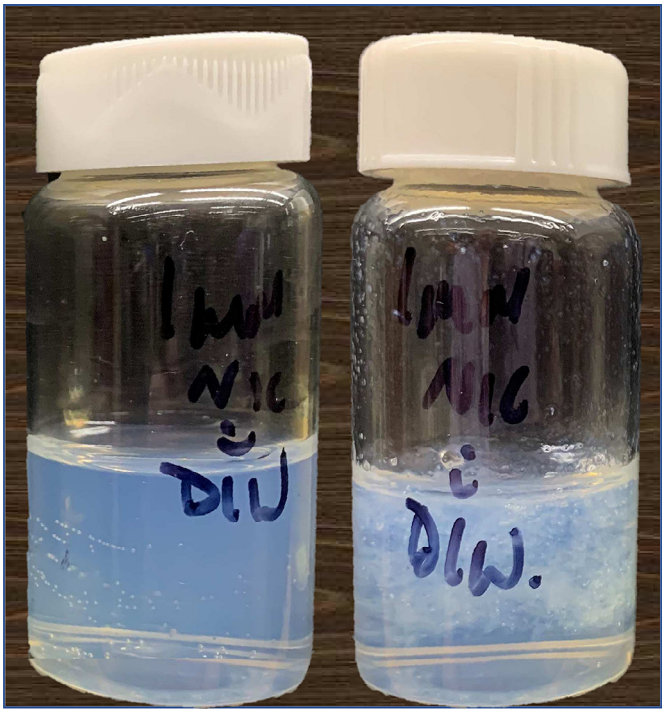


Fig. 13. Niclosamide precipitated into DI water at a final 1 mM niclosamide concentration forms a nanoparticle suspension (left); but, when hand shaken, converts to a visible, "fluffy" precipitate (right).

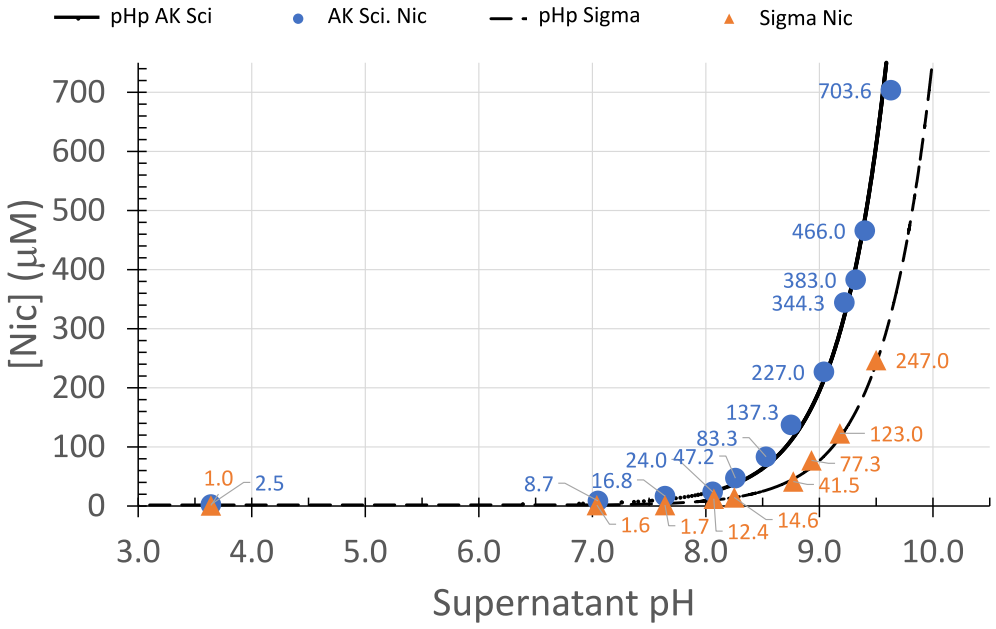


Fig. 14. Equilibrated dissolution of Niclosamide in Tris Buffers over a range of pH. Plotted is the concentration of niclosamide measured by UV/Vis, [Nic] (μM) vs Supernatant pH as. data and pHp theory: (***blue*** symbols) AK Sci Niclosamide, (solid line) pKa = 7.12, intrinsic solubility [AKSci] = 2.53 μM; compared to (***orange*** symbols) Sigma Niclosamide, (dashed line) pKa = 7.12 intrinsic solubility [Sigma] = 1.0 μM.

Here then are some brief details, in the form of a personal storyline, of what I discovered by doing a preformulation drug characterization, and not immediately jumping to a LPDMC-cyclodextrin-spray-dried "nano-micro-medicine".

8.3. The development of a simple niclosamide solution-based formulation for a nasal preventative and an early treatment throat spray

I decided to do the only sensible, scientifically-rigorous thing and start with niclosamide in DI water. This is the most basic "preformulation drug characterization" you can do, —drug and water (and a few % of residual ethanol). Using the rapid solvent exchange technique, precipitating excess (1 mM) niclosamide into water from ethanol solution actually gave nanoparticles of 70 nm diameter or so, that grew slowly over a period of a few hours into the 100 nm range. As shown in Fig 13 ***left***, they were quite stable, likely electrostatically stabilized because of their small mutually repulsive negative charge (from the fraction of deprotonated niclosamide salt) in an almost infinite-Debye-length solution. If left standing they could stay like that for days, unless the vials were hand-shaken (Fig. 13 ***right***) resulting in massive aggregation as a visible, "fluffy" precipitate of large sheet-like structures of the nanoparticles when viewed under the optical microscope.

Being a weak acid with a calculated pKa of ~7.12, I tried precipitating niclosamide from ethanol solution into increasing-pH solutions. Tris buffer was the only one that would go from 7 to 9.[26] As is well appreciated in first year college pharmacy, the solubility of a weak acid can be increased by increasing the pH above its pKa, as the more soluble and negatively charged salt starts to dominate, as shown earlier in Fig. 5.

So that's where I started. As we all know, Henderson and Hasselbalch[27] gave us an equation for us to use to determine the fractions of each protonated and unprotonated species as a function of pH, that can be adapted to give the maximum amount of solute, in this case niclosamide, in solution at any pH (as shown in Fig. 5.) Exploring this pH-dependence both as supersaturated solutions made by powder dissolution from different commercial sources [185], I discovered that it wasn't just as simple as –"*it all depends on pH*". The original niclosamide polymorph played a significant role in how much niclosamide was actually in solution, —and therefore potentially bioavailable in the nasal spray.

8.3.1. pH-dependent solubility: Maybe this is all we need

The main finding is shown in Fig. 14. As predicted from the Henderson-Hasselbalch and pHp theories, the calibrated UV–Vis measurements for the amount of niclosamide that were dissolved into aqueous buffered solution increased with increasing tris buffer suspension pH. For the first supplier of niclosamide (AK Sci, CA) (data as blue symbols; pHp theory as solid line) solubilities ranged from an intrinsic solubility of 2.53 μM at pH 3.66, to 30 μM a nasally-safe pH 8.3 just by slightly increasing the alkalinity of the buffer. The concentration could be raised to 200 μM at pH 9.0, and 300 μM at pH 9.2 where the concentration vs pH curve becomes quite steep. At an orally-safe pH 9.63, where the unprotonated salt dominates, the amount of niclosamide in solution can be increased to 703.6 μM.

Applying the precipitation pH (pHp) theory with the measured intrinsic solubility of 2.53 μM measured at pH 3.6 where the protonated acid dominates, the best fit to the data was for a niclosamide pKa of 7.12. What this data shows is that for this particular source of niclosamide that (as discovered later) appears to be mostly anhydrous, we can get significant amounts of niclosamide in solution all in simple tris buffer, with no other added solubilizing surfactants or cyclodextrins or nano- or micro-medicine scaffolds. Thus, the amount of niclosamide in simple aqueous solution could be 30 times its 1 μM IC_{100} that completely inhibits SARS-CoV2 viral replication (see all references above in Table 1) in a 30 μM niclosamide solution nasal spray at a nasally-safe pH 8.3. It could be almost 100 times the IC_{100} if used as an early-treatment 300 μM niclosamide throat spray. Curiously, for the other supplier (Sigma), the equilibrated suspension concentrations were significantly less (data as orange symbols; pHp theory as dashed

[26] Here's a handy buffer recipe generator: https://www.aatbio.com/resources/buffer-preparations-and-recipes.

[27] In 1908, Lawrence Joseph Henderson wrote an equation that described the use of carbonic acid as a buffer solution. Later Karl Albert Hasselbalch reworked that formula in logarithmic terms. This came to be known as the Henderson-Hasselbalch equation. https://study.com/academy/lesson/the-henderson-equation-definition-examples.html; https://en.wikipedia.org/wiki/Lawrence_Joseph_Henderson; https://en.wikipedia.org/wiki/Karl_Albert_Hasselbalch.

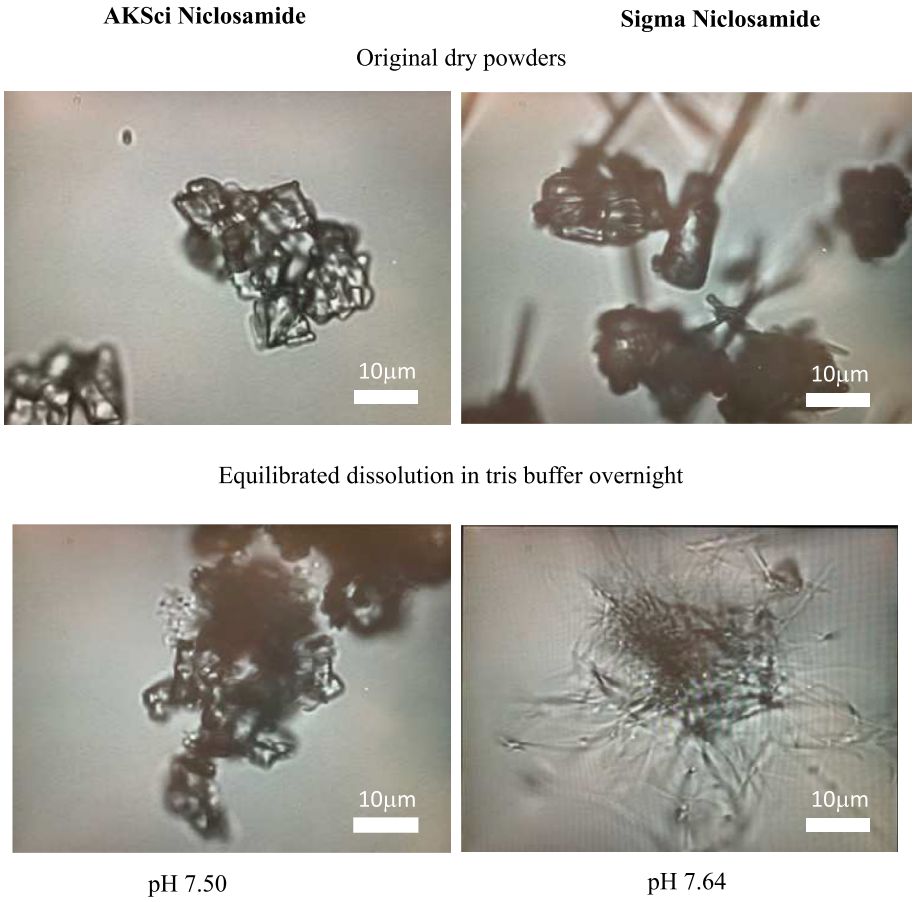


Fig. 15. Optical microscope images: (top row) original dry powders of AKSci niclosamide and Sigma niclosamide; (bottom row) excess niclosamide from equilibrated overnight dissolution showing that the AKSci material (left) was largely unchanged, but the Sigma material (right) converted to almost complete spiky-needle shapes reminiscent of the low solubility, most stable, monohydrate polymorph [261].

line). This shift to the right was satisfactorily fitted across the whole pH range by pHp theory using the same pK_a of 7.12, but a lower intrinsic solubility of 1 μM.

Interestingly, then, as shown also in Fig. 14, there was some dependence of solubility on what was assumed to be the original polymorphic nature of the niclosamide obtained from each of the suppliers (AK Sci and Sigma). In this first paper, the pH-dependence of the solubility of all polymorphs (and solvates) was shown (for the first time) to exist across the whole pH range [185]. Also, while not having access to x-ray, Raman, Thermal Gravimetric Analysis, and other structure techniques, could I simply evaluate these suspected polymorphs microscopically?[28]

Shown in Fig 15, (top row) are typical bright field microscopic images of the original dry powders. The AKSci niclosamide had an appearance of aggregated, smaller, block-like crystals, but the Sigma niclosamide had some blocks but also with spikey needle shapes [185].

Such needles have been seen and characterized before by van Tonder et al. [261]. These needles represent the low solubility, most stable monohydrate (with a ~ 1 μM intrinsic solubility). But then, as shown in Fig 15 (bottom row), when the excess materials were stirred overnight in pH buffer solution to measure dissolution and reach an equilibrium concentration, the excess, undissolved, AKSci. material was largely unchanged, but the Sigma material appeared to completely convert and grow much more spiky needles that also accompanied a lowering of the amount of niclosamide in solution (as shown above in Fig. 14, orange triangles).

It appeared then that the AKSci material was an anhydrous niclosamide with a higher intrinsic solubility that was reflected across the whole pH range. In contrast, the Sigma niclosamide was an initial mixture of anhydrous blocks and monohydrate needles. Speculating, it could be that, during synthesis and post synthesis handling, for the AKSci material, only the anhydrous niclosamide was formed. In contrast, for the Sigma material, both the anhydrous polymorph and the lower solubility monohydrate had been formed. Or, some of the anhydrate had been converted to the monohydrate by humidity and time. What's more, during the dissolution experiment they both seemed to initially (over the first 1–2 h) dissolve to a similar extent (just the block like morphology?), but when the excess material was left in contact with the equilibrating dissolving suspension overnight, the amount of dissolved niclosamide in the Sigma sample was reduced as it all converted to that lower solubility material.

The lesson here is that "*niclosamide is not niclosamide is not niclosamide*". These simple pH-dependent dissolution and morphology experiments led to the realization (and hypothesis to be tested in new work) that, depending on the supplier and the original polymorph, and the length of time your niclosamide formulation is in contact with an equilibrating aqueous solution at any pH, it could equilibrate to the lowest solubility polymorph, and so reduce the bioavailability of the niclosamide in solution in a nasal spray, throat spray or inhalant, or any niclosamide formulation.

[28] In addition to my DLS being only feet away from the nano and microparticle making system, I have an inverted microscope that I use daily to look at all samples even if the DLS tells me they are nano and below the wavelength of light, —some aren't! I also have a UV–Vis, a pH meter, and a stir plate. That's basically all I need to get these formulations rolling and provide interesting data that can then generate deeper analyses with other collaborators.

The data though shows that, for the anhydrous (AKSci) niclosamide, the amount of niclosamide in solution achieves 24 μM to 47 μM in the 8.1 to 8.3 pH range. While for the monohydrate (Sigma) niclosamide the amount is only 12 μM to 14 μM. The anhydrous suspension does eventually covert and so the only way to maintain the higher solution concentration is to filter out and remove the equilibrating solids. As discussed below for optimization of the formulations, if we want an additional depot of material, then the equilibrated solution concentration will likely be in that 12 μM–14 μM concentration range, where, by a slight increase in nasally safe alkalinity, we can increase the bioavailability of niclosamide in solution by over an order of magnitude compared to the original 1 μM solubility and the 1 μM efficacy against viral infection.

So, this is how we can create higher bioavailable concentrations of niclosamide in simple sprayable solutions at pH 8.3, which is in the safe and upper pH range of the nose, and at pH 9.3, which is fine for oral because green tea is pH 9 and the popular alkaline water is also pH 9. Also, as above, the S-protein cleavage needs a pH in 6.2–6.8 range which is close to the nasal epithelial mucous range. However, our buffered pH 8.3 solution of 20 μM niclosamide increases the local pH and so could directly impact viral RNA entry just by the pH alone, at least for a while.

8.3.2. Niclosamide can be extracted from already approved and commercially available tablets

When we approached the FDA with potential formulations of niclosamide, they wanted us to start afresh with this "new formulation", even though the Bayer Yomesan is taken orally and so exposes the buccal mucosa to a 2-g tablet of niclosamide,[29] and what we had was, ostensibly, just a pure buffered solution of niclosamide. Our 100 μL spray of a 20 μM solution is just 0.65 micrograms of niclosamide. Thus, my next thought was, what if I can show that niclosamide can be extracted from commercially-available and regulatory-approved niclosamide oral tablets (already containing pharmaceutical grade material) that could serve as a preventative nasal spray and early treatment oral/throat spray? Would this give a pathway to a more expeditious testing and regulatory approval?

So, back to the lab for another 4 months where I set out to make measurements of supernatant niclosamide concentrations (again by calibrated UV–Vis) for the dissolution of niclosamide from commercially available Yomesan crushed into a powder and added in simple dissolution experiments into Tris Buffer (TB) solutions. Knowing from the previous experiments [185] that niclosamide solubility had not only a pH-dependence but also the potential for polymorphic changes, the parameters tested were: time, (0–2 Days); concentration (300 μM – 1 mM), pH (7.41 to 9.35), and anhydrous/hydrated state. Optical microscopy was again used to view the morphologies of the initial crushed powder and the dissolving and equilibrating undissolved excess particles in order to identify and detect morphologic changes that might occur.

The results can be divided up into two main studies [187]. –concentration dependence at one pH, pH dependence over a range of concentrations.

8.3.2.1. Concentration-dependence for the extraction. Starting with Yomesan niclosamide-equivalent concentrations of 300 μM, 600 μM and 1 mM, niclosamide was readily extracted from powdered Yomesan in Tris Buffer, Peak dissolved niclosamide supernatant concentrations of 264 μM, 216 μM and 172 μM were achieved in 1 h, 1 h and 3 h, respectively. These peaks though were followed by a reduction in supernatant concentration to an average of 112.3 μM +/− 28.4 μM after overnight stir on Day 2 showing that polymorphic changes had occurred and reduced the amount in solution.

8.3.2.2. pH-dependence. pH dependence studies showed that for, nominal pHs of 7.41, 8.35, 8.85 and 9.35, peak niclosamide dissolved concentrations were 4 μM, 22.4 μM, 96.2 μM, and 215.8 μM, respectively, and so somewhat in line with the pure niclosamide values measured earlier [185]. And, wanting to see if there were any polymorph-morphology conversions, the Day 2 values all reduced to 3 μM, 12.9 μM, 35.1 μM, and 112.3 μM, respectively, confirming that there were.

In the absence of gravimetric thermal analysis to measure any water of hydration, I came up with a simple heat-treatment protocol of heating the original Yomesan powder slowly to 200 °C. I thought I could maybe catch some dehydrating water vapor on a cover slip placed over the mouth of the vial. It worked! and was able to identify the dehydration-condensation transitions of van Tonder's H_A polymorph at 105 °C and the H_B polymorph at 170 °C agreeing with van Tonder and de Villers et al.'s, [53,261] values for niclosamide and its hydrous to anhydrous transitions.

Also, this dehydrated niclosamide showed the highest high 3-h concentration (262 μM) and the least day-2 reduction (to 229 μM). This again, indicated that the presence, or formation during exposure to buffer, of lower solubility polymorphs were responsible for the reductions in total solubilities. It behaved a lot like the anhydrous AKSci material. The unheated Yomesan niclosamide though behaved like the Sigma sample, where the excess, undissolved material converted to the low solubility monohydrate polymorph upon exposure and dissolution in buffer solution.

These morphologic changes were confirmed by optical microscopy, that, as in Fig. 16, (t = 0 mins) showed initially featureless particulate-aggregates of niclosamide but then converted to the spiky needle morphology starting during dissolution (t = 180 mins) progressing at t = Day 2.

Thus, over time with continued stirring and dissolution of the excess material, (Fig. 16, t = 180 mins) the drug particles grew multiple needle-shaped crystals and by, the second day of equilibrating dissolution (Fig. 16, t = Day 2), formed needled-masses. This was especially seen in the presence of tris buffered sodium chloride, where new red-colored needles were rapidly made. In a series of preliminary experiments, I also detected an excipient that interfered with the UV/VIS, it was vanillin! So, screening this out with control solutions of vanillin, showing that it rapidly dissolved in less than 30s, and painstakingly and, as one reviewer said, "meticulously" evaluating all possible aspects, I could get usable UV–Vis spectra. These spectra were therefore baseline-corrected, 30-s subtracted spectra from which the supernatant concentrations could be obtained from microfiltered samples giving the dissolution extraction of niclosamide.

Anticipating that this discovery could generate available niclosamide worldwide if it were *scaled up,* I showed how to make a 1 L solution of niclosamide achieving 165 μM supernatant niclosamide in a 3 h dissolution of just one fifth (100 mg niclosamide) of a Yomesan tablet. Basically, as shown in Fig. 17 A and B, a compounding pharmacy or company manufacturing could crush up the tablets using a simple pill crusher. They could then extract the niclosamide from the tablet material into a simple buffered solution at pH 9.3 (Fig 17 C and D), and then adjust the pH down and dilute as needed to make the pH 8.3 20 μM niclosamide nasal spray solution.

These comprehensive results now provide a guide as to how to utilize commercially available and approved tablets of niclosamide to generate aqueous niclosamide solutions from a simple dissolution protocol.

8.3.3. Summary guide to making the extraction-formulation

The procedure for crushing a tablet, dissolving it in pH buffer and some data is shown in, Fig 17 along with a filtered 1 L sample. Fig. 17 ***A)*** shows how a 500 mg niclosamide Yomesan tablet can be readily

[29] Although with its limited solubility the mucosa may only be exposed to a say 5 μM solution of 30 mLs is 50 μg niclosamide and so only 0.0025% of the available niclosamide from the 4 × 500 mg tablets. I always thought maybe the tablet being "thoroughly chewed in the mouth" is simply feeding the worm itself and it receives solid niclosamide that then dissolves within the worm's tissues. Not heard anybody talk about that.

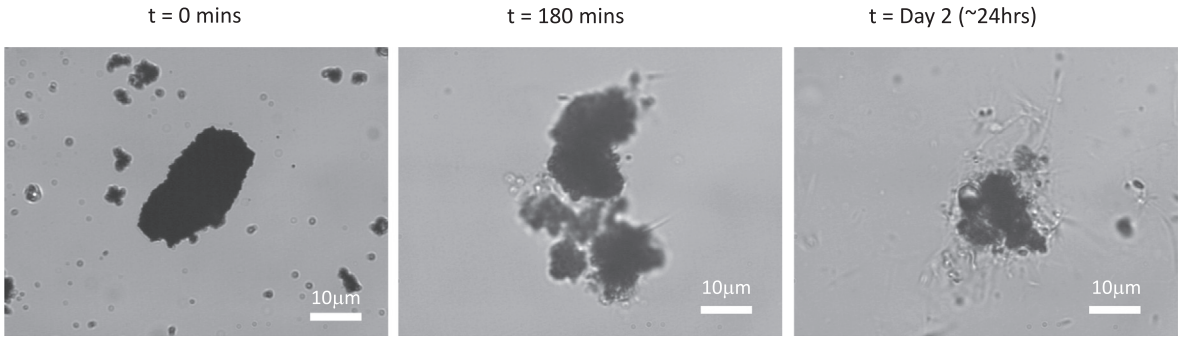


Fig. 16. Dissolution of 600 μM Yomesan niclosamide at nominal pH 8.85. typical Optical microscope images of particles before ($t = 0$ mins), during ($t = 180$ min) and after equilibration (Day 2). The growth of the characteristic monohydrate needles is evident.

crushed using an *Easy-Grip Pill Crusher* giving, as depicted in Fig. 17 ***B)***, to give 637.7 mg of a fine powder, i.e., because of the excipients, Yomesan is 0.784 mg niclosamide per 1 mg of tablet. Then, in Fig. 17 ***C)***, when a 300 μM equivalent of this niclosamide powder is dissolved into tris buffer at pH 9.3, dissolution proceeds in a logarithmic fashion to a relative maximum of ~170 μM niclosamide in 3 h. Beyond this time the excess material would start to convert into the lower solubility monohydrate and drive the amount of niclosamide in solution down. By filtering the suspension to provide a particle free solution of the aqueous buffered niclosamide, the elevated concentration is preserved.

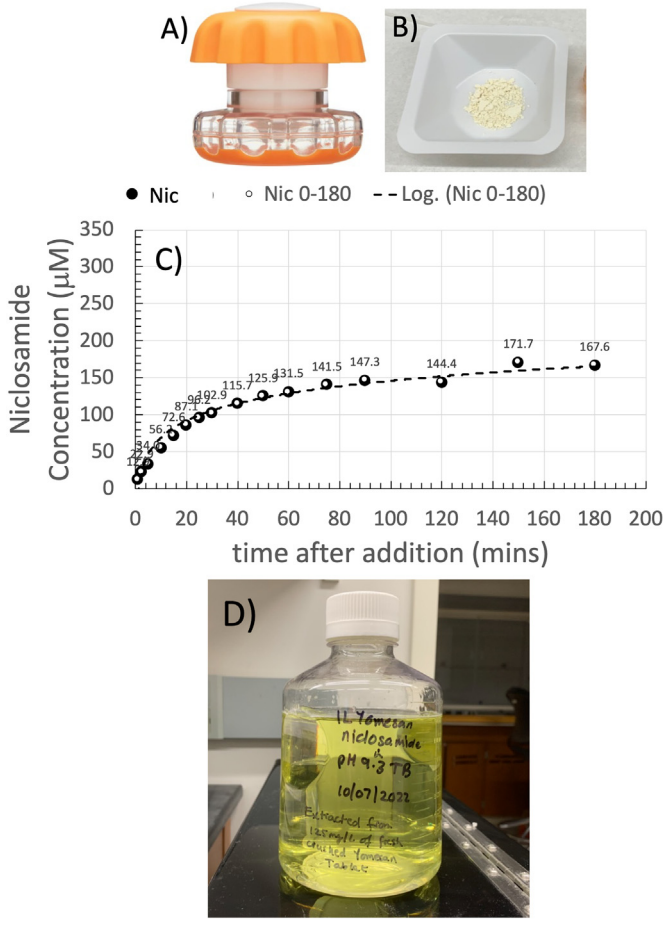


Fig. 17. ***A)*** Preparation of **c**rushed Yomesan tablet using an Easy-Grip Pill Crusher, to crush whole Yomesan, tablets; ***B)*** 637.7 mg of a 500 mg niclosamide crushed Yomesan tablet, (0.784 mg niclosamide per 1 mg of tablet). ***C)*** Dissolution of Yomesan-niclosamide (filled black symbols) at 1 L scale dissolved over a period of 0–180 min giving, with a logarithmic dependence (dashed line and open white symbols) to the dissolution; ***D)*** the filtered 1 L sample of 165 μM niclosamide in final pH 9.33 showing the yellowness of the solution for niclosamide ready for dilution and rebuffering to 20 μM at pH 8.3.

The solution was therefore scaled up to a 1 L volume by dissolving just 125 mg of Yomesan powder (1/4 of a tablet) added to 1 L of nominal pH 9.36 Tris buffer in a conical flask stirred at 500 rpm with a magnetic stir bar for 3 h. Fig 17 ***D)*** shows the resulting yellowness of the Yomesan-niclosamide solution giving a filtered 1 L sample of 165 μM niclosamide in a final pH 9.33 ready for dilution and rebuffering to 20 μM at pH 8.3. Again, this is something any local compounding pharmacy and/or any company with lab-regulated approval could make (including Bayer).

8.3.4. How much niclosamide solution could this simple procedure make?

Thus, if ***¼*** of a tablet can give ***1 L*** of ***165 μM*** filtered niclosamide solution at ***pH 9.33***, then this gives ***8.25 L*** of a niclosamide solution that is diluted to ***20 μM*** and rebuffered to ***pH 8.3*** where the niclosamide is well solubilized at this nasally-safe pH. ***1 tablet*** of Yomesan can therefore give ***33 L***, and just one ***4-tablet pack*** of Yomesan could readily make ***132 L*** of this 20 μM niclosamide solution giving ***13,200 × 10 mL*** bottles of nasal spray. ***One million*** bottles is obtained from just ***76 packs*** of Yomesan and would provide ***100 million*** single sprays at ***100*** μL/***dose*** for distribution. This could mitigate a host of respiratory infections as a universal preventative-nasal and early treatment oral/throat sprays throughout the world. Even at the retail price (from Belgium) of ~ €6 (Euros) per 4 tablets, ***€304*** would make ***1 million*** spray bottles for distribution to parts of the world where vaccines are scarce, anti-virals are absent, and preventatives (other than masking) are non-existent.

Therefore, with this publication [187], I am ready to now ask Bayer or any other generic niclosamide company, institute, or government lab to take this on and provide simple niclosamide solutions for testing and eventually, if successful, preventative nasal sprays and early treatment throat sprays for COVID19 and other respiratory infections worldwide at reduced reinvested profit, open source pharmaceuticals, starting generic.

8.3.5. Optimizing the formulation

I realize that a simple niclosamide solution may or may not be the most optimal nasal or throat spray. For one, the nasal epithelium is a direct route to the blood stream. Other nasal solutions of, for example Midazolam,[30] show a peak plasma concentration at ~15 min after dosing [124] and so begin to deplete the concentration of the drug in the nasal epithelium. The question for a 100 μL dose of a

[30] A weak base but with similar micromolar solubility (30 μM) and LogP (3.9) for its unprotonated species at neutral pH.

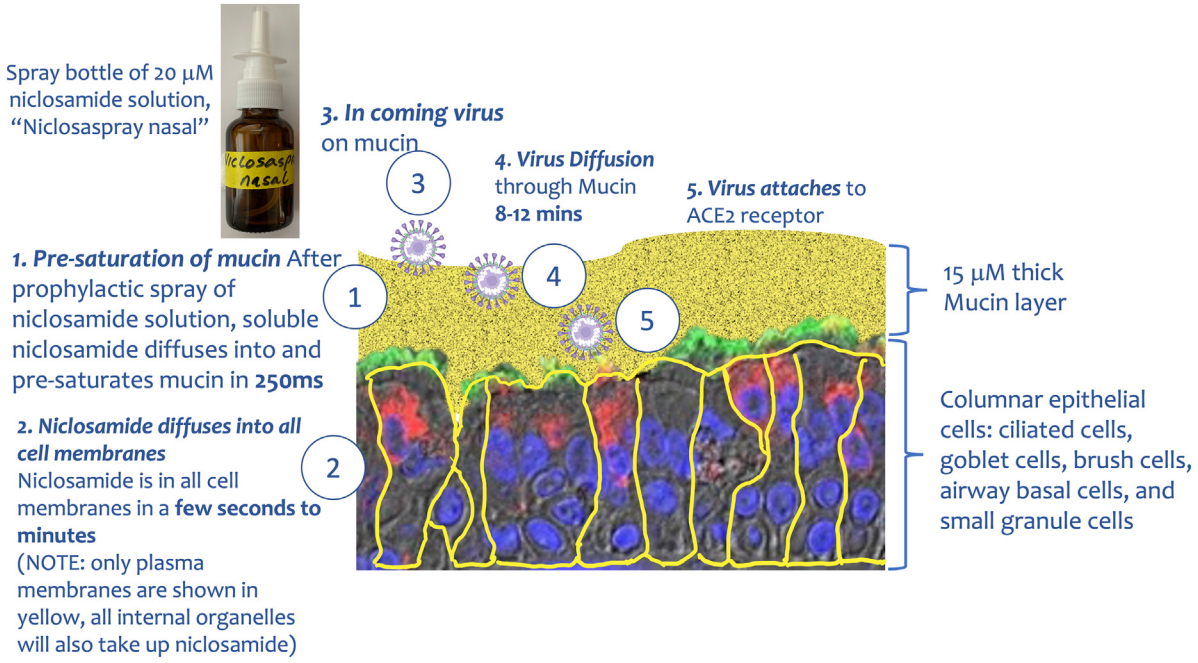


Fig. 18. Pre-saturation of nasal epithelium with niclosamide and initial stages of virus infection. Schematic adapted from Hou et al. [104] of stained real epithelium, showing, in yellow, 1. Pre-saturation of the mucin with niclosamide solution (yellow shading); 2. Niclosamide (sketched yellow lines) in the plasma membranes of at least the top layer of epithelial cells and also organelles (not shown); 3. Incoming virus on mucin; 4. Virus diffusion through mucin; 5. Virus attachment to ACE2 receptors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

20 μM solution of niclosamide is, how long after intranasal dosing would the concentration of niclosamide in the nasal epithelial cells get down to the efficacy concentration of ~1 μM? (another opportunity for a new study)

So, I will be soon back in the lab again, exploring how to optimize the formulation so that it can have additional dissolvable niclosamide that can continually supply the bioavailable solution for as long as it takes for the mucus to be moved and cleared to the back of the throat and out of the nasopharynx. With a dose of niclosamide present, that reduces ATP in the cells, this could actually take a while longer than the normal (~4–5 h for the whole journey of ~5 cm, at a mucociliary transport is *1–2 mm/h* anterior and *8–10 mm/h* posterior [112]) That is, we have seen in Air Liquid Interface cultures that have microvilli, niclosamide slows down any microvilli activity that would normally sweep the mucus retrograde, and may even reduce the mucus secreted from the goblet cells (see above, ***5.6 Proposed idea***).

It is actually common for many nasal sprays to comprise soluble and insoluble portions. Good examples are Nasonex [12,70,163] and Flonase [36,200,241], where the drugs (mometasone furoate and fluticasone propionate) are active in the nanomolar, soluble up to a few micromolar, and are present at 1 mM.

The idea then is to have a safe (20 μM) solution with an additional undissolved but dissolvable niclosamide depot, (up to say 1 mM niclosamide) to act as a preventative prophylactic for any person who is about to go into a high-risk virus setting.

8.3.6. Ongoing cell studies

We do have on going cell studies of our own, funded by a 2-year grant from the American Lung Association with my colleague Dr. Christina Barkauskas as PI and her senior technician Zach Kelleher [16]. Rather than utilizing the usual immortalized cell lines (Vero, Calu-3, etc.) we are putting our efforts into primary human Nasal and Bronchial Epithelial cells where such respiratory infections start. New data is looking at how niclosamide affects ATP and cell respiration through cell titer glo and seahorse assays, as well as cytoplasmic and endosomic pH. And we also have promising preliminary data in vitro on the way niclosamide can inhibit SARS-CoV-2 viral infection in hNE, hBE and A549 cells. Publications on all of this will be forthcoming.

8.4. The Niclosaspray preventative: How might it work with the virus

8.4.1. Nasal spray, niclosamide's presence in the mucosa, and initial virus infection

Finally, how I envision things going for the preventative is presented schematically in Fig. 18. as adapted from Hou et al. [104]. The red colour highlights SARS-CovV-2-infected ciliated cells in a COVID-19 patient's bronchi in the first monolayer of columnar cells with cell-type-specific markers (green) determined by dual-immunofluorescent staining (acetylated a-tubulin cilia marker).

Knowing that it was safe to hNE and hBE cells up to at least 100 μM (for a 24 h exposure) I, as the inventor, thought it was incumbent on me to test it on myself and get at least an "*n-of-1*". So, I do protect myself.[31] As I get out of my car in the parking lot at Walmart or am about to go into Duke or a clinic or anywhere where there are people and a risk of super spreading, I spray the niclosamide solution from my little "Niclosaspray nasal" bottle. I usually do 2–3 squirts of 100 μL of the 20 μM solution in pH 8.3 buffer up each nostril, as "*added protection behind the mask*".

As shown in the schematic, Fig. 18 ①, it is expected that using the spray at pH 8.3 will pre-saturate the mucin with the 20 μM niclosamide solution (colored yellow). I calculated, based on molecular diffusion, that niclosamide is through the 15 μm-thick mucin layer in, at most, ***250 m seconds***. Using the hypo-osmotic 20 μM tris buffer solution, so that the osmolarity gradient favors water, and so solution, -transport into the nasal epithelium, when I spray it intranasally, I can even feel a slight tingle as the epithelium momentarily swells, ("*If you can feel the*

[31] My wife is immunocompromised and so I can't bring it home!

tingle, you know it's working"). This passes in a second or so as, I assume, the cellular osmolarity is readjusted.

Then, in, Fig. 18 ②, given a LogD of ~3 at this pH 8.3, meaning a partitioning of ~1000 times more in the lipid membranes than in water, niclosamide is expected to be in all cell membranes in a few seconds to minutes. In the figure, for clarity, only the plasma membranes are schematically depicted in yellow, but it is expected that internal organelles (endosomes, lysozomes, mitochondria, endoplasmic reticulum, Golgi) will also take up niclosamide. Thus, niclosamide is already present in the plasma membrane (that makes the endosomes) and is already at work dissipating any pH gradients across these organelles.

If a virus enters the nasopharynx it lands on the niclosamide saturated mucin Fig. 18 ③. The SARS-COV-2 virus particle is measured to be 80 nm – 120 nm in diameter as detected in an electron-microscopic examination of cell culture supernatant fluid [90]. A Stokes-Einstein-equation calculation gives this particle a diffusion coefficient in water for this size range of 5.4×10^{-8} cm^2/s to 3.6×10^{-8} cm^2/s, respectively. Olmsted et al., [204] found that similarly small virus-like particles, (e.g., the 38 nm diameter Norwalk and 55 nm diameter HPV particles), surprisingly, diffused in mucus (cervical) as fast as they diffused in saline buffer. Thus, if the corona virus also does not interact with the mucus, as shown in Fig. 18 ④, it would pass through a hydrated 15 μm thick mucin layer by diffusion in ***~20s to 30s*** ready to bind to the cell receptors.

Thus, as in Fig. 18 ⑤, the virus attaches to the ACE2 receptors. Data from Hou et al. [104] shows that the virus seems to preferentially infect the ciliated cells and not the nasal submucosal glands. Other data by Zhu et al., [293] indicates that virus can be found at the apical surface of both ciliated cells and secretory cells, plus, the observation of inclusion bodies formed by viral components in the cytoplasm, and therefore infection of both cell types.

Then, as described in detail above (in section ***5.3*** and Fig. 7) niclosamide has all its intracellular effects. Having suggested detailed ***Proposed Ideas*** earlier in the "*Niclosamide in Viral Infection*" section, I'll just refer you back to that section, that can now be viewed in context of the Fig. 7 schematic, and here I will just summarize what I think could be done.

8.1 Proposed ideas (all aspects of host cell-viral biology in hNE and hBE cells). Calling all researchers and experts to apply what you know and carry out as many experiments as you can in human nasal and bronchial epithelial cells. Pick any of the pathways you know about and evaluate if, how, and to what extent (IC_{50}s and IC_{100}s), any of these pathways are positively or negatively (cytotoxicity and lethality, not just "viability") affected by niclosamide getting into the plasma membrane and all these intracellular membranes. Ideally, in order to prepare for the study of respiratory viruses, it would be good to focus specifically on human nasal and bronchial cells as opposed to the more robust VeroX, etc.
Study the following concentration and time dependences without and with niclosamide: clathrin-dependent endocytosis, endosome-acidification, lysosome-permeabilization, intracellular-acidification, mitochondria and cell metabolism, ATP production and local ATP concentrations, and autophagy. Then, again in hNE and hBE cells, if you have access to a BSL3 or can work with lower Biocontainment viruses like influenza, evaluate, again, without and with niclosamide, the concentration and time dependence for all these processes that could influence: viral transport across the mucin, binding to the ACE2 receptors, membrane-viral spike protein fusion, clathrin pit formation, uncoating and RNA release (involving ubiquitin, Histone deacetylase 6), transcription-translation, viral manufacturing in endoplasmic reticulum-refashioned vROs, viral assembly in Golgi, secretion and the immunology of any "dud" virions that could act as their own in situ vaccine, the autophagocytic removal of the viruses, and the inhibition of syncytia.

As far as I know, hardly any of this has been done in hNE and hBE cells. We are certainly considering all of these but, with limited resources and only one main senior technician (Zach Kelleher) in the Barkauskas lab, we invite anyone to work with us, or take the lead yourselves.

If I have been anything in this research business over the past 46 years, it has been collaborative, inclusive not exclusive. So please do reach out and let's collaborate, and can we at least try, at cost, or reduced reinvested profit, open-source pharmaceuticals, start generic.

9. Final thoughts and an invitation for further studies

9.1. Niclosamide is like Kinam

As we can now all appreciate, niclosamide operates so far upstream in the cell that it influences every other pathway. For niclosamide, this is mainly antagonistic (inhibitory) and can be used for good in diseased or unhealthy cells for the benefit of the host. For Kinam, I think he's also upstream, but it's more of an agonistic role, supporting, enabling, mentoring, researching, inventing, teaching, giving seminars, editing papers for others, and publishing his own. If my interactions with Kinam over chapters, discussions, and a glass of whiskey is anything to go by, then I imagine Kinam has similarly operated "upstream" and selflessly influenced every person, students, post docs, faculty, (university administrators?), authors, grant review panels, even publishers, for the benefit of themselves, and society.

9.2. Further studies especially into the clinic and onto the suffering patients

Since the early 1990's (and motivated long before that in 1978) I have been focused on developing more clinically effective formulations for cancer and now for viral infections. After Thermodox came niclosamide as the main molecule of study because of all the unique properties and cellular influences it can have in all those conditions and diseases as reviewed above and by so many others. "*It just needs testing*" has been my latest plea [185,187] and now you know why. I would encourage you to join us. As listed above in with the text, there are multiple "***Proposed Ideas***" that could easily each become full-blown studies for med chem, drug formulation, *in vitro* cell efficacy, toxicity and pathways and *in vivo* preclinical studies for formulation validation, leading to INDs for clinical testing. I hope some of you do take these on and build that career around niclosamide.

9.3. So let me know

I wrote this article to generate interest and collaborations and enable you to develop your own ideas, obtain funding, and do your own experiments and use niclosamide as a career builder.

I hope that I have brought to your attention an increasingly diverse literature that you can easily now look up yourselves online and read, learn, think, have those new ideas, research, invent and innovate. And as we all know, this takes funds, and so I will offer to help any of you get funded to study niclosamide in all its manifestations (so please do get in touch).

Some of this was purposefully written as a story because, if and when you live it, that's what all research is, a story, a timeline, an arrow into the future. And it's not finished yet, it is waiting for you to write the next chapters. It is each of you that I have imagined are sat beside me as I wrote, thought, schemed, and looked up old and new references. I tried to provide a readable and enjoyable document that tells the science, a personal story (for me and others) that motivates your interest to join me before I run out of time. As I dedicate this for Kinam, please know that you are our legacy.

CRediT authorship contribution statement

David Needham: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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Appendix

Niclosamide: A Career Builder

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A1. Lyerly-Chen-Mook: Niclosamide inhibits the Wnt pathway

As they reported in their 2009 paper, (Chen, Wang et al. 2009) Wei Chen's lab carried out a high-throughput drug screen focused on the plasma membrane proteins that initiated the pathway. The screen was an image-based GFP fluorescence assay that used Frizzled-1 endocytosis as the readout. With it they screened the over 1200 FDA-approved drug and drug-like compounds from the Prestwick Chemical Library, screened at 12.5 μM concentration. This primary screen revealed one hit, niclosamide ("Prestwick 01D11"), that inhibited Wnt/Frizzled signaling induced by the Wnt full agonist, and in a dose-dependent way with an IC_{50} of only 0.5 ± 0.05 μM. This concentration of activity of 0.5 μM to 1 μM (or even less) will crop up again and again as niclosamide was screened and studied in many different pathways, diseases and conditions, even viral infection.

Looking into this in much more detail, Chen and Mook continued working on niclosamide in preclinical studies in colorectal cancer, and it was around this time that Dr. Lyerly had recommended niclosamide as a molecule for me to consider as a focus of my own drug delivery efforts. They presented new data at a 2104 AACR meeting (Mook, Zhao et al. 2014), "*Interrogating the mechanism of Wnt pathway inhibition by niclosamide*", where they confirmed that that niclosamide decreased β-catenin levels and inhibited Wnt/β-catenin signaling and suppressed colon cancer cell growth *in vitro* and *in vivo*.

A1.1. Niclosamide structure–activity relationships for the Wnt pathway

Focusing on the most successful derivatives, Mook and Chen (Mook, Wang et al. 2015) conducted a numerical assessment of the inhibition of Wnt/β-catenin transcription by their TopFlash assay and made substitutions in a series of 35 different niclosamide derivatives, both in the anilide (25 derivatives) and salicylamide (10 derivatives) rings. Judged via the IC_{50} for inhibition of this assay, as shown in Fig. A1, niclosamide itself (A) came in at 0.34 μM.

Of the *anilide substitutions*, only the trifluoro-derivative (B), with a similarly moderate electronegativity to the nitro group, gave a TopFlash IC_{50} of 0.29 μM and so was actually slightly better than niclosamide. The only other substitution that was close to this was the similarly electronegative 2, 4 di-chloro anilide (C), at 0.42 μM.

Substitutions on the *salicylamide ring*, especially at the 2-OH position, gave compounds with comparable IC_{50}s like the methanoate (D) at 0.32 μM and octanoate (E) at 0.23 μM.

What they concluded about these the various substitutions and derivatives was that,

> "*the '4-nitro substituent can be effectively replaced by trifluoromethyl* (Fig. A1 B), *or chlorine* (Fig. A1 C) *and that the potency of inhibition was dependent on the substitution pattern in the anilide ring*".

Interestingly, "*Non-anilide, N-methyl amides and reverse amide derivatives lost significant potency….*"

i.e., taking off the nitro group and leaving a bare benzene ring (IC_{50} = 11.81 μM), substituting a carbamide ($CONH_2$) (IC_{50} = >12 μM) or a methyl sulfonyl SO_2-CH_3 (IC_{50} = 7.66 μM) all reduced the electron withdrawing power that was otherwise provided by the nitro group and these derivatives lost their ability to inhibit Wnt/β catenin transcription in the TopFlash assay.

Most interestingly,

> "*… acylated salicylamide* (Fig. A1 D, *and* E) *derivatives inhibited signaling with potency similar to non-acyl derivatives*".

When they focused on derivatives that increased oral absorption and circulation pharmacokinetics *in vivo*, it was the octanoate derivative of niclosamide (Fig. A1 E), (DK-520) that significantly increased both the plasma concentration and the duration of *in vivo* plasma exposure of niclosamide when dosed orally.

Thus, while niclosamide is poorly absorbed when taken orally (as reviewed in the main text, section *4. Niclosamide in Cancer*) and pharma tend to want to make hydrophobic drugs more water soluble to improve oral absorption, it was making niclosamide more hydrophobic, not less hydrophobic, that showed increased oral absorption. This probably sent it through a different absorption pathway, that of the lymphatics, via the chylomicrons (Gershkovich and Hoffman 2005, Gershkovich, Fanous et al. 2009). In the Gershkovich model, he finds that, "*The most important physicochemical property that affects the affinity to chylomicrons was found to be LogD at pH at pH 7.4*"

As shown in Fig. A1 E, this octanoate derivative, DK-520, was formed when the phenolic OH on the salicylamide ring was esterified with octanoic acid giving a much less soluble derivative. Chem axon calculations show that its solubility in water is predicted to be 2.24nM, and so ~1,000 times less soluble than niclosamide, with a decent LogP of 6.2. When dosing a corn oil solution of DK-520 orally into mice at 200 mg/kg, they were,

> "*… delighted to find DK-520 provided significantly increased plasma exposure of Niclosamide when compared to published studies of 200 mg/kg Niclosamide dosed orally.*"

Pharmacokinetically, the C_{max}, the Area Under the Curve (AUC) for plasma concentration versus time, and the duration of exposure of niclosamide obtained by dosing DK-520 at 200 mg/kg were all increased compared to niclosamide, and this oral dose was well tolerated when dosed daily for three weeks. Furthermore, the plasma levels of Niclosamide were above the IC_{50} inhibition of Wnt signaling

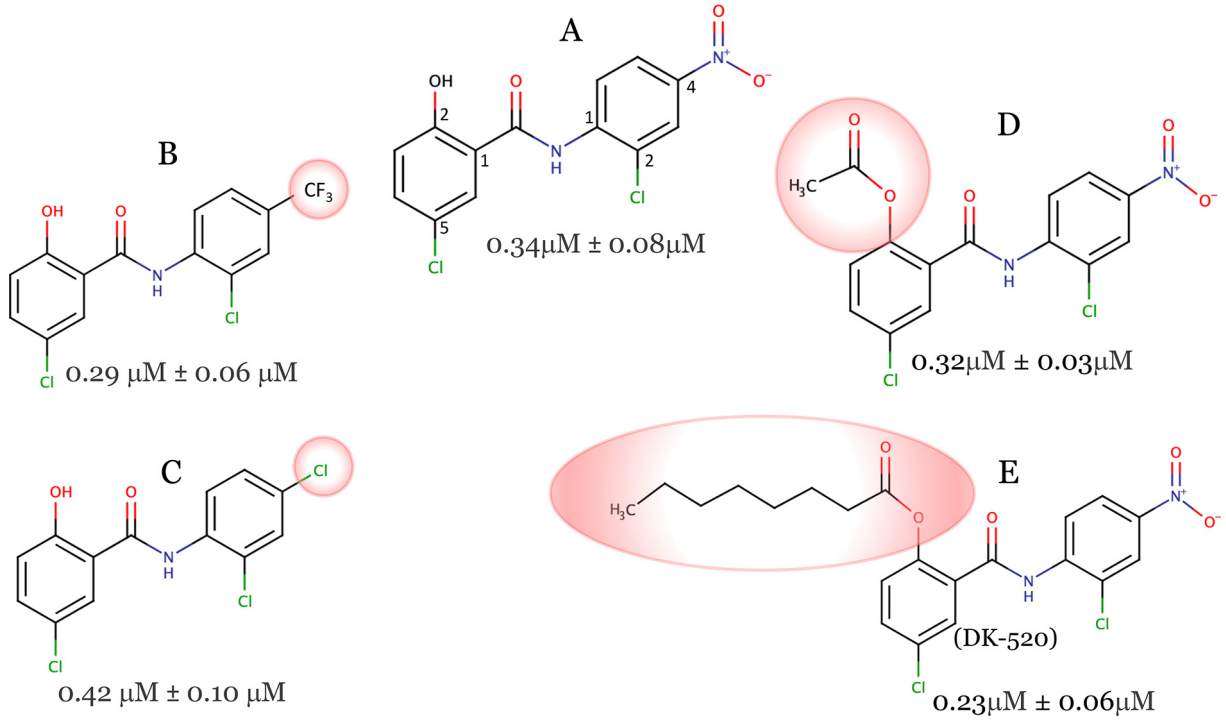

Fig. A1. Structure–activity relationships for some of Mook and Chen's (Mook, Chen et al. 2013, Mook, Wang et al. 2015) successful derivatives of niclosamide. A) Niclosamide; B) trifluoro; C) chloro; D) methyl ester; E) octyl-ester. Also shown are IC_{50}s for the 50% Inhibition of Wnt/β catenin transcription by TopFlash.

in the TOPFlash assay for nearly 24 hr, whereas the plasma levels of niclosamide dosed as a solution at the same 200 mg/kg by oral gavage were only above the IC_{50} for less than 1 hr.

This all might seem a bit too detailed (chemically). However, the reason I introduce this complex chemistry, structure-activity relationships, and focus on inhibition of a particular (Wnt) pathway is two-fold:

1) these data show the importance of:

- substituting groups with comparable electronegativity to the nitro group that preserve or even enhance the weak acid status of the compound;
- substituting with groups that do not have the same electronegativity, or
- making the alkyl esters that appear to, ostensibly, abolish the weak acid character but in fact could create a different route for oral absorption.

These alkyl substitutions are not as simple as they might first appear, especially when the compounds are used *in vitro* in cancer cell culture or *in vivo* in the blood stream and hydrolysis or enzymolysis may de-esterify them.

2) This is one very good example of a research group that were experts at and focused on a particular protein-gene pathway, carrying out a pathway-specific multi-drug screen.

They discovered that niclosamide was active, and following up with a very comprehensive structure-activity study that revealed a few derivatives that showed equal or slightly better promise to niclosamide, and many that didn't.

We are therefore left with the question: "*Was niclosamide acting specifically and dogmatically on one or more proteins or genes in this canonical Wnt/b-catenin signaling pathway? and if so, which one(s)?*" Just like in "Dem Bones", (where the Toe bone connected to the foot bone, Foot bone connected to the heel bone, Heel bone connected to the ankle bone, …. …) this is a complicated pathway where: the ***Wnt glycoproteins*** bind and activate the ***Frizzled and LRP5/6 receptors*** in the cell membrane, that activates cytosolic ***Dishevelled***, leading to internalization of the ***Frizzled receptor***, and stabilization and translocation of ***cytosolic β-catenin*** into the cell nucleus, which activates the transcription factor ***LEF/TCF*** that transcribes ***Wnt/b-catenin target genes***, leading to ***tissue development*** and ***homeostasis***, ***stem cell maintenance*** and ***renewal*** in its normal function, or in cancer, the abnormal activation of the ***Wnt/β-catenin*** pathway promoting ***Cancer Stem Cell*** (CSC) progression leading to ***deterioration*** and ***metastasis*** of cancer (Zhang and Wang 2020).

Or, is the story not necessarily about this pathway *per se*, binding to proteins and or genes etc., but maybe something completely unorthodox that is perhaps adjacent and probably upstream? As in the main text, niclosamide acts on membranes as a proton shunt dissipating important pH gradients. And so, the answer could be, it is not acting directly on any aspect of this (and other) pathways, but indirectly by reducing ATP in the cell, and acidifying the cytoplasm by releasing H^+ from lysozomes such that many proteins are not functioning in their ideal pH range.

A2. We actually did cure cancer with one of these highly toxic chemo drugs

We did actually solve this problem of local anti-cancer drug delivery in Mark Dewhirst's Hyperthermia Program, so I think it is worth mentioning here how one might go about doing this. By inventing (Needham 2001) and developing (Needham, Anyarambhatla et al. 2000, Needham and Dewhirst 2001) a thermal sensitive liposome (Needham 2013b) that could release the drug in the microvasculature of a warmed tumor using clinically-attainable hyperthermia, we successfully delivered one of the most toxic anti-cancer drugs around, Doxorubicin, mainly to the tumor itself.

A2.1. A Little Background on our LTSL-Dox (that became Thermodox)

As I related back in 2016 in the chapter in Anya Hillary's edited book, (Needham 2016) "*Lessons Learned*" our LTSL-Dox was licensed by Celsion in 1999 but, unfortunately, 23 years later, it is still not approved. Even though Celsion took it through two phase III human clinical trials for Hepatocellular Carcinoma *(HCC)*, as they admitted in a personal communication, "*Hepatocellular Carcinoma with Radio-Frequency Ablation in China was probably not the best choice*". To their credit Celsion did set up Celsion-GMBH in Germany, and there is some slow progress testing in breast cancer as above, urology, sarcoma, and bladder.[32]

I know this is all really hard, and a difficult and costly process, so I am actually grateful they are still pursuing it all. Given our data though and even some of the Progression Free Survivals in the ill-fated HCC studies, Thermodox, what is clearly a "*more clinically effective nanomedicine*" tailored specifically to the job in hand, stands a good chance of being effective, if done properly.

A2.2. Preclinical trials

As presented in two papers in Cancer Research in 2000 (Kong et al. 2000; Needham 2000) the LTSL technology concept (as Dox-LTSL) was evaluated in terms of tumor regrowth. The study dosed 5mg/kg Dox-LTSL in tumor bearing mice concurrent with a 1 hr heating of the flank tumors to a mild HyperThermic (HT) temperature of 42°C. These growth delay studies showed that ,for an implanted tumor (FaDu, a squamous cell carcinoma), there was no regrowth in 11 out of 11 tumors out to 60 days. By comparison, an unheated saline control grew to 5 times the original tumor volume (of 5–7 mm diameter) in 10 days. Also, while free drug could only delay the growth by 3.5 days, HT alone did have a significant effect, causing a delay in growth of 10 days, and so Hyperthermia does have some positive effect. When the two approved treatments (i.v. Dox + HT) were combined, i.e., heating to 42°C with injected drug, the growth delay was 14 days. The more traditional non-thermal sensitive liposomes NTSL (like Doxil) had some effects (11-day growth delay) at normothermic temperatures (37 °C), showing how the EPR effect must have been operating for this long-circulating liposome, in this particular implanted tumor.

As might be expected, Dox-LTSL at normothermic temperature only produced a 1-day growth delay, –its lack of effect was comparable to saline and so, being encapsulated, and with a short, encapsulated half-life, it was worse than free drug. When the tumor was heated to 42°C for 1 hr, NTSL had an even greater effect, causing a growth delay of 22 days and this was probably a direct result of the increased extravasation enabled by mild HT, which is known to enhance vascular permeability (Gaber, Wu et al. 1996, Kong, Anyarambhatla et al. 2000). The main result though came with the LTSL cohort, where the tumor was heated to 42°C, the Dox-LTSL was injected, and the tumor heating was continued for 1 hr (Dox-LTSL + HT) and all 11/11 tumors remained regressed out to the full endpoint of the study, which was 60 days,

Kong's paper, measured Doxorubicin in the treated and excised tumor (Kong, Anyarambhatla et al. 2000), total DOX concentrations extracted with chloroform and silver nitrate included the DNA-RNA bound and total fractions of the drug, respectively. Tumors treated with LTSL-Dox + HT showed a significant difference in DOX concentration when extracted with chloroform and silver nitrate (25.6 ng/mg i.e., ~25mg/L which equals ~50 μM doxorubicin the tissue) compared to without silver nitrate (13.1 ng/mg; $P < 0.02$). This indicates that there was a substantial amount (~ at least 50%) of DOX bound to DNA and RNA in these tumors. This is actually quite clear from the confocal images shown later in Fig. A2 B, where the focal red dots are presumably

[32] Although, as of two days ago June 21st, 2023, their web site https://celsiongmbh.com/ was no longer available.

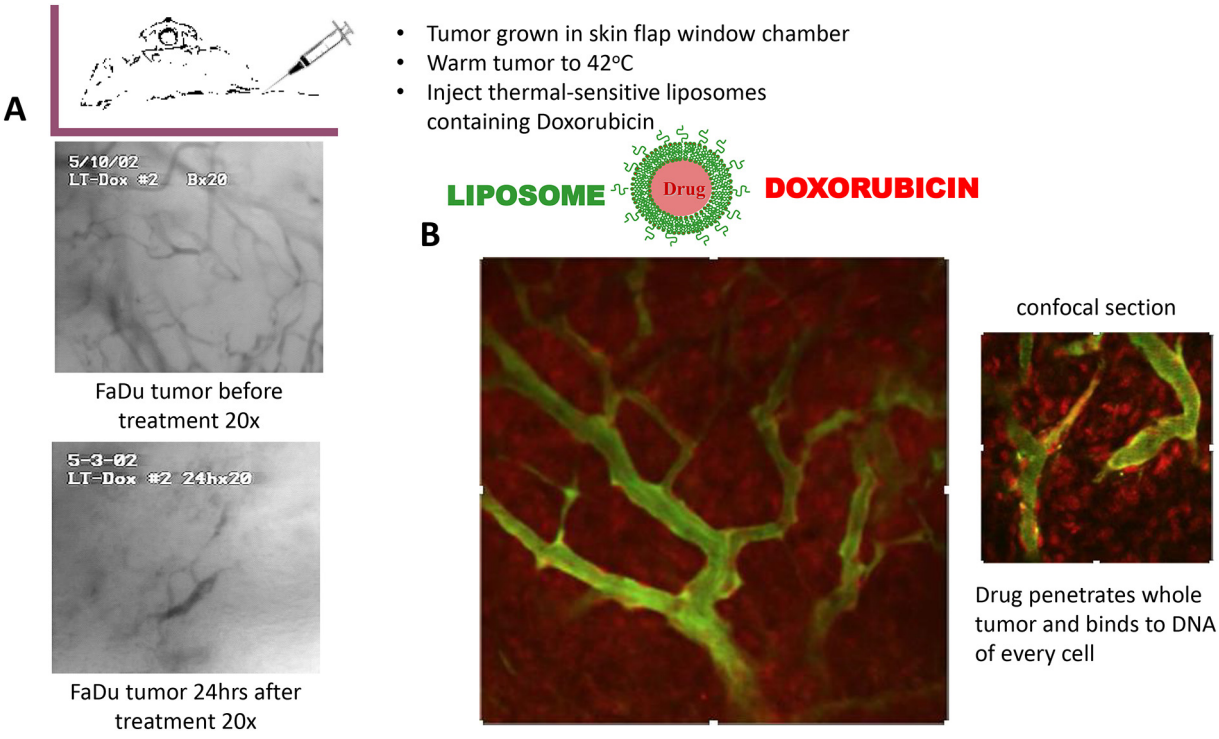

Fig. A2. The only way to get a chemotherapeutic drug throughout entire tumor is to release it in the blood stream of the tumor. A) Window chamber images of a FaDu tumor before treatment and 24 hrs after treatment, – all the blood vessels have been shut down (Chen, Krol et al. 2008); B) Tail-vein injection of thermal sensitive liposomes (fluorescent green membrane, red drug) showing fluorescent confocal images during release and accumulation of doxorubicin throughout the whole tumor within 20 minutes of starting the infusion. From (Manzoor, Lindner et al. 2012).

fluorescent red doxorubicin concentrated in each cell nucleus. Doxorubicin alone gave only 1 ng of doxorubicin/mg of tissue, showing just how effective the LTSL-Dox was at delivering drug throughout the tumor interstitium, and just how poor i.v. doxorubicin actually is, i.e., 1ng/mg vs 25.6 ng/mg of tumor tissue., respectively.

Thus, this hyperthermia-triggered drug release "nanomedicine" strategy was shown to inhibit tumor regrowth in mice (Kong, Anyarambhatla et al. 2000, Needham, Anyarambhatla et al. 2000), and in canine patients (Hauck, LaRue et al. 2006), including a video by the Australian Broadcast Corporation where a dog called "Tucker" was cured of his sarcoma, using our Heat Sensitive Liposome treatment carried out for the first time in 2001. (See https://www.youtube.com/watch?v=UdLEMmRo9oY).

Later, Ashley Manzoor (Manzoor, Lindner et al. 2012) - Mark Dewhirst's graduate student at the time, showed similarly large amounts of doxorubicin accumulated in an already warmed (40.7°C to 41.8°C) window chamber model with LTSL-Dox in the blood stream. This is why we specify that the tumor and its blood vessels need to be warmed first to 41 °C to 42 °C no matter what the hyperthermia device or mechanism. It is the blood vessels that feed the tumor and are such that live tumor cells are fed by oxygen and nutrients delivered by convective transport, with final transport being over small 100 μm diffusion distances. Thus, as I have said repeatedly, "*the only way to get drug throughout a whole tumor is to release the drug in the blood vessels of the tumor*". Here is that data.

As shown in Fig. A2 (A) by the Fan Yuan lab, tumors did not grow, and after 24 hrs their blood vessels had all but disappeared. And then in the later work using a fluorescence rat skin flap window chamber, Manzoor, warmed the tumor to 41 °C to 42 °C and injected the thermal sensitive liposomes containing doxorubicin into the tail vein of the rat with a flank tumor (Manzoor, Lindner et al. 2012). As shown in (Fig. A2 (B), within 20 minutes, the confocal video showed that the tumor filled up with the red fluorescent drug, and every cell's DNA was pink.

Basically, then our recommended protocol is: the tumor is warmed to 41 °C–42 °C, the Low temperature thermal sensitive liposomes containing doxorubicin (LTSL-Dox) are injected, the tumor is kept warm for 30 mins. Thus, the injected LTSLs circulate around the body, and, while they do leak drug slowly in the blood stream (half-life is 1 to 1.5 hrs), they release their drug mostly in the warmed tumor vasculature, within 2 seconds of reaching the critical temperature of their gel-to-liquid phase transition at 41 °C to 42 °C. As the gel phase, mostly DPPC liposome goes through the phase transition, the grain boundaries melt and their permeability to encapsulated drug solution is enhanced by the presence of the 10 mol% lysolipid, see these papers for more information (Needham and Dewhirst 2013, Needham, Park et al. 2013).

A2.3. Clinical trials

Carried out in ~2008, and reported fully in 2014, we also showed activity in human female patients with Recurrent Chest Wall (RCW) cancer after mastectomy, (Zagar, Vujaskovic et al. 2014). Thus, when tested in clinical trials, as shown in Fig. A3 (C) a woman's chest wall cancer after mastectomy disappears. In this "DIGNITY" phase II trial for Chest Wall Recurrence after mastectomy a female patient (Fig A3 (A)) with a chest wall cancer is shown receiving hyperthermia treatment. The chest wall cancer is shown in Fig A3 (B) prior to treatment. After 4 cycles of the thermal-sensitive liposomes, the widely disseminated chest wall tumor had completely disappeared. While the maximally tolerated dose is 55mg/m^2, she had a Complete Response at 30 mg/m^2.

Our recommended protocol from our preclinical studies therefore was:

- Warm tumor to 41 °C–42 °C
- Inject liposomes
- Keep tumor warm
- Kill tumor
- (Get it approved)

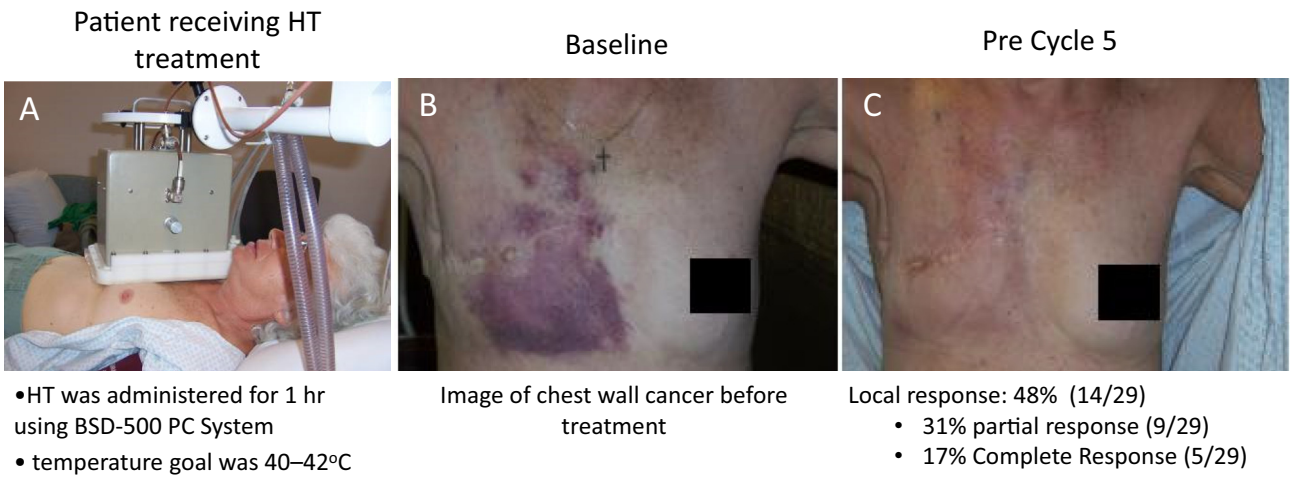

Fig. A3. "DIGNITY" phase II trial for Chest Wall Recurrence after mastectomy –started in 2006. A) Patient receiving Hyperthermia treatment of her cancer; B) Photographic images of the same patient with chest wall recurrence of breast cancer; C) Same patient after 4 cycles (thermogram in pre cycle 5) the widely disseminated chest wall tumor had completely disappeared for a Complete Response 30 mg/m^2.

Unfortunately, the above protocol, was not adhered to by the Licensee of the technology, Celsion Corporation in their Phase III clinical trials using the Radio Frequency Ablation (RFA) heating system in Primary Liver Cancer (Hepatocellular carcinoma –HCC). Their protocol and results were:

- Inject liposomes first and lose most of the drug
- Warm tumor but using a central-ablation heating mode
- Temperature fluctuates and so does not reach or maintain release temperature especially at the periphery where recurrence occurs
- Don't kill tumors, (well, kill some of them, but not enough to show positive Progression Free Survival)

As a consequence, Thermodox was not approved after being licensed to Celsion in Nov 1999, and 23 years of various trials including 2 in phase III (Tak, Lin et al. 2018, Celsion 2020)

For a full account of what happened in terms of clinical development, see these chapters I wrote for Kinam's and Anya's books. They describe the thermal sensitive liposome (by reverse engineering it) (Needham 2013a), what happened in that series of clinical trials and the lessons learned (Needham 2016), and why it is apparently so difficult to develop clinically effective formulations (Needham 2020).

For a more detailed review and in-depth analysis of the current status, as well as potential challenges faced by continued clinical translation of thermosensitive liposomes, do see Christine Allen's (Dou et al) very important paper "*To heat or not to heat: Challenges with clinical translation of thermosensitive liposomes*" (Dou, Hynynen et al. 2017). They provide an excellent in-depth (and independent) analysis that, combined with my observations above, gives a very good overview of the reasons Celsion failed to achieve approval after two Phase III trials that did not meet the required PFS endpoints. Their figure, as reproduced in Fig. A4, basically sums this up.

A2.3.1. High frequency ultrasound?

New studies now in Phase 1 trials (as mentioned in the main text), are using High Frequency Ultrasound, (HiFu) to heat the tumors. While I hope this shows promise, one problem I see here is that HiFu may also not actually warm the tumors to the correct temperature for LTSLdox release, that requires at least 41 °C–42 °C to be optimal. While transient peaks can spike to 43 °C, there are prolonged cooling periods between treatment cycles and so the average temperatures are much lower. The Coussios group in Oxford (Lyon, Gray et al. 2018), recently carried out an open-label, single-centre, phase 1 trial using Thermodox in order to examine HiFu capabilities. The study was on unresectable and non-ablatable primary or secondary liver tumours. As shown in their Fig. 3: "*Illustrative controlled hyperthermia by focused ultrasound*", a 90.9 cm^3, (4.3 cm diameter) was exposed to focused ultrasound at 115 W in a 70% duty cycle in linear mode. Taking only 39.5 °C as their "release threshold" (when optimally it is 41 °C–42 °C), the recorded tumor tissue temperatures were always below 40 °C, and so suboptimal. Similarly for a smaller 68.3 cm^3 tumour volume (3.3 cm diameter) and increasing power to 125 W and duty cycle to 77%, the average temperature was still only between 39.8 °C and 40.8 °C (reading off the graph) for the remaining 35–80 min. Maybe conditions and the technique can be improved, but as it stands, HiFu has not achieved a continuous and stable 41–42 °C due to its inherent transient peaks and prolonged cooling periods between treatment cycles. And so, like RFA, that may well provide

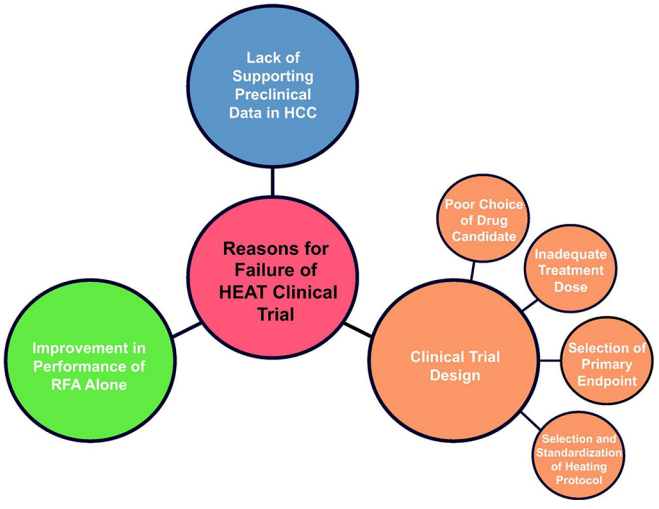

Fig. A4. (Fig. 3. from Dou et al, (Dou, Hynynen et al. 2017)). *Potential reasons for failure of the HEAT clinical trial. The major causes resulting in failure of the HEAT clinical include poor choice of drug candidate, inadequate treatment dose, selection of primary endpoint, selection and standardization of the heating protocol, improvement in performance of RFA alone, and lack of supporting preclinical data in HCC.*

ablation at 55 °C but can also char the tissue reducing thermal diffusion, HiFu does not achieve the required temperatures in the tumor margins where relapse occurs at least in their liver tumors.

A2.3.2. And what about Prostate and Chest Wall, that can be reliably heated?

There was early positive data on Prostate (~2002-2003) heated by the Medifocus, Prolieve, trans-urethral heating catheter established for benign prostate hyperplasia (BPH) that can heat the margins of the prostate to 43 °C.

Also, as shown above, Recurrent Chest-Wall (RCW) of breast cancer after mastectomy, was heated by a BSD applicator and cured. So, why was LTSL-Dox not approved 15-20 years ago? It seems that a company decision by Celsion was made to abandon these patients because of low incidence in preference for the much greater number of cases and remuneration from Hepato-Cellular Carcinoma (HCC). The Global burden of liver cancer is such that, in 2020, an estimated 905,700 people were diagnosed with, and 830,200 people died from, liver cancer globally (Rumgay, Arnold et al. 2022).

Given our *in vitro* phase transition and drug release data and preclinical data showing drug throughout the tumor in 20 minutes (see again Fig. A2 B), is likely that the LTSL-Dox itself didn't fail; rather the use of relatively uncontrolled Radio Frequency Ablation (RFA) technique failed by not heating the right sized and heatable tumors and their margins to a high enough 41 °C–42 °C temperature to release the doxorubicin in the tumor vasculature.

Prostate cancer and its treatments can disrupt normal urinary, bowel, and sexual functioning (pcf.org 2023), and, in RCW, patients with tumors greater than or equal to 4 cm or at least 4 involved nodes experience local recurrence rates more than 20% (Katz, Strom et al. 2000). Thus, a return visit to Prostate and Prolieve, and RCW and the BSD applicator are certainly warranted, since these cancers can be readily heated thought the tumor mass to 43 °C, and, especially, RCW is still not effectively managed with surgery, chemotherapy, hormone therapy, radiation alone, or radiation with hyperthermia, and so Thermodox could at least enhance these more traditional treatments.

A3. The drug development and testing process for new drugs

Before I try to address the reformulation of existing drugs that are in need of more clinically effective formulations, I thought it might be instructive to briefly explore what happens from the start of drug development for a new drug, by reviewing the story of Lapatinib, GSK's tyrosine kinase inhibitor for cancer. Lapatinib was brought to my attention by a Duke Clinical Researcher, Neil Spector MD,[33] who had been the director of Exploratory Medical Sciences-Oncology at GlaxoSmithKline and then transferred to the Duke Cancer Center in 2006; I met him in ~2010.

A3.1. Preamble

I imagine that most people will be familiar with traditional drug development and testing, starting from scratch that focuses almost entirely on the new compound. It appears that any "formulation issues" that might impair or reduced its clinical effectiveness are only considered later, and it is usually formulated by default as an oral tablet. Thus, there seems to be a total disconnect between the drug and its eventual "formulation" in traditional preclinical testing and this might be the reason why 90% of drug candidates in clinical trials fail, (Sun, Gao et al. 2022). Fogel (Fogel 2018) has some suggestions as to what to do about it, but still, many drugs also fail to provide minimal additional efficacy even when approved. According to Downing et al (Downing, Shah et al. 2017), a third of the drugs the FDA approved between 2001 and 2010 were involved in some kind of safety event after reaching the market. And in an article by Darrow Kesselheim (Darrow and Kesselheim 2017), *"20 percent of expedited drugs and 41 percent of non-expedited drugs offered zero or negative incremental health gains over older comparators"*. They suggest that there should be a greater focus on efficacy and so this brings an opportunity for a more effective reformulation. While this is a topic for another day, these numbers are actually quite shocking, and one wonders "*what were they thinking?*"

A3.2. The drug development and testing process starting with a new drug: Lapatinib

As we all know, while it is rare to start from scratch, (there is always some evidence even if it's from prehistory use or "old-wives-tales") this is a very complex and involved process. The drug I chose for this exploration is ***Tykerb*** (Lapatinib) that was specifically designed by Rusnack et al at GSK (Rusnak, Lackey et al. 2001). Each stage of this development process that was successful is denoted with a (✓); the parts that were not successful (or at least optimal), and prompted Neil's request for reformulation, are denoted with ($\mathcal{X}$)

A3.2.1. New drug is designed

As described by Rusnak et al in 2001, (Rusnak, Lackey et al. 2001), GW572016 was designed with novel and robust chemistry for a particular cellular pathway. It targeted the epidermal growth factor receptor (EGFR) in HER2 cancers with a tyrosine kinase inhibitor that competed for ATP and would block downstream signaling and so would be antiproliferative, although not curative. A series of compounds were made (GW2016, OSI-774, and ZD1839), as powders and likely stored in vials. (✓)

A3.2.2. Drug is dissolved in DMSO and tested in vitro against kinase domains

Serial dilutions of GW2016 beginning at 10 μm were added first to intracellular kinase domains of EGFR, ErbB-2, and ErbB4, purified from a baculovirus expression system. Domains of VEGF were also tested. It was found that "*GW2016 is a potent inhibitor of the ErbB-2 and EGFR tyrosine kinase domains with IC_{50} values against purified EGFR and ErbB-2 of 10.2 and 9.8 nM, respectively*", and >300-fold more selective for EGFR and ErbB-2 over other kinases tested. (✓)

A3.2.3. Drug is dissolved in DMSO and tested in vitro against human tumor lines

GW2016 in DMSO was then added to a series of human tumor cell lines overexpressing either EGFR or ErbB-2 or negative controls. "*Treatment with GW2016 resulted in IC_{50} values of ≤0.16 μm on the EGFR- and the ErbB-2-overexpressing tumor cell lines*" Importantly for therapeutic index considerations, the average of the tumor cell IC_{50} values, GW2016 was ~100-fold more potent on the tumor cell lines than on the normal fibroblast cells. (✓)

A3.2.4. In vivo validation in mice

GW2016 was tested *in vivo* for growth of BT474 and HN5 human tumor xenografts. Drug administration was done per orally (p.o.) by oral gavage, 30 and 100 mg/kg, twice daily for 21 days in a vehicle of sulfo-butyl-ether-β-cyclodextrin 10% aqueous solution (CD10). GW2016 was a potent inhibitor of growth for the human tumor xenografts. A dose-responsive showed complete inhibition of tumor growth at 100 mg/kg dose, with <10% weight loss in treated animals over the 21-day treatment. They also confirmed efficacy in assays of outgrowth, cell cycle, and effects on signal transduction. (✓).

A3.2.5. Huma clinical testing

Human studies were started by determining the safety, tolerability and pharmacokinetics of single and multiple oral doses given to healthy

[33] Neil unfortunately passed away in 2020, see "Gone too soon: Dr. Neil Spector Passes Away", http://dukecancerinstitute.org/news/gone-too-soon-dr-neil-spector-passes-away.

subjects (Bence, Anderson et al. 2005). Data showed dose-related headache, diarrhea, rash, cold symptoms, gastrointestinal symptoms from single and multiple oral doses of GW572016, that were nevertheless considered tolerable.

Human trials then continued with a phase I study in 2005, (Burris, Hurwitz et al. 2005), in heavily pretreated patients with metastatic carcinomas. In an interesting and rare personal view of *"The Discovery of Lapatinib (GW572016)"*, by the discoverers, Rusnak and Gilmer, (Rusnak and Gilmer 2011), ten years on, they relate, in their own words, that:

> "*The selectivity of lapatinib for HER2 and EGFR kinase domains and its activity in HER2-overexpressing cell lines (e.g., breast and gastric cancer) and EGFR-overexpressing cell lines (e.g., head and neck cancer) provided a foundation to test lapatinib in selected patient populations. The U.S. Food and Drug Administration's approval in 2007 of lapatinib, in combination with capecitabine for treatment of advanced or metastatic HER2-overexpressing breast cancer, provided another option for patients whose disease progressed on trastuzumab (a humanized monoclonal antibody directed against the extracellular domain of HER2). Ongoing clinical trials are examining lapatinib activity in HER2-overexpressing breast cancer, HER2-overexpressing gastric cancer, and head and neck cancer*". (✓)

And indeed, on-going trials in breast cancer were carried out, including a phase II study reported in 2009 for a Lapatinib monotherapy in 126 patients with HER2-overexpressing relapsed or refractory inflammatory breast cancer (Kaufman, Trudeau et al. 2009). They each received 1500 mg as oral tablets once daily in a non-randomized, open-label, phase II study.

As they reported, the results were, "*No patients had complete response. 49 patients (39%) had partial response. Median progression-free survival was 14.6 weeks, with median duration of response of 20.9 weeks. 130 (92%) of 141 (total) patients had at least one adverse event; 45 (32%) had serious adverse events, the most common were dyspnoea (eight patients) and pleural effusion (six). Five patients had fatal adverse events that were possibly treatment related*". (✗)

To be fair, they do also say that "*The cause of these events is difficult to discern because of the poor clinical outcome inherent in this heavily pretreated population of patients with inflammatory breast cancer*" and was also possibly related to underlying disease. Nevertheless, the conclusion and recommendation was that "*Lapatinib monotherapy is a potentially effective treatment for relapsed or refractory HER2+ inflammatory breast cancer*".

To his credit, Neil then went on to examine patient data in more detail to see if he could figure out what happened and what could be done to optimize the treatment. In a subsequent paper *"Lapatinib Plasma and Tumor Concentrations and Effects on HER Receptor Phosphorylation in Tumor"* (Spector, Robertson et al. 2015) he and an extended team at Duke, local experts in Durham NC, Illinois, GSK, Pennsylvania, Miami, and Texas, examined PK and tumor drug delivery data. They recognized that while cytotoxic drugs had been the main stay of cancer treatment and, as in Doxorubicin's case, were highly toxic to everything (*when your white cell count gets too low, we'll stop for a while*) tumor targeted therapies represented a paradigm shift to cytostatic control. Dosing was optimized based on a biologically effective dose, rather than the historical maximum tolerated dose, but they could still be toxic and dose limiting. So, that was considered an advance.

In what was quite a difficult-to-coordinate study at multiple institutions, they sought a deeper understanding of the plasma-tumor relationship, tumor efficacy from plasma, and tumor sampling in mice and humans. Mice were dosed with an oral suspension in water with 0.5% hydroxymethylcellulose and 0.1% Tween-80, at 100 mg/kg every 12 hours (6 doses), or 200 mg/kg every 24 hours (3 doses). Human patients receive oral lapatinib at 1000 mg once-daily (QD), 1500 mg QD or 500 mg twice-daily (BID) for 9 days prior to definitive surgery (mastectomy, lumpectomy).

Perhaps surprisingly they found that the concentrations of lapatinib in plasma were actually less than drug levels in the tumors. "*In mice, lapatinib levels were 4-fold higher in tumor than blood with a 4-fold longer half-life. Tumor concentrations (~ 900 nM or 500 ng/mL) exceeded the in vitro IC_{90} for inhibition of HER2 phosphorylation throughout the 12-hour dosing interval. In patients, tumor levels were 6- and 10-fold higher with QD and BID dosing, respectively, compared to plasma trough levels*".

Neil was therefore interested in a more optimal dosing focused on the site of action to avoid inappropriate dose escalation. It was while he and his team were contemplating doing this study, and he was wrestling with the issues in the previous human trial data, that we met in ~2010. The conversation went something like this:

(NS): *Dave, can you help us with a new formulation of Lapatinib? Like, put it in a liposome?*

(DN) *"Sure Neil, but why would you want to put such a hydrophobic drug in a liposome?"*

(NS) *"Oh, I don't know, but put it in something"*

And that's what started my journey of seeking and coming up with a new way to formulate similarly low solubility drugs (bricks) as even lower solubility prodrugs (as rocks), for our *Bricks to Rocks technology* that forms the section in the main text 7.4 "*Make the drug look like the cancer's food": they all have to eat*.

A3.2.6. Summarizing the Lapatinib drug development

So, there you have it. A well-established pharmaceutical company (GSK) generates a new drug, (GW2016), demonstrates binding against the molecular target, shows efficacy in cells, and validates, to some extent, in preclinical animals. They generate GMP oral tablets and carry out, undoubtedly, very expensive multiple clinical trials that show no complete responses, and only modest partial responses, and in an admittedly heavily pretreated population of patients with inflammatory breast cancer, five patients on a trial had *fatal adverse events that were possibly treatment related.* And then, because I was known (at least within Duke) for coming up with a liposome for treating cancer, one of the lead clinicians, who was still stumped as to what to do to deliver this very effective 10nM-efficacy drug to a patient's tumor, asked me if I could reformulate it. As a result of this conversation, I was motivated to try and solve it. It turned out that I didn't solve it for Lapatinib, (and now there are better TKIs anyway), but I did for niclosamide.

One final point here is that Lapatinib and all TK Inhibitors are not cures, they are cytostatic drugs, and so are taken daily to control tumor growth, but not eradicate it. As a result, though, cancer could be managed as a chronic disease.

A3.3. Neil's plea stimulated new ideas

As a result of Neile's plea for Lapatinib, in 2011, I started exploring the whole literature on hydrophobic drugs and how one might go about reformulating them, and, as per Neil's request, delivering them to tumors. I generated an unpublished 42,000-word, 60-page tome called ***The Formulation of Hydrophobic Anti-Cancer Drugs. Part II. Engineering Design for the LDL-like Drug Nanoparticle*** (Needham 2012).

In reviewing the Lapatinib literature I came across, what was for me then, (but probably not for any card carrying Pharmacokineticist who knew about these things) a curious observation: –the systemic exposure to *Lapatinib* was increased when the drug was administered with food (Ratain 2007). A randomized, cross-over, food-effect study (Burris, Hurwitz et al. 2005, Reddy 2007) demonstrated that both peak concentration and area under the concentration–time curve were increased markedly when a single 1500mg dose of *Lapatinib* was taken with food as opposed to when fasting. Both were increased further by patients who ate a *"high-fat meal"*; an increase in the AUC was 325% and 167% when the oral drugs were taken with high- and low-fat meals, respectively. This was what first got me thinking about hydrophobic drugs

and going deeper into the cancer cell literature I focused on what cancers feed on to survive, grow, replicate and become more aggressive – LDLs and VLDLs and also albumin.

The bottom line was I discovered that:

- rapidly growing cancer cells have high numbers of LDLRs, some 4-100x greater than on normal cells.
- Numerous malignancies are known to over-express LDLR including brain, colon, prostate, adrenal, breast, lung, leukemias, and kidney tumors.
- As a result, cancers are known to take-in more LDL than normal cells, and
- in patients with cancer, their Low-Density Lipoprotein (LDL) count is even known to go down.
- An abundance of LDLR is also a prognostic indicator of metastatic potential, and a propensity to store cholesteryl ester is a sign of the aggressiveness of a patient's cancer.

With 52 pages of text, 50 figures, and 219 references, my unpublished white paper (Needham 2011) (I wrote it for my own benefit) sought to review the LDL –Nature's own hydrophobic delivery system. I tried to answer basic questions such as, *"What makes the LDL so effective at reaching its normal targets (adrenals, muscle, liver) and also cancer cells?"*, and *"How are its contents processed?"*. What I learned created an endogenous inspired formulation for hydrophobic anti-cancer drugs, that formed the basis for our Bricks to Rocks Technology (B2RT) of converting the poorly absorbable low solubility "bricks" of the cancer drug delivery industry into even more insoluble "rocks", so that they could be precipitated and controlled as nanoparticles of the same size as LDL:s and VLDLs and so "*make the drug look like the cancer's food*", – they all have to eat.

Thinking about how to develop and test these formulations then generated of the next story, the *Laboratory to Clinic Translational Development for Cancer*, and my new focus on niclosamide and what it would take to reappropriate and more importantly reformulate existing drugs that could perhaps provide more clinically effective results if reformulated for i.v. administration, as that "cancer's food" . See the main text for details of the B2RT formulation and in vivo studies in mice and canine patients *7. The Niclosamide Stearate Prodrug Therapeutic (NSPT) for Cancer (Osteosarcoma).*

A4. The drug development and testing process for reappropriated drugs

Here, I would like to introduce what I see and have experienced as the drug development and testing process from my own experiences based on reformulating two drugs, Doxorubicin and Niclosamide, as described at length in the main text and some in this Appendix. This will establish a structure for new studies in various diseases and conditions that could help generate and build those new careers.

In quite stark contrast to new drug development (see above for Lapatinib in *A3.2*), the repurposing process, for me, is not just reappropriating an existing tablet but is all about a true reformulation, that provides a more clinically effective drug or prodrug matched to a particular administrative route. Of course, in an ideal world, ALL DRUGS need a clinically effective formulation. Here, though, we are likely dealing with the same (or new) indication but now with a new delivery route. Maybe the original drug failed as an oral tablet, or is not optimal as for example a cremophor emulsion (paclitaxel), or as a liposome (Doxil), and so needs that clinically more effective formulation (Needham 2020).

A4.1. Laboratory to clinic translational development for cancer: repurposing and reformulating existing drugs

Shown in Fig. A5 is what I have called the *Laboratory to Clinic Translational Development for "X"*, where "X", for me, has largely been for *Cancer*. The schematic depicts what I have experienced in the bench-to-bedside development and testing of Thermodox (Needham, Anyarambhatla et al. 2000, Needham and Dewhirst 2001, Needham 2013b, Needham 2016) and now our *Bricks to Rocks Technology* (B2RT) for cancer (Needham 2020, Reddy, Kerr et al. 2020, Eward, Needham et al. 2023) as well as developing a new preventative nasal spray and early treatment throat spray for COVID19 and other respiratory infections (Needham 2022, Needham 2023, Needham, Kelleher et al. 2023). They all need testing (properly). For "Repurposing and Reformulating Existing Drugs", the scheme starts at the top, and circles around each of the tasks in the drug development and testing scheme.

A4.1.1. Failures in the clinic

We start with number 1, current treatments in the clinic where there are clinical successes but with known deficits (including marginal efficacy, poor bioavailability, or toxicity concerns). As lab researchers, where do we find this out? On-line notifications from MedPage today or Cancer network? Scouring the Literature? Well sure, but I would encourage you to develop a relationship with one or more of the clinical fellows in your medical center (see my next "***As an Aside").*** As I advised in my last chapter, *"Development of clinically effective formulations for anti-cancer applications: why it is so difficult?"* (Needham 2020), for Kinam's second edition of his edited book "*Biomaterials for Cancer Therapeutics: Evolution and Innovation*", in one of my notes to students: Align your research locally. As above (*A3.2.5*) I was approached by Neil Spector back in 2010, asking if I could help reformulate the drug, Lapatinib (Spector, Robertson et al. 2015), that they had trialed for GSK, but had largely failed.

As an Aside: *Seek to talk with a clinician first*: One lesson learned from this is that, as basic researchers in drug development, it is the clinicians that can give you an initial go-no-go on the advanced formulation, is it even administrable? and in what population(s) of patients? and for what cancers? They are also your path to translation and impact. But beware and treat them gently. I have it on good authority from one of my close clinical collaborators that most are increasingly overwhelmed and under stress. "*Every month we are squeezed tighter and tighter such that the minutiae become increasingly prominent and obstruct the higher-order tasks. Modules, meetings, ever-more forms to document various aspects of teaching and/or patient care. Faculty spend less time doing research and writing papers and more time doing...other...stuff*".
Here's a paper describing that stress pre-COVID (Kumar 2016) and it has become worse since then (Ali 2022). So, this stress is not just from their clinical care, but increasingly by so many more meetings, administrative duties, forms to fill in, clinical trials to run (sometimes futile but lucrative, especially for the hospital system and medical school). They may also be trying to establish or keep a research lab, but, if the grant goes down, their effort is reassigned to the clinic, and they never recover.
They are, however, the ones on the front line, who care with an oath, who know what has not been working for their patients, who know most about what has failed in those trials, and what is needed (like Neil did 13 years ago). They could also be not only the people who can tell you this, they could also have research labs doing preclinical testing, or companies screening drugs for

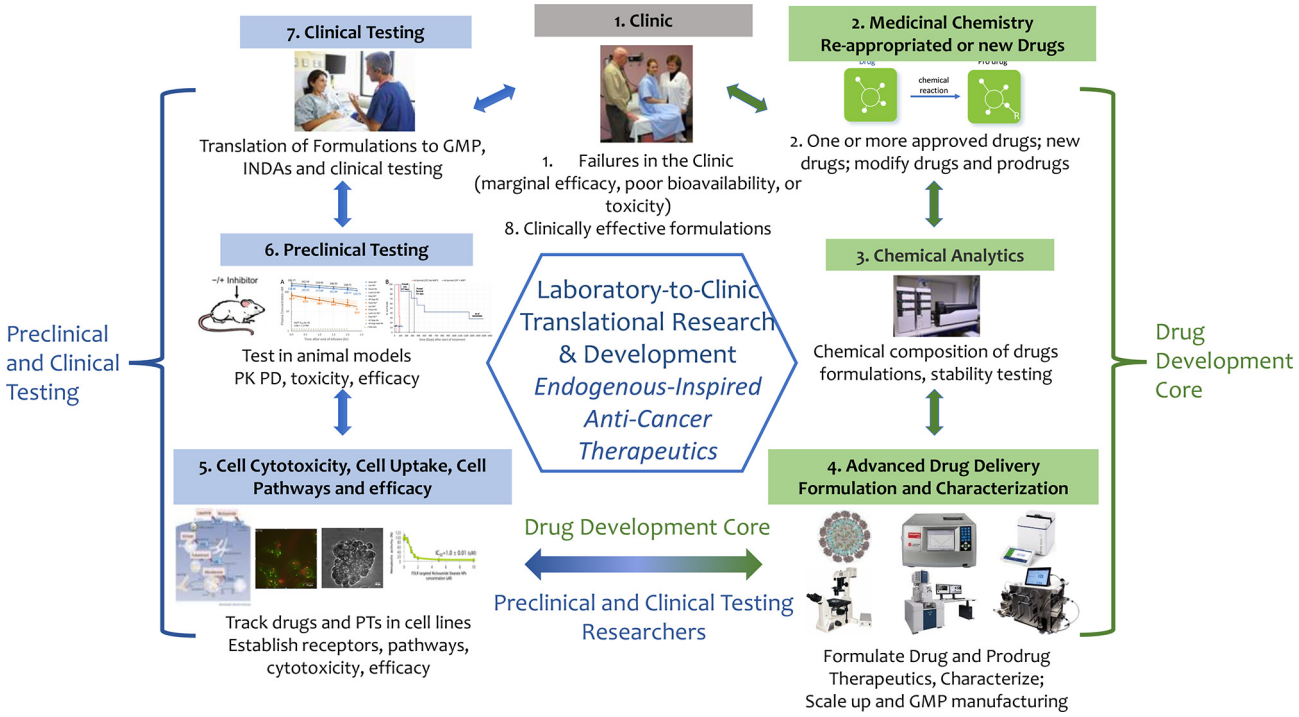

Fig. A5. Laboratory-to-clinic translational research and development – endogenous-inspired anti-cancer therapeutics.

patient's cancers. Importantly, they could also be the people who would eventually test the new formulation as PIs, when we come full circle, on any investigational new drug application (IND). If you want to know what drugs to formulate, do not ask a Pharma company, ask a clinician.
As we will see, niclosamide (as with many drugs) despite overwhelming cellular data that it has broad range activity in many diseases and conditions, it has failed to be reappropriated as an oral tablet in the clinic beyond its original anti-helminthic application. Niclosamide is prime, is crying out, for more clinically effective formulations to be tested in the clinic. So, drug researchers and clinicians, please make an extra effort to get together.

A4.1.2. Medicinal chemistry: re-appropriated or new drugs

For cancer, many of the chemotherapeutics given by intravenous (i.v.) infusion are limited by systemic toxicity or, if given as tablets, can often be limited in efficacy by poor oral bioavailability. As discussed by Altunel and Hsu et al (Altunel, Roghani et al. 2020),[34] the failure rate for new cancer drugs is more than 80% in Phase II and 50% in Phase III (Arrowsmith 2011a, Arrowsmith 2011b). One other reason for the high failure rate is that, while pathway studies, combinatorial chemistry, and drug design have led to the development of new drug candidates, the majority of clinical failures are still due to poor drug-water solubility (Kalepu and Nekkanti 2015) and hence inadequate efficacy (Fogel 2018). Since the preferred route for most drugs is oral, poor solubility leads to low absorption and low bioavailability resulting in suboptimal drug delivery and poor clinical outcome (Merisko-Liversidge, Liversidge et al. 2003). With 40% of approved drugs and 90% of molecules in the discovery pipeline being poorly water-soluble (Kalepu and Nekkanti 2015), there is an urgent need to develop and test new strategies that achieve greater bioavailability, more specific (passive) tumor targeting and reduced systemic toxicity.

Thus, there are many drugs that already exist and are given in a way that produces toxicity or is not very efficient. Reappropriation-by-Reformulation of existing drugs could be a major focus, but also, as new drugs come available, or are discarded because, again, of poor oral bioavailability. Because of this discovery- and physicochemical-trend towards increasing hydrophobicity, new drug candidates cannot even be successfully validated in animals. My advice here is to find, or again, be motivated by your local medicinal chemist(s) to redesign new prodrug formulations. For the medicinal chemists consult reviews on structure-activity-relationships of niclosamide that could show promise but have been lost in the literature or create new ideas for an optimized "niclosamide", see the main text (*3.5 Brief Historical Perspective on structure-activity relationships (SARs)*) and above, (*A1.1 Niclosamide structure–activity relationships for the Wnt pathway*) and below, ***A6. Medicinal Chemistry of Uncouplers).***

A4.1.3. Chemical analytics

Chemical analytics is important for determining and confirming the purity and chemical composition of the new prodrugs, as well as the analysis of prodrugs in the new formulations, including stability testing. This is usually more of a facility at the institution than a research lab, but it is well worth engaging them, paid on a grant. They are often run by very accomplished analytical chemists with experience in the pharmaceutical industry, supporting process chemistry, QC, and formulation. They have good experience in quantitative and qualitative analysis using a wide range of techniques, including, chromatography, spectroscopy, mass spectrometry, and physical-biophysical tests. As a result, they are often only too keen to advise on many aspects of not just the analysis but also on the expected data required in a GMP/GLP-compliant environment. So, these, ostensibly, "contracted-facility" analytical chemists can be a hidden gem in the process.

[34] David Hsu, MD, PhD at Duke, is one of my new collaborators on a new R21 submitted to NIH taking the Bricks to Rocks Technology as an SN38-prodrug for colorectal cancer.

A4.1.4. Advanced nanoparticle formulation and characterization

While this is about providing, say, an advanced *nanoparticle* formulation for the drugs and prodrugs, a forgotten "art" is the pre-formulation characterization of the drug itself. As one of the main themes for this article, I (and others (Niazi 2007)) think it is necessary, even beneficial, to understand the drug or prodrug first before choosing one of many potential "nanomedicine-scaffolded-formulations". As in many discussions with Kinam, and as I spell out in that chapter (Needham 2020), many of the starting points for the myriad new "nanomedicines" focus more on the development of the liposome, polymer, micelle, dendrimer, or chitosan (LPMDCs) rather than on the drug itself. As I started out describing in the beginning of Part II, "Nanomedicine" more broadly refers to the cellular and molecular world of disease at the scale at which it occurs. However, recently, the term has become synonymous with a variety of different nanoparticle or macro-molecular formulations where the drug is the after-thought. It seems to be all about what the LPMD or C can do, and it is the "novel formulation" and not necessarily its actual "effectiveness" that the researchers, grant reviewers, and journals are most excited about. As I ranted on in that chapter, the criteria for publishing in the literature seems to be the potential "promise" of a new formulation rather than the problem it will actually solve for a particular drug and disease or condition in the clinic (if it ever gets there).

A4.1.4.1. A preformulation drug characterization could reveal a simple solution or suspension. I therefore reiterate here that it is the physicochemical properties of the drug that should be our starting point for any nanomedicine formulation; any additional matrix or encapsulation materials should only be included if it enables and enhances its processing or performance-in-service. Actually, I would encourage all drug delivery people to check out this excellent book by Sarfaraz Niazi (Niazi 2007)[35] "*Handbook of Preformulation Chemical, Biological, and Botanical Drugs*". Professor Niazi, (who seems like quite a character). In it, he posits a similar tenet to mine,

"*Drug discovery precedes preformulation studies but often a preformulation feedback helps faster drug discovery*". And, speaking about the working relationships between "*the two major groups of scientists: those at the drug discovery end and those at the drug delivery end*" he gives the preformulation group a very important role, and so I quote it here for your information and amusement.

> "*Whereas scientific camaraderie, or perhaps stubbornness, at the two ends of new drug development has historic roots, it is the preformulation group of comrades that brings peace to the table. It is often humbling for the drug discovery group to bring out a novel molecule with remarkable potential only to be shot down by the formulation group as a worthless exercise in taking it to a deliverable form. The preformulation group works with both ends and helps reduce the overall cost and shrink the timeline of drug development*".

While he writes mainly on the more traditional formulations for solid dosage forms, solution formulations, emulsion formulations, freeze-dried formulations, suspensions, topical and pulmonary delivery, there is nevertheless the same interest, concern, and knowledge-application as should also go into the more Advanced Formulations and "Nanomedicines".

So, before we go developing our favorite LPMD or C, listen to Sarfaraz when he says that

> "*The goals of preformulation studies are to choose the correct form of the drug substance, evaluate its physical and chemical properties, and generate a thorough understanding of the material's stability under the conditions that will lead to the development of a practical drug delivery system*".

And as I say, "*As we know, in any materials science and engineering project (and drug formulation qualifies as an engineered material), chemical composition, structure and properties are inextricably linked*".

For more on the material science and engineering of my first inventions, do see my first chapter for Kinam, (Needham 2013a), "*Reverse engineering of the low temperature-sensitive liposome (LTSL) for treating cancer*", as well as these papers (Needham and Dewhirst 2013, Needham, Park et al. 2013). In them I also try and show how this process of materials engineering design can be used to not only learn about a particular product or natural system, but can also generate new ideas, invention, and innovation. I might, (in my retirement) draft a book or monograph along the same lines as Niazi, but for "*Handbook of Preformulation for Advanced formulations*" and our prodrug therapeutics in particular. (See also in this *Appendix, A8. How to Reverse Engineer Anything*).

A4.1.4.2. Iterative design, formulate, and characterize. With all that said, here in this part of the process, we attempt, often iteratively, to design, formulate drugs, and also prodrug-therapeutics, and come up with ways to make them in their hypothesized optimal form, characterizing them in terms of size and physical chemical stability. Characterization can certainly include all the analytical chemistry and for nanoparticles, such as Dynamic Light Scattering (DLS) as well as electron and optical microscopy. Optical microscopy? Yes, even though the DLS tells us that the nanoparticles might be less than 200 nm, and so are not viewable by visible light, some of the particles might be. I also often alter the conditions of the precipitation to purposefully get larger micro particles that can be seen. That is, even the larger particles, if single, can give some indication as to what the nano ones are like before going to perhaps less convenient electron microscopies.

So, personally, I start with the parent drug and characterize it and what it makes under a series of precipitation conditions, which can often give larger particles, aggregates, and crystals. It's good to know how the parent and prodrug compare under these same conditions. Here too, I would encourage any budding drug delivery person to strike up a good scientific relationship with the PK/PD core that may be a core in your cancer center (see later, A4.1.6.). And finally, working with your local university- or even external -compounding pharmacy, we would also want to translate the methodologies to scale-up and GMP manufacturing.

In this scheme then, we would consider advanced drug formulation to go hand in hand with medicinal chemistry and analytics. Rather than drugs being made and the formulation-people having to figure out how to formulate and deliver them (often with not much luck), in this scheme, drugs are now designed to match the formulation, delivery route, as well as the target and so enable efficient and successful drug delivery to that target.

A4.1.5. Cell cytotoxicity, cell uptake, cell pathways and efficacy

It is usual that the drugs we might be wanting to reformulate have already been tested in cell studies. So, as the cell biologist or drug delivery person, make sure you scour the literature for all that previous information (IC_{50}s, IC_{100}s, mechanisms). As reviewed in the main text, there is a huge amount of literature on niclosamide in many and varied scenarios. For example, regarding some of the mechanisms and cell pathways affected by niclosamide, one being the cell's mitochondria, new evidence is focusing on how mitochondria do a lot more than make ATP, and in cancer there is, as is loosely termed, "altered metabolism". We might ask, how does that affect what niclosamide does and our choice of assays (recipes from Promega) that we use to measure them? (The seahorse assay is one of my current favorites). I know this is difficult for those of us not well-trained in the acronystic field of biochemistry, but we have to try. The drug delivery people, especially, really do need to know a lot, across the board, –connecting medicinal chemistry, to cell and preclinical and even clinical studies.

[35] Ph.D., SI, FRSB, FPAMS, FAACC Adj. Professor, Pharmaceutical Sciences, University of Illinois, Chicago https://www.niazi.com/.

Crossing into other fields is what enables not just *multidisciplinary research*, but actual and functional *interdisciplinary research*. So, I do try to make the effort.[36]

As in the traditional drug discovery and development route outlined above (*A3.2* and ***Lapatinib***), it is here that the parent drug (usually just added from, and maybe precipitated from, DMSO into the cell culture medium) is tested as a control, and then we include our specific prodrug formulation. Both are tracked in diseased and normal cell lines in order to establish and characterize the intracellular pathways and cytotoxicity. For what could be a nanoparticle we are also very interested in their entry into the cytoplasm via receptor-mediated or clathrin-independent endocytosis, using, for example, fluorescent assays.

Perhaps an underappreciated aspect of cell-drug testing is that the drug could easily be 99% bound to albumin, and at 10% Fetal Bovine serum, there is a swamping 70μM albumin in the cell culture. Therefore, it is as an albumin-complex that the drug is taken up by the cells, perhaps more avidly in cancer cells than any normal cell controls. Also, as more and more drugs being tested are less and mess soluble in water, just because they dissolve in DMSO does not mean they are free when squirted into the cell culture medium. They can precipitate during that solvent exchange, and so what is the actual concentration of the drug in the cell culture that you write on the x-axis of any cell viability plot?

While cell studies in a culture dish are a far cry from an *in vivo* tumor interstitium we would want at least some idea of cell-uptake amounts and kinetics for one or more pathways, carried out to determine the cellular fate of the new formulation, before preclinical testing. Of course, there are now 3D cultures (mammospheres, and Patient Derived Organoids (Altunel, Roghani et al. 2020), etc.) that can give some idea of the transport (or not) of the "nanomedicines", –there may even be a cut off in size to diffusive intracellular access.

A4.1.6. Preclinical testing

Here, as with the traditional drug development, tests are made in appropriate animal tumor models of the same tumor systems as the cell work, including quantitative measurements of PK, PD, toxicity, and efficacy. The difference though is that we test the actual formulation, not some oral gavage or intraperitoneal, subcutaneous, or i.v. injection of the drug in dimethyl acetamide and PEG.

Because we are dealing with nanoparticles of prodrugs, we have to work within the limitations of the delivery routes. For cancer, it is well recognized that i.v. nanoparticles need to be small enough to access the tumor interstitium via what we expect are leaky vessel walls, (the EPR effect). Given that LDLs seem to be the "cancer's food", and so, would seem, by definition to able to passively access the tumor interstitium, the preclinical animal models need to represent metastatic tumors that could potentially have leaky walls, are feeding to grow, and where the most aggressive cancer cells (–the tumor initiating cells, stem cells..) are accessible and therefore killable by *"making the drug look like the cancer's food"*.

As an example, we recently submitted to NIH as an R21 for *colorectal cancer* (Needham David and Hsu 2023). Specific aim number 1 proposed to do a head-to-head between irinotecan (prodrug of SN38) and our new SN38 Prodrug Therapeutic nanoparticles. Specific Aim #1 was to prepare and characterize the new formulation *in vitro*, and then new *in vivo* preclinical studies formed the basis for the next two specific aims, as:

SA2: To evaluate in vivo circulation pharmacokinetics and maximal tolerable dose of (the new formulation), and the standard of care drug in a non-tumor bearing model.

SA 3. To assess drug-accumulation, drug-sensitivity, and anti-tumor efficacy of (the new formulation) vs standard of care drug in vivo in sensitive and resistant PDX models of colorectal cancer.

That is, we would want to test if and to what extent our new formulation will promote longer circulation and show lower toxicity than Standard of Care (SoC) irinotecan. The obtained PK parameters in SA2 (C_{max}, AUC, clearance,) then guide the dosing regimen design (multi-dose MTD and dosing frequency) for the anti-tumor efficacy studies in SA 3, that looks to determine if we can achieve greater drug accumulation, increase drug-sensitivity, and greater reductions in tumor volume than the SoC drug (not funded, let me know if interested).

One additional piece of advice is to also form a good working relationship with the PK/PD person. They have usually *seen 'em come and seen 'em go* and have a wealth of information that might not be published regarding the drugs themselves, as well as information on other "formulations" they have tested for others (and that may have just generated manuscripts but never made it through into any clinic anyway). It is these important folks who will measure the same things for your formulation and that will guide your, and your preclinical collaborator's, ultimate dosing regimen design (multi-dose MTD and dosing frequency) for the subsequent anti-tumor efficacy studies and any *in vivo* efficacy study. I think you are getting the idea, building a career is also about building good-working relationships.

A4.1.7. Clinical testing

While I understand the Phase I, II, III sequence, I have to admit, as "just the drug delivery guy" the whole workings (or not) of clinical trials are a bit of a black box to me. I do appreciate the FDA and its mission to keep us safe, but have you seen the paper-work and evidence in a company or investigator-sponsored IND that has to be produced to get a preIND letter, never-mind the whole IND including the CMC?[37] I still don't know how to navigate all of this, that obviously needs funds, partners, and resources. I do know a lot about what doesn't work (see my Lessons Learned in Anya Hillary's edited book (Needham 2016)). Hopefully, your lead clinician has some experience, and your local Office of Regulatory Affairs and Quality (ORAQ) can definitely advise.

A5. So, what is oxidative phosphorylation again and how does niclosamide influence it?

In order to understand the effect of Niclosamide on normal cells and then on cancer cells we have to understand the process of Oxidative Phosphorylation and how it is linked to glucose metabolism or both. While this is probably in all standard biochemical text books, a fascinating review by Santo-Domingo and Demaurex (Santo-Domingo and Demaurex 2012), called "*The renaissance of mitochondrial pH*" sought to remind us that, while most studies only record changes in membrane potential to track the metabolic state of mitochondria, we should not ignore the contribution of pH gradient ΔpH_m that, "*drives the fluxes of metabolic substrates required for mitochondrial respiration and the activity of electroneutral ion exchangers that maintain mitochondria osmolarity and volume*", and also "*plays an important and underappreciated role in physiological and pathological situations such as apoptosis, neurotransmission, and insulin secretion*". Hence the importance of niclosamide is that it dissipates that gradient.

They go on to describes how generating and maintaining a proton gradient across the inner mitochondrial membrane (IMM) couples the oxidation of carbohydrates and fat to the synthesis of ATP. This is illustrated in Fig. A6 as indicated by the red downward arrow of H^+ going through the lilac ATP Synthase. In normal operation, this electron chain and all its series of biochemical conversions succeeds in generating the proton gradient of ~1 pH unit as protons are pumped from the

[36] When I went up for tenure in 1993, my Chair Bob Hochmuth who had hired me 6 years earlier, said in my supporting letter, "*He crosses boundaries easily, sometimes not recognizing they are even there*". Which I took to mean "*He is undaunted and bravely pursues the science wherever it leads*". Or "*He has no formal training, but with dumb luck, he gets there anyway*". On reflection, I think it's a bit of both.

[37] We had to write a a 55-page preIND letter for our simple nasal spray of a drug already given as 2gm doses but used in the spray at less than 1 microgram per spray. The FDA does not do math, they want evidence.

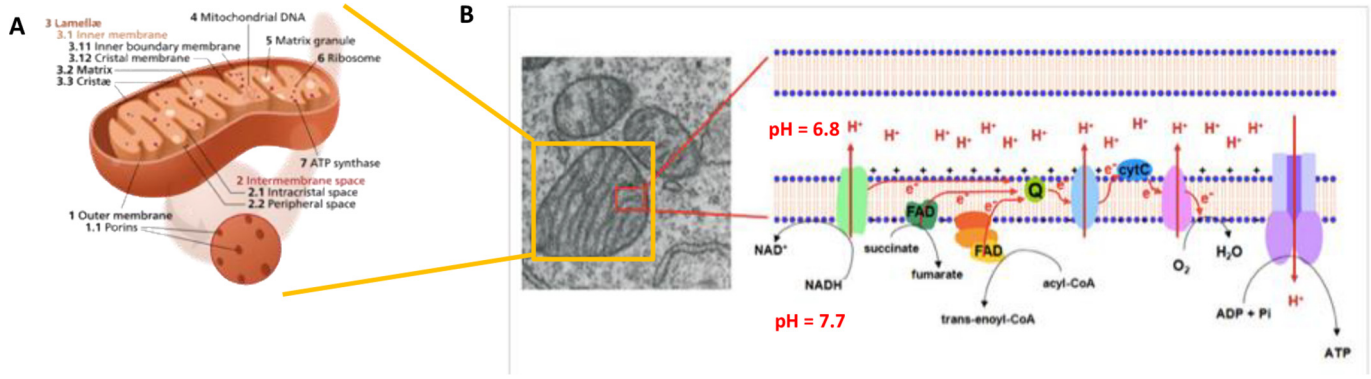

Fig. A6. ***The Mitochondria and its electron transport system (ETS) in the inner mitochondrial membrane*** **A)** *A mitochondrion and its components* (Wikipedia) (Wikipedia)**:** 1 Outer membrane with 1.1 Porin; 2 Intermembrane space with 2.1 Intracristal space, 2.2 Peripheral space; 3 Lamella with 3.1 Inner membrane, 3.11 Inner boundary membrane, 3.12 Cristal membrane, 3.2 Matrix, and 3.3 Cristæ; 4 Mitochondrial DNA; 5 Matrix granule' 6 Ribosome; and 7 ATP synthase. **B)** *The electron transport system (ETS) in the inner mitochondrial membrane* (Nature Education) (Da Poian, El-Bacha et al. 2010)**,** showing, an electron micrograph of a human cell section of three mitochondria and a schematic of "the mitochondrial membranes in purple and pale orange; NADH dehydrogenase in light green; succinate dehydrogenase in dark green; the complex formed by acyl-CoA dehydrogenase, electron transfer flavoprotein (ETFP), and ETFP-ubiquinone oxidoreductase in yellow and orange; ubiquinone in green labelled with a Q; cytochrome c reductase in light blue; cytochrome c in dark blue labelled with cytC; cytochrome c oxidase in pink; and the ATP synthase complex in lilac. The flux of electrons is represented by red arrows and e-, and the flux of protons is represented by red arrows and H+. Nomenclature: "IN", is into to the complex from the inter-membrane space, and "OUT" is out from the complex into the mitochondrial matrix.

matrix to the inner membrane space (IMS) by the respiratory chain complexes. As such, the pH of the inner membrane space is 6.8 and that of the matrix is pH 7.7. It also generates a mitochondrial membrane potential of ~180 mV as the matrix becomes more alkaline than the IMS.

More specifically, Alasadi et al at Rutgers in 2018 (Alasadi, Chen et al. 2018) used the Seahorse oxygen consumption rate (OCR) assay, and confirmed (in this case) that Niclosamide ethanolamine (a slightly more soluble salt of niclosamide) uncoupled mitochondria at 2.0 μM. To quote them: "*Inside mitochondria, as illustrated in (their) Fig. 1a, acetyl-CoA is metabolized to CO_2 through TCA cycle, and energy is extracted and stored in the form of high-energy electrons in NADH and $FADH_2$. The electrons then feed into the electron transport chain (ETC) residing in the mitochondrial inner membrane, which pumps protons out across the membrane and generates a proton gradient. Protons enter the mitochondrial matrix through ATP synthase, driving ATP synthesis. Usually, the ETC activity is coupled to the energy requirement of the cells. When the energetic requirement is met, ETC and oxidation of acetyl-CoA are shut down, along with pyruvate flux into mitochondria. Mitochondrial uncoupling is a process that leads to proton influx across the mitochondrial inner membrane without passing through ATP synthase*".

As illustrated in Fig. A7, they go on to say, "*this process de-couples mitochondrial oxidation from ATP synthesis, leading to a futile cycle, i.e., complete oxidation of acetyl-CoA without generating ATP. As a result, the energy efficiency of mitochondria is compromised. To meet the cellular energy demand, the flux of pyruvate into mitochondria is expected to accelerate, which promotes the complete oxidation of glucose. This mode of metabolic change induced by mitochondrial uncoupling could potentially diminish the anabolic effect of aerobic glycolysis*".

In experiments by Possmayer and Grab (Possmayer and Grab 1994) H^+-ATPase from chloroplasts was isolated, purified, and reconstituted into liposomes made from phosphatidylcholine/ phosphatidic acid.

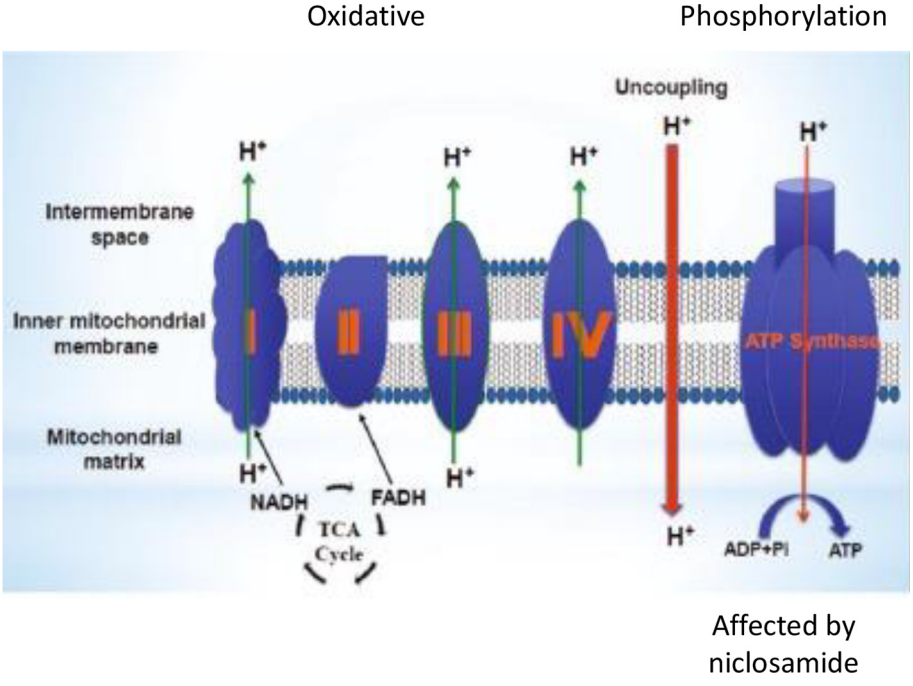

Fig. A7. Schematic representation showing mitochondrial uncoupling process that is affected by niclosamide (Alasadi et al, (Alasadi, Chen et al. 2018)).

The rate of ATP synthesis was measured after energization of the proteo-liposomes by an acid-base transition as a function pH_{out} (Mitochondrial Matrix) and pH_{in} (Inner Mitochondrial space). Mimicking our current situation of Niclosamide dissipating the "in" to "out" gradient, at any given Inner Mitochondrial pH, the rate decreased sigmoidally with increasing Mitochondrial Matrix H^+ concentration.

In summary: Mitochondrial uncoupling is a process that facilitates proton influx across the mitochondrial inner membrane without generating ATP, stimulating a futile cycle of acetyl- CoA oxidation. This is what niclosamide and other protonophores do and this can then influence all other down-stream processes in the cell that use ATP, or viruses that need it and perhaps more to replicate, or in cancer alter the mitochondrial system (see below *A7*) to such an extent that it induces Apoptosis.

A6. Medicinal chemistry of uncouplers: a brief review of (Childress, Alexopoulos et al. 2018)

Niclosamide is just one of many small molecules that can uncouple OXPHOS. In fact, there are also endogenous uncouplers (Uncoupling proteins, or (UCPs)) that are responsible for a basal leak of protons of ~20%. These UCPs can be actively regulated to alter ATP production naturally, in situations such as non-shivering thermogenesis in adipose tissue and more widely to prevent hyperpolarization and decrease mitochondrial superoxide production.

So, for the medicinal chemist there could be projects in the design, synthesis, and development of optimum uncouplers for OXPHOS that could revive these apparently lost compounds for cancer and other diseases and conditions. In introducing niclosamide and developing our discussion in the main text, I have already referenced the compounds by Williams and Metcalf (Williamson and Metcalf 1967) and Terada (Terada 1990), as well as the few successful ones by Mook et al (Mook, Wang et al. 2015). And here is another comprehensive list of uncouplers given by Elizabeth Childress et al, (Childress, Alexopoulos et al. 2018) in their mini-perspective, "*Small Molecule Mitochondrial Uncouplers and Their Therapeutic Potential*". It is an excellent review (and references therein) of much of what we have been talking about regarding uncouplers of OXPHOS. It makes for very interesting reading with details of some molecular properties like pKa, and calculated LogP, minimum effective concentrations at inhibiting OXPHOS, toxicity, and comments about each drug, uses, successes and failures, and a section on synergistic drug delivery to increase uncoupling potential. So, FYI, here is a brief review of their review.

They have an interesting section on the therapeutic potential of mitochondrial uncoupling, including: increased nutrient oxidation as a therapeutic strategy to treat obesity and related metabolic diseases; and to prevent Reactive Oxygen Species (ROS) production which has been linked to ischemia—reperfusion injury, inflammation, insulin resistance, and neurodegeneration. Because uncoupling stops ROS at source, it has advantages over antioxidants that scavenge ROS that have already been produced.

As for uncouplers per se, it is well worth checking out their Table 1, where they list 21 prototypical uncouplers. They group the uncouplers into categories of prototype uncouplers; repurposed FDA-approved drugs (including niclosamide as niclosamide ethanolamine); repurposed chemicals that were found to have uncoupling actions mostly by chance; chemical library screens for drug discovery that has led to the identification of numerous mitochondrial uncouplers, including small-molecule and natural-product library screens; and an interesting section on synergistic drug delivery to increase uncoupling potential.

Highlights (with minimum effective concentration and toxicity) include:

- ***Prototype Uncouplers*** includes the classic uncoupler DiNitro Phenol (DNP) (5μM, toxicity 0.8 – 3 g human) and 4 derivatives. These derivatives include two molecules you may have heard of if you are already into cell and mitochondrial respiration and use the seahorse assay, namely hydrazone 5 (carbonyl cyanide p-trifluoromethoxy) hydrazine, (FCCP) and its analogue carbonyl cyanide m-chlorophenylhydrazone (CCCP). These are potent uncouplers (EC_{50} = 0.04 μM and 0.1 μM, respectively. However, none are successful therapeutics. Given the lack of regulated control, its over-use as an anti-obesity drug, its narrow therapeutic window, and off-target effects, people died (see Royal Pharmaceutical Society's recommendations, https://www.rpharms.com/about-us/news/details/RPS-welcomes-Home-Office-move-to-classify-DNP-as-a-poison, and https://www.rpharms.com/about-us/news/details/rps-reiterates-call-for-ban-on-dangerous-dnp).
- ***Repurpose FDA-approved drugs*** lists just two, bupivacaine and niclosamide ethanolamine. While Bupivacaine (0.125 mM, toxicity 4-12 mg/kg rats) has some uncoupling activity, it relies on certain conditions and has significant toxicity. Niclosamide is also reviewed as niclosamide ethanolamine (1μM, acute toxicity of 500 mg/kg to 10 g/kg in cats to rats (Andrews, Thyssen et al. 1982)), referencing studies where it was shown to uncouple mitochondrial respiration in cells (NIH-3T3) and isolated rat liver mitochondria at concentrations as low as 1 μM.
- Repurposed chemicals include:
  - o TTFB (4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole) (100 nM, toxicity LD50 1.6 mg/kg rat), that was discovered in studies of the biological activity of substituted benzimidazoles (Beechey 1966) that has some toxicity but also has an uncoupling preference for brain mitochondria but has not been pursued as a therapy for metabolic or neurodegenerative diseases and so could represent a targeted therapy for the future;
  - o A short-chain alkyl derivative of Rhodamine 19 (C4R1) (1 μM, toxicity >60 μM) acts as a mild uncoupler of mitochondria and confers neuroprotection, tested in mice for stroke and obesity and suppresses their appetite;
  - o Ellipticine, (0.1μM, toxicity >50μM) in anti-cancer tests it inhibits the topoisomerase II-β pathway (–is the mechanism directly via drug stabilization of a cleavable complex formed between topoisomerase II and DNA (Tewey, Chen et al. 1984) in isolated DNA or indirectly via mitochondria in cells?) and induces endoplasmic reticulum stress;
  - o The salicylanilide (SF-6847 or S13) (32nM, toxicity 0.1μM) as reported by Williamson and Metcalfe (Williamson and Metcalf 1967), and Terada (Terada 1990) finds its way into the table, as the most potent uncoupler, (CBCN-salicylanilide, uncouples oxidative phosphorylation at a concentration of one molecule per respiratory assembly under optimal conditions (Kaplay, Kurup et al. 1970), but not much seems to have been done with it since the 1970s;
  - o tyrphostin A9/SF-6847, or [(3,5-di-tert-Butyl-4-hydroxyphenyl) methylidene]-propanedinitrile) (0.02 μM, toxicity 0.1 μM) is reported as a tyrosine kinase inhibitor, with very potent protonophoric uncoupling activity (20nM), but not really pursued because of cytotoxicity above 0.20 μM, (assuming oral-delivered) hepatotoxicity above 0.97 μM, and a mouse LD50 of 29 mg/kg. If delivered more effectively to tumors could it have its exceptional cytotoxicity there. We'll come back to this later because it has the electron withdrawing CN groups, a bulky hydrophobic ring with the two trimethyls so it's quite hydrophobic (LogP 4.22, or 5.56 by Chem Axon) and it has a de-protonatable phenolic OH with a reported pKa of 6.7 (or 8.79 by Chem Axon) and already a quite low solubility of 17μM.

- Making the S13-Stearate puts its aqueous solubility at a very low (calculated) 0.15 pico molar (0.15×10^{-12}M), with a LogP of 11.91, this compares very favorably to what we have already made for niclosamide as the niclosamide stearate (solubility 0.16pM i.e., 0.15×10^{-12}M, LogP = 10.66) and as presented in the main text, is the focus of our cancer treatments and studies in mice and canines.

- Chemical Library Screens for anticancer activity led to the discovery of the 1,3- bis(dichlorophenyl) urea (SR4) (>3μM, toxicity >25 μM) which, while not as potent as some, it did bring a new mechanism when tested in cancer cells, that of mitochondria-induced apoptosis, associated with a depolarization of the mitochondrial membrane. The next 3 are no better than niclosamide in terms effective dose, but BAM15 ((N5,N6-bis(2-Fluorophenyl)[1,2,5]oxadiazolo-[3,4-b] pyrazine-5,6-diamine) (0.27 μM, toxicity >50 μM) was found to have mitochondrial uncoupling activity equivalent to niclosamide with an EC_{50} of 0.27 μM in L6 myoblasts as determined via a Seahorse XF flux analyzer. It was broadly active when tested in hepatocytes, myoblasts, cardiomyocytes, and fibroblasts.
- Natural product isolates included usinic acid (0.75 μM, hepatotoxicity >1 μM) is not a good candidate for metabolic or neurodegenerative diseases, but as a natural product it is included in unregulated weight-loss supplements.

As with many of the compounds, Pharma is looking for these kinds of drugs to be taken orally and hopefully they go systemic, and so selectivity for mitochondria has usually not been established. Childress et al conclude that the main barrier to clinical translation is safety. If oral dosing results in sufficient absorption leading to systemic distribution many of these drugs bring various toxicities. Attempts to improve mitochondrial selectivity to avoid off-target actions on other organelles, and or reduce toxicity invariably reduce efficacy.

It is here that new opportunities present themselves for more disease-site-specific formulations that reduce systemic toxicity and target tumors or do not even create systemic toxicities because they are administered locally, e.g., as in our nasal and throat sprays of niclosamide solutions.

A7. Warburg and beyond: (Cassim et al)

It turns out that, there is life beyond the Warburg effect as comprehensively reported by Cassim et al (Cassim, Vučetić et al. 2020) (and references therein). Cancer cells do not exclusively depend on aerobic glycolysis to satisfy their bioenergetic and anabolic demands. Furthermore, "*mitochondria have now been recognized as important mediators of cancer behavior in all steps of tumorigenesis*". So, it is not that Warburg was wrong, just not completely right. It is irrefutable that cancers do take in more glucose than normal tissue cells, (^{18}FDG is a marker for cancers).

Here are some excerpts from the Cassimi paper (Cassim, Vučetić et al. 2020) that are quite important and crucial to understand (and explore further) if we are going to use niclosamide for cancer. This is an exceptional manuscript that goes into great detail about the long-standing Warburg effect and then reviews the whole role of mitochondria in cancer with particular attention to the cancer cell-intrinsic/extrinsic mechanisms by which mitochondria influence all steps of tumorigenesis. They even ask the question (and answer it) "*Is the Warburg effect dispensable for cancer?*"

Briefly stated, some of their most important conclusions are:

- Dispelling the "Warburg myth" and bringing it to an up-to-date reality, tumor cells do not exclusively depend on aerobic glycolysis to satisfy their bioenergetic and anabolic demands,
- Mitochondria play a key role in tumorigenesis,
- A full genetic disruption of the Warburg effect of aggressive cancers does not completely suppress tumor growth,
- As per Warburg though glucose is taken up more in cancer than normal tissue cells, and this enhanced glucose consumption is used as a source of carbon for anabolic processes and biomass that are needed to favor the growth of rapidly proliferating cells and promote nucleotides, lipids, and proteins,
- Besides having fundamental bioenergetic functions, mitochondrial metabolism provides appropriate building blocks for tumor anabolism, controls redox balance, and coordinates cell death,
- Cancer cells display an extraordinary metabolic plasticity, and other sources of carbon (than glucose) can be used including, lactate, pyruvate, citrate, glutamine, folate, and fatty acids since the ultimate goal of a tumor cell is nothing more than cellular growth and division. Such synthesis can also occur in the mitochondria.
- The substantial flexibility that mitochondria confer to neoplastic cells, such as modifications in fuel choice utilization, bioenergetics, oxidative stress, and susceptibility to cell death, allows the survival of these cells in the face of hostile fluctuating microenvironmental conditions

One statement that really caught my attention was when they finished off with:

> "…. *significant issues in translating these preclinical drugs (that were evidenced to be highly effective in eradicating tumorigenic cancer cells) towards clinical settings still remain, as their use would necessarily affect normal cells, including beneficial anti-cancer immune cells. Therefore, sophisticated therapeutic strategies in order to precisely modulate mitochondrial functions in a distinct cellular type will have to be defined in the future*".

A7.1. Block glucose uptake, Na+ gradients?

Another activity that niclosamide has in mollusks is that it blocks glucose uptake (Maltas 2014). Could this also be important to cancers that also feed on glucose? And if so, what is the mechanism? Maybe niclosamide could counter sodium-glucose transporters, also known as Na+/glucose cotransporters or symporters (SGLTs), where the energy for active glucose transport is provided by the sodium gradient across the cell membrane. In addition to proton gradients, since niclosamide can also be present as a sodium salt, could niclosamide also dissipate Na^+ gradients through membranes as a sodium ion shunt? Alasadi (Alasadi, Chen et al. 2018) has also speculated that targeting aerobic glycolysis, which in turn diminishes the production of reducing agents and building blocks for cancer cell biosynthesis, can be an effective and likely a universal anti-cancer strategy.

A8. How to reverse engineer anything

As you develop your ideas, I would recommend carrying out a formal reverse engineering of these systems that could be, or are, affected by niclosamide. This reverse engineering process is one that I have used and adapted in for both research (Needham 1999, Mills and Needham 2004, Needham and Dewhirst 2013) and teaching for the past 20 years. It is a formal use of the materials engineering design methodology (Ashby 2006) that I adapted for any macro (physiology), micro (cell biology) or nano (molecular biology) system in health, disease, or affected by drugs, toxins or other compounds (pharmacology and pharmacy). If anybody is interested in this process please do reach out to me and I'll walk you through it for anything you are interested in, as I do in my course that I developed and taught at Duke, "*How to Reverse Engineer Anything for (your) Invention and Innovation*". I actually did describe this process for one of my own inventions, "*Reverse Engineering the Low Temperature Sensitive Liposome (LTSL)*" (Needham 2013c). It was Chapter 12 in Kinam's first edition of his edited book *Biomaterials for Cancer Therapeutics (Park 2013)*. Basically, we would use the scheme to show the normal behavior of a particular biological system and then the altered mechanism(s) that niclosamide induces, and I guarantee going through this process will give you new ideas, inventions, and innovations.

A9. "Nano" in clinical trials

For completion and to complement the section in the main text 6.1.1 Clinical Trials for "nanoX"

here are the additional trials searching for "nano":

- No Studies found for: *nanocomposites, radiochemotherapy | Cancer*
- 4 Studies found for: *inorganic nanoparticles*
- No Studies found for: *DNA nanostructures*
- 3 Studies found for: *polymeric nanoparticles | antibiotics*
- 5 Studies found for: *polymeric nanoparticles | Cancer*
- 8 Studies found for: *carbon nanotubes*

Of the 4 studies on inorganic nanoparticles, there was a Phase 1 study on 90 participants in Brazil started January 13, 2022, entitled, "*Assess Safety, Reactogenicity and Immunogenicity of the VACCINE RNA MCTI CIMATEC HDT (HDT-301) Vaccine Against COVID-19*". This trial was designed to assess novel Lipid-Inorganic Nanoparticle (LION) formulated replicating RNA-based vaccine that encodes for a full-length spike (S) protein of the SARS-CoV-2 virus. Expected to end October 2020

Encouragingly, a follow-on study is listed, "*Safety and Immunogenicity of the RNA MCTI CIMATEC HDT Vaccine, designed to compare the safety and immunogenicity of two dose levels of the MCTI CIMATEC HDT RNA Vaccine against two authorized COVID-19 vaccines"* in Brazil (Comirnaty - Pfizer and Covishield - Oxford/AstraZeneca) in 300 participants. It was due to start September 2022, but is not yet recruiting, so hopefully they will make some progress.

Still on inorganic nanoparticles, there was as third trial, *"Clinical and Genetic Study of Familial Sarcoidosis (SARCFAM) at Hospices Civils de Lyon"*. This trial was actually looking to understand the respective role of *inorganic / nanoparticles* and genetic background in creating chronic diseases but does not seem to be recruiting since 2016.

Then there was the 2010 study, "*Wear Characteristics of Denture Teeth*" to evaluate the wear characteristics of new resin denture teeth (nano particles - hybrid composite) made by an injection technique that was terminated (Enrollment difficulties and drop-outs).

For *carbon nanotubes*, there was a successful trial that started in 2013 and a subsequent publication (Qian, Tucker et al. 2012), that replaced the rotating mammography x-ray tube with a specially designed carbon nanotube (CNT) x-ray source array. The rest are more device oriented, or to detect biomarkers in exhaled breath of Parkinson's patients, and as a hernia prosthetic material. But there was nothing on nanotubes as a drug delivery therapeutic.

For *polymeric nanoparticles | antibiotics*, (2018) these were in post operative pain and oral health. There were however 5 studies found for: *polymeric nanoparticles | Cancer*, including: Cetuximab in ethylcellulose in polymer particles for colorectal (2010); Quercetin in PLGA for oral docetaxel (2022), presumably IV injected for advanced solid malignancies,

In one, NLG207 at 12 mg/m^2 in combination with enzalutamide was not well tolerated in patients with mCRPC following several lines of the standard of care therapy.

I examined each of these motivations including a series of papers in ACS Nano's Anniversary edition (ACSNano 2017), containing several on drug and agent delivery:

- *Designing core shell gold and selenium nanocomposites for cancer radiochemotherapy* (Chang, He et al. 2017);
- *Virus-inspired membrane encapsulation of DNA nanostructures to achieve in vivo stability* (Perrault and Shih 2014);
- *Multifunctional inorganic nanoparticles for imaging, targeting, and drug delivery* (Liong, Lu et al. 2008)
- *Surface charge-switching polymeric nanoparticles for bacterial cell wall-targeted delivery of antibiotics* (Radovic-Moreno, Lu et al. 2012)
- *Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery* (Liu, Sun et al. 2007)

I know, it is probably unfair to single out just a few papers, but the point is, as Kinam points out, the literature is full of papers on "nanomedicines" with potential that are simply published but are unlikely to go forward unless someone leads them.

A10. Kaplan Meier and more

Finally, here is a section that I think is really important for drug delivery folks to understand and appreciate, what is really in a Kaplan Meier plot and how does it compare with survival of people who do not have cancer?, That is, what is the head room we are dealing with in terms of cancer survival and what would a cure actually look like?

I was at a cancer center meeting earlier this year and the talks were focused on the theme of "translation". The plenary was an excellent talk by Norman Edward "Ned" Sharpless MD,[38] called "*Translational Science Journey*". It focused on using Cell Cyclin Dependent Kinase Inhibitors (CDKI) that could be utilized to lessen the severity of the toxicity of the chemotherapeutic drugs that patients are given in an attempt to treat their cancers (Roberts, Kumarasamy et al. 2020).

As recently reported by Zhang et al, (Zhang, Zhang et al. 2021), the first, 1990s-generation of pan-CDK inhibitors, (Flavopiridol and Roscovitine, etc.) were designed to block the cell cycle and inhibit cell proliferation by inhibiting the CDK enzyme activity. However, poor selectivity and high toxicity, led to inevitable harmful effects on normal cells and so most of the pan-CDK inhibitors failed in their clinical trials. Later versions, though, have really taken hold, like Palbociclib –the first and most popular CDK4/6 inhibitor, with $2.135 billions of global sales in 2016, and is expected to reach $7 billion in 2022 (Zhang, Zhang et al. 2021). Interestingly, since haemopoietic stem cells don't proliferate that often anyway, and are usually stuck in G1, this clever use of CDKIs also helped to reduce myelo-suppression and neutropenia, as well as the gut. Taking an oral dose generates the inhibitor-drug in the blood stream that lasts longer than the highly toxic chemo, thereby putting normal cells to sleep while the cancer drug is absorbed, distributes, is metabolized, and excreted. Then, as the CDKI reaches its diminished presence, the normal cells wake up unharmed. It actually doesn't work that well for paclitaxel that seems to bind permanently to all tubulins, but it does have some positive effects for other toxic chemo, which is certainly something.

Another excellent talk was by Andy Armstrong,[39] a well-known and respected Prostate Cancer clinician and researcher at Duke. His talk featured their ground-breaking work on using an effective liquid biopsy (Armstrong, Luo et al. 2020) to stratify prostate cancer patients depending on variants from Circulating Tumor Cells (CTCs) and hence help choose the best drug treatments, like enzalutamide and abiraterone and follow-on taxane chemotherapy. Their conclusion was, *"Detection of AR-V7 in CTCs by two different blood-based assays is independently associated with shorter PFS and OS with abiraterone or enzalutamide, but such men with AR-V7–positive disease still experience clinical benefits from taxane chemotherapy. And there were some real gains"*.

A10.1. How far could we still go in prostate cancer?

Speakers at the meeting invariably represented the outcomes of the trials in the well-known and often-utilized Kaplan Meier plot of % survival versus time after start of treatment. One study showed enzalutamide plus androgen deprivation therapy (ADT) significantly prolongs survival versus placebo plus ADT in patients with metastatic hormone-sensitive prostate cancer (mHSPC) (Armstrong, Azad et al.

[38] Ex-director of UNC's Lineberger Cancer, ex-director of the NCI, ex FDA commissioner, so quite an accomplished cancer researcher and government servant.

[39] Duke Professor of Medicine, Professor in Surgery, Professor in Pharmacology and Cancer Biology, and Associate Director, Clinical Research in Prostate & Urologic Cancers in the Duke Cancer Institute.

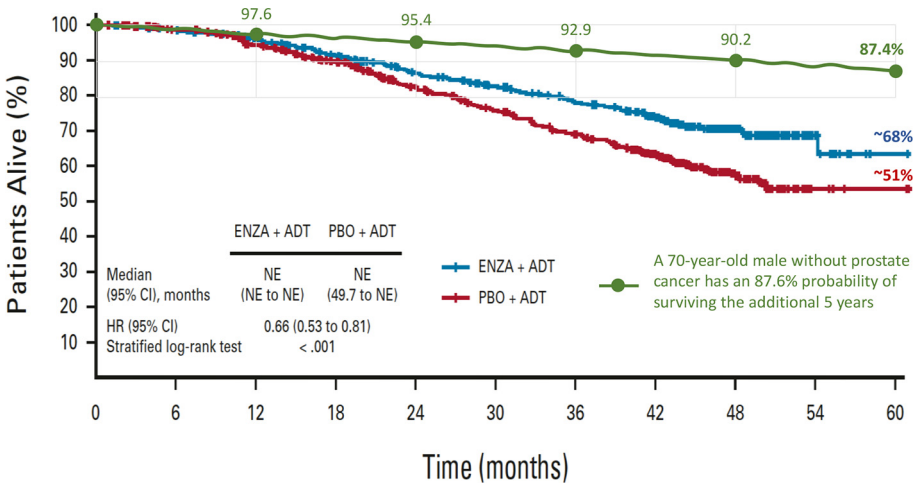

Fig. A8. Efficacy analyses (intent-to-treat population). Kaplan-Meier estimate of final OS analysis (Armstrong, Azad et al. 2022), showing that % survival for prostate cancer patients enzalutamide plus ADT significantly prolongs survival versus placebo plus ADT in patients with metastatic mHSPC with overlaid data from US Social Security "period life" Actuarial Life Table (US_Social_Security 2022) for the general population, (men without prostate cancer, age matched to the average age of the trial, of 70 yrs. old (green symbols).

2022). As shown in Fig. A8 for, on average, 70 yr. old patients, it produced positive results over a 5-year survival of ~ 64% still alive at 5 yrs. compared with ~51% for patients given placebo.[40]

This also got me thinking, if Enzalutamide extends the life of survivors compared to placebo, then how does enzalutamide compare to age matched controls of the general population? Has anybody actually shown a drug-treatment KM plot with a population that doesn't have the cancer, or a population that we treat, and they are actually cured? It's a long shot I know, but this gives us an idea of the best it can be, right Kinam? "*Be the best you can be*" (definitely see *Dr. Kinam Park, "30 Years of Research on Drug Delivery: A Personal Reflection" a video at this link* https://www.youtube.com/watch?v=CTtr8I9WqNY. It also gives us a sense of the extent of room for improvement.

I know this is all retrospective and obviously not part of any trial, but I think I might have figured out, at least a first cut at, how to do this. I went to the social security web site and found the Actuarial Life Table for 1-year survival for all ages (US_Social_Security 2022). I then picked the average age in the Enzalutamide trial, which was 70 yrs., and used a spread sheet to calculate the "Non-prostate-cancer" normal 1-year survival from the percentages for a 70-year-old man compounded for each year over 70 matching the 5 years of the trial. That is, I took the 97.37% chance for a 71-year-old to reach 1 yr. and scaled that 1 yr. number to give his chance of surviving 2 years, and so on. I then plotted that on the prostate cancer KM plot from the paper, as shown in Fig. A8.

The result is really quite interesting, and telling. Basically, a 70-year-old man (like me), would have a 97.58% chance of surviving 1 additional year, and an 87.4% chance of surviving to 5 years, without prostate cancer. What we can see is that, while Enzalutamide had a quite positive effect improving the 5-year chance of survival from 51% (placebo + ADT) to 68% with Enzalutamide + ADT, the non - prostate cancer (normal) person has an 87.6% chance of surviving 5 years. I did this to show myself (and also the readership) that yes indeed, there is quite a bit of head room for improvement compared to a 70-year-old who does not have prostate cancer, or what the data would look like if we had, say, a cure.

I then looked at that prospective trial (Armstrong, Luo et al. 2020) associated with detecting the circulating tumor cell AR-V7 variants and testing outcomes for abiraterone or enzalutamide in the PROPHECY trial for hormonal treatment metastatic castration-resistant prostate cancer. Here, the data shows a much more rapid decline in % overall survival for both of these populations of men. This data plus the overlay of the general US-SS population is shown in Fig. A9.

Overall Survival did improve with AR-targeted therapy due to selection based on the AR-V7 RNA liquid biopsy assay, with median survivals for those with AR-V7 positive being 11.1 months versus AR-V7 negative disease at 24.8 months. Ultimately, however, declines in Overall Survival were only down to only 7% at 33 moths and 8% at 43 months, respectively. The US-SS actuary data is 90% survival at 42 months for a prostate-cancer-free male. And so, for this prostate cancer population treated with abiraterone or enzalutamide, with follow on taxane, there is a lot of head-room for improvement.

Where could that improvement come from? The IC_{50} value in LNCaP cells for enzalutamide was measured to be 152 nM, (Semenas, Dizeyi et al. 2013) and the competitive IC_{50} binding to the Androgen Receptor for Enzalutamide in LNCaP/AR cells was 36 nM (Tran, Ouk et al. 2009). Therefore, this is a very potent drug, and so it's not the drug that is the problem, (apart from the developed resistance), it's the drug delivery. Thus, is there room for improvement by a reformulation effort, with a new medicinal chemistry approach that makes these kinds of drugs much more insoluble to make pure drug or prodrug nanoparticles for i.v. injection-infusion and a potentially more optimal drug delivery? Possibly. (See again our Bricks to Rocks Technology Section that again, forms the section in the main text 7.4 *"Make the drug look like the cancer's food": they all have to eat.*)

A10.1.1. Niclosamide has activity in Prostate Cancer

Where might we look for other possibilities of improving this treatment? As usual, I searched for "niclosamide" and "prostate". Actually, I found this some years ago. It's a paper by Liu et al (Liu, Lou et al. 2014) *"Niclosamide inhibits androgen receptor variants expression and overcomes enzalutamide resistance in castration resistant prostate cancer",* Almost 10 years ago now they identified niclosamide as a novel inhibitor of AR variants. We already showed it had efficacy in prostate as free drug and in the form of our NSPTs (Karimi 2020), and so now it just needs testing. One of the main issues seems to be that inhibition of the androgen receptor (AR) by second-generation anti-androgens for metastatic castration resistant prostate cancer (mCRPC) inevitably leads to the development of resistance. Niclosamide targets the cell membranes so there is less, or even no, expectation of resistance. (see ***7.5 Proposed Idea*** in main text.)

[40] Which I always think is a cruel thing to do, –you think you are getting a drug that could help you and your cancer and that could improve your 5-year chance of survival from 51% to 68% but, you are actually not?

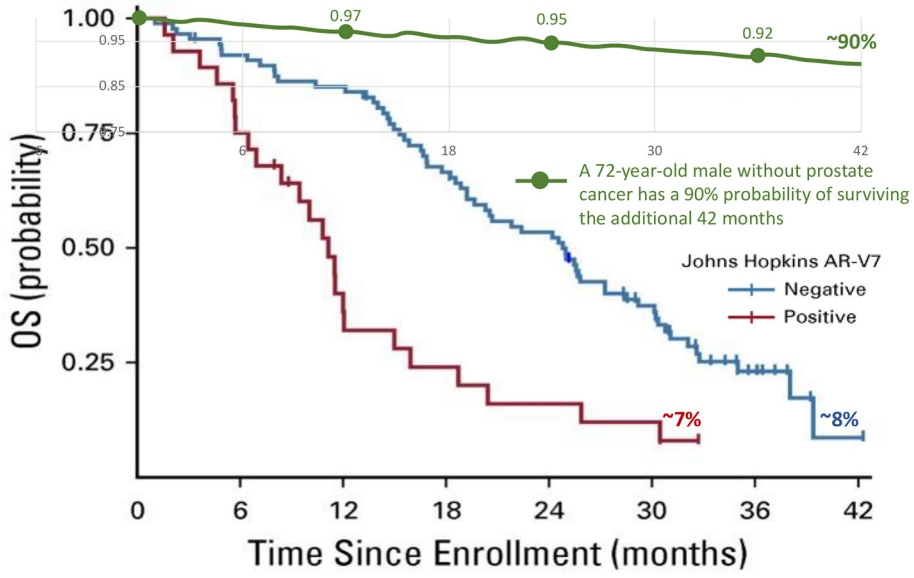

Fig. A9. Kaplan-Meier plot of outcomes in men with metastatic castration-resistant prostate cancer treated with abiraterone or enzalutamide in PROPHECY: overall survival (OS) by pre-treatment Johns Hopkins circulating tumor cell (CTC) androgen receptor splice variant 7 (AR-V7) detection criteria (Armstrong, Luo et al. 2020). Overlaid is a separate plot of with data from US Social Security "period life" Actuarial Life Table (US_Social_Security 2022) for the general population, –men without prostate cancer, age matched to the average age of the trial, of 72 yrs. old out to 42 months (green symbols).

A10.2. How far could we still go in breast cancer?

What about women and breast cancer? The same social security web site also has the 1-year survival for all ages of women. So, I picked Triple Negative Breast Cancer, and found a 2019 paper by Houts et al, (Houts, Olufade et al. 2019) "*Treatment patterns, clinical outcomes, health resource utilization, and cost in patients with BRCA-mutated metastatic breast cancer treated in community oncology settings*". And show their KM plot in Fig. A10. They start by saying,

> "*In 2018, it was estimated that 40,920 women in the USA will die of breast cancer, making it the second leading cause of cancer death for women after lung cancer. Despite advances in the treatment of breast cancer, the prognosis for metastatic breast cancer (MBC) remains suboptimal with median survival of* ***20.5–60*** *months in first-line treatment Phase III trials depending on subtype, and slightly less in routine clinical practice*".

Obviously this is a very complex disease with many parameters and conditions. Basically, in their micro abstract, the methods and numbers were: ***Method:*** *Reviewed medical records for outcomes, resource utilization, and costs in 114 community patients with BRCA mutated metastatic breast cancer.* ***Patients:*** *57 hormone-positive (HP); 57 triple-negative (TN).* ***Results:*** *Median Progression Free Survival: 12.1 months HP; 6.1 TN. Median Overall Survival: 38.4 HP; 23.4 TN. Patients with TN disease need better therapeutic options.*

The average age of the women in the trial was 49.5 yrs. As shown in the whole KM plot in *Fig. A10* the media survival was ***38.4 months*** for Hormone Positive and just ***23.4 months*** for Triple Negative. So, the average age when they succumbed meant that only half of the women reached their 53rd and 52nd birthdays, respectively. The 16-year survival probability was only 19.1%.

Applying the age matched control calculation from the US-SS Actuarial Life Table (US_Social_Security 2022) but now for the average age of 50 years for women and taking it out to 200 months (16.7 years), a 50-year-old woman without breast cancer or one who is cured, has an 84.3% probability of surviving and additional 16.7 years. There is clearly, again, a huge amount of head room for much more clinically-effective drugs and/or formulations. But, again, is there any evidence in the literature that niclosamide could help out here with TNBC? Actually, there is.

A10.2.1. Niclosamide has activity in TNBC

It turns out that niclosamide has activity here too as shown in cell and preclinical models of TNBC. As reported by Yin et al, (Yin, Gao et al. 2016) "*Niclosamide sensitizes triple-negative breast cancer cells to ionizing radiation in association with the inhibition of Wnt/β-catenin signaling* concluding that, "*our study provides rationale for further preclinical and clinical evaluation of niclosamide in TNBC management*".

In other studies, drug combinations (that are probably needed) also included niclosamide. For example, as reported by Zhao et al, (Zhao, Hu et al. 2021) paclitaxel and niclosamide decreased the expression of Ki67 and CD44 to inhibit cell proliferation and migration and induce apoptosis in TNBC.[41]

Also, as reported more recently by Liu et al, (Liu, Chen et al. 2016), niclosamide showed IC_{50} cytotoxicity (by alarma blue –resazurin solution) in cisplatin-sensitive (231-CS) TNBC cells at ~1.5 μM, and in platin-resistant (231-CR) cell line at ~3 μM. And cells were more sensitive to the combination of cisplatin and niclosamide with IC_{50}s of 0.5 μM and 1.5 μM respectively for the sensitive and resistant cell lines. The combination also inhibited cell proliferation and reversed the epithelial to mesenchymal transition (EMT) inhibiting Akt, Erk, and Src signaling pathways. In even more recent studies Liu et al, (Liu, Ding et al. 2021) they demonstrated that, *the inhibitory effect of niclosamide was mediated by apoptosis induction and Bcl-2 downregulation*. They also see niclosamide as something that should be tested when they concluded that, *Taken together, the results of the present study suggested that niclosamide combined with cisplatin may be considered as a novel treatment for chemoresistant HER2-positive breast cancer*.

We, ourselves, have already shown that NSPTs have activity in TNBC cell lines as Amina Arslanagic showed in her MS and PhD theses and a CLINAM presentation (Arslanagic 2014, Arslanagic, Hervella et al.

[41] They used a fairly complex formulation comprising injectable paclitaxel (PTX) nanocrystals (PTX-NCs) and Niclosamide (NLM) nanocrystals (NLM-NCs) co-loaded PLGA-PEG-PLGA thermosensitive hydrogel (PNNCs-Ts Gel).

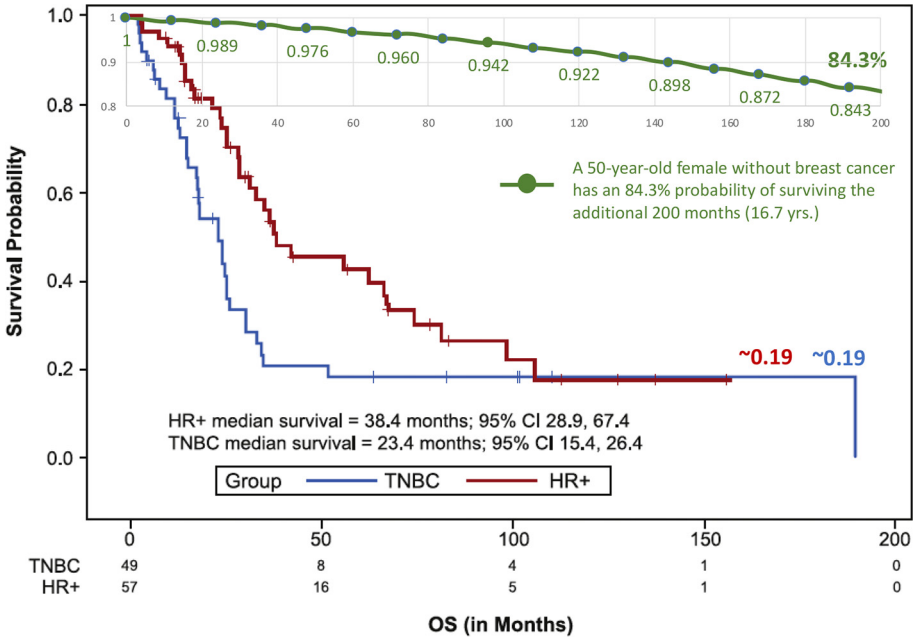

Fig. A10. Kaplan–Meier plot for median overall survival from initiation of first line treatment for patients with TNBC and HR+ BRCA-mutated metastatic breast cancer adapted from Houts et al(Houts, Olufade et al. 2019) with overlaid data from US Social Security "period life" Actuarial Life Table (US_Social_Security 2022) for the general population, (women without breast cancer, age matched to the average age of the trial, of 50 yrs. old (green symbols).

2016, Arslanagic-Kabiljagic 2019), where non-targeted NSPTs were taken up in breast cancer stem cells of triple negative origin. In the TNBC cell line MDA-MB-231 she evaluated metabolic activity using a cell titer blue assay at different treatment times (24h and 72h) in 2% FBS. IC_{50} values were 0.9 μM for a 24h incubation and, for a 72h incubation, the IC_{50} values were reduced to 0.3 μM. In a cell death assay, 72 hr exposure to niclosamide induced 60% cell death that occurred at 5 μM and a maximum cell death of 70% occurred by10 μM; the NSPTs also reached a maximum of 60% at 10 μM. And so, niclosamide alone has activity on TNBC. These studies were then followed by our Niclosamide Stearate nanoparticles that showed similar IC_{50} values of 1 μM for a 24h incubation, and a slightly lower 0.8 μM for 72h, which showed that the prodrug nanoparticles were as good as free drug but could now be administered *in vivo* by i.v. injection as a more stable formulation.

What is also important in both the Liu and Arslanagic data is that "normal" cells like MCF10 and HBL100 and MCF12A, showed a much greater resistance to cytotoxicity and cell death. In Liu's studies the normal cells at 5μM niclosamide only reached a 60% cell viability (which may not mean death) for a 48 hr incubation. In Arslanagic's studies, when MCF12A cells were incubated with niclosamide for 72 hr, the maximum cell death was only 30% at 2 μM. Interestingly for the NSPTs it was only 5% at 10 μM. Thus, making the niclosamide as the NS prodrug therapeutic showed reduced lethality in these "normal" cells, and so, when tested *in vivo*, we might see a reduced off-target toxicity. As always, it's just a matter of testing them in these and other preclinical models of TNBC and then with one or more other drugs (see ***7.6 Proposed Idea*** in main text).

I again encourage carrying out these kinds of preclinical studies with the inclusion of one or more local clinicians. From my experience, (as above) this is so that they can get in on the ground floor and start thinking about what the clinical studies would look like from square one. While it might not work with many, the more we can engage the clinicians in such preclinical studies, the more they may carve out the time to collaborate and start thinking with us more about reformulating existing drugs that already work in cell culture and just need optimized delivery.

My naive "age-matched-control" Kaplan Meier exercise above was a very illuminating and actually quite shocking revelation. I therefore encourage you all to do this age-matched comparison for other data you might find in the literature for yourself. And for the clinicians, maybe this could be a standard and sobering addition to all Kaplan Meier plots of drug data vs placebo or other standard of care treatments that are at least shared with the industrial sponsors. What I hope we have seen and appreciated is that these comparisons underline the fact that todays' oral tablets that are prescribed from the various pharmaceutical companies are just not achieving all they could do, motivating all of us in our research labs to consider a REFORMULATION of existing therapeutics, to at least try to make them more clinically effective.

As an Aside: (not really an aside but…)
I have a friend of a friend that I connect with on social media from time to time and update her on all my efforts in cancer. She is always very supportive. Then she got diagnosed with cancer herself a few months ago. She is Irish and has a way with words.
She sent me this last June 2022.
" I have lung cancer… fags of course and smoking more than ever now…. I will have 30 radiation treatments and 2 types of Chemo once a week ….. to walk away from treatment, I would get 6 months….. oncologist says it's been caught early and is localized and, all going well, I should get 5 years plus….. life's a bitch….. I start next week".
My response was all I could muster.
"I am so sorry to hear this. But... I know you are strong, and don't take shit from anybody, so don't take shit from a few unruly cancer cells, show them who's boss. And that survival rate is for everybody, not you. They say, the five-year survival rate for lung cancer is 56 percent for cases detected when the disease is still localized (within the

lungs). So that puts you at the top end of 56% well beyond 5 years and more. I know you can do this; YOU CAN DO IT"

Then, I just received this a few months ago (March 11th, 2023), so ~9 months after the initial diagnosis and treatments.

"Hi David…. I just want to say that's it's been a privilege knowing you of sorts.. I have secondary cancers now.…the worst one is in the middle of my hip bone/groin as they are finding it hard to stop the pain. It's also in my liver etc.,…… I have refused chemo this time as the most it would do would help with pain... I have a hospice nurse and my own doc now and it's all about my quality of life which at the moment is zero as I can't walk….. my husband is great cooking nice meals etc., etc., but I can see the tiredness in him now…. I'm going into the hospice on Monday to see if they can come up with a pain killer that will work for me….. I keep going into toxicology which is not nice….. anyway, enough of that, that bastard has caught up with me….. you continue with the wonderful work you are doing and hopefully someday. Take care of yourself and family, you beautiful man…… x"

I responded with some heartfelt words and an offer of,

"I understand if it's not possible, but I would love to say Hi if and whenever you feel up to it".

I have heard from her, saying,

"I am taking so many drugs at the moment that I feel dazed but hopefully we will have that chat soon….. mind yourself".

Maybe we all have stories like this, maybe not, but in one paragraph she encapsulated the problems with today's cancer treatment options, their toxicities, and their failures. Surely, we can do better than this.

A10.3. Financial toxicity

I really can't leave this section without making a brief comment on, and bringing to your attention, a very important toxicity of today's, especially, anti-cancer medications, – financial toxicity. As academic researchers, even if we measure the toxicity of the drugs on our cells and animals and put it in our papers on the new nanomedicines we develop or for clinical trials of the tablets, the final cost that a corporation may assign to our inventions is probably not at the forefront of our minds. But it is for the patients who have to pay it, or not get treated, or suffer the consequences. Even if that treatment is not very effective, at least they would have the choice of suffering the debilitating chemo side effects or going into hospice care (as above).

As recognized by the National Cancer Institute in their article, ***The Imperative of Addressing Cancer Drug Costs and Value*** by Barbara K. Rimer, Dean of the University of North Carolina's Gilling's School of Global Public Health, –the panel's chair, their report "*recommends six critical actions to maximize the value and affordability of cancer drug treatment and to support investments in science and research that will drive future innovation*" (Rimmer 2018).

They start with, "*As a panel, we began discussing the possibility of focusing our next report on cancer drug prices because it seemed that, everywhere we turned, we heard stories—some heartbreaking—about the impact of the rising cost of cancer care and the escalating costs of cancer drugs*". I am sure they won't mind me copying and pasting their info graphic and spreading the word, in Fig. A11.

I encourage you to read it, especially the part where they say, "*Many unanswered questions remain regarding the best ways to prevent, detect, and address financial toxicity among individuals with cancer. In our recommendations, we stress the important opportunity for researchers to close these knowledge gaps*". That's us.

Bottom line: Most cancer drugs launched between 2009 and 2014 were priced at more than $100,000 per patient for one year of treatment. More recently, we've seen launch prices of more than $400,000 for a year of treatment and the new CAR T-cell therapy can cost between $500,000 and $1,000,000 (plus the cost of a hospital stay) (Robinson and Goodell 2023).

(Rhetorical question): Is it time to collectively figure out a way to use public funds, (like were spent on Enzalutamide, but are now contributing to the profits of Japan's Astellas Pharma and Pfizer, that costs US Patients about $156,000 per year on average) and go –at cost, non-profit, reduced-reinvested profit, open-source pharmaceuticals, start generic? I'd say yes, especially if we are smarter about this whole drug delivery thing and our reformulation of existing drugs that are less than optimal as oral tablets. If so, we wouldn't have to pay for the 24 out of 25 failures that pharma routinely cites as why it's a $1-2 Billion+ for human phase testing, and not the $19M – $50M it really could be especially with a system of at-cost testing or reduced and reinvested profit.

In my previous chapter for Kinam (Needham 2020), I did try and address this issue then, citing the Tufts Center for the Study of Drug Development's (CSDD) latest assessment that the average cost (pretax) of reaching marketing approval in 2016 was just under $2.6bn (DiMasi, Grabowski et al. 2016). Unfortunately, around nine out of every ten drug candidates that make it to trials fail to win approval. The 2018 paper by Moore et al (Moore, Zhang et al. 2018) did an analyses of a total of 138 pivotal clinical trials that provided the basis for approval of 59 new therapeutic agents by the FDA from 2015 to 2016, finding that the median estimated cost of such trials was in fact only $19.0 million. This $19 million median figure represents less than one percent of the average total cost that is assigned to developing a new drug. I'll leave it up to the readership to explore these numbers further and ask, can we collectively answer that question and figure out how to do it?

As mentioned in the main text, someone already is, –The Global Coalition for Adaptive Research (GCAR) https://www.gcaresearch.org/

"The Global Coalition for Adaptive Research (GCAR) unites physicians, clinical researchers, advocacy and philanthropic organizations, biopharma, health authorities, and other key stakeholders in healthcare to expedite the discovery and development of treatments for patients with rare and deadly diseases. As Sponsor of innovative trials, including master protocols and adaptive platform trials, GCAR is dedicated to the advancement of science by modernizing clinical trials that support more efficient, less costly drug development. Adaptive platform trials can accelerate the time from discovery in the lab to implementation in the clinic resulting in better treatments and lives saved".

To learn more and maybe find your own representative, see their "About us" and especially their ***Trials Leadership***, *a committed group of oncologists, statisticians, pathologists, neurosurgeons, imaging experts, advocates, and researchers from academia, industry, and government for all its collaborations and initiatives*. https://www.gcaresearch.org/steering-committees/

I have already contacted this group and found a clinician at Duke who is associated with them. I would encourage others to do the same.

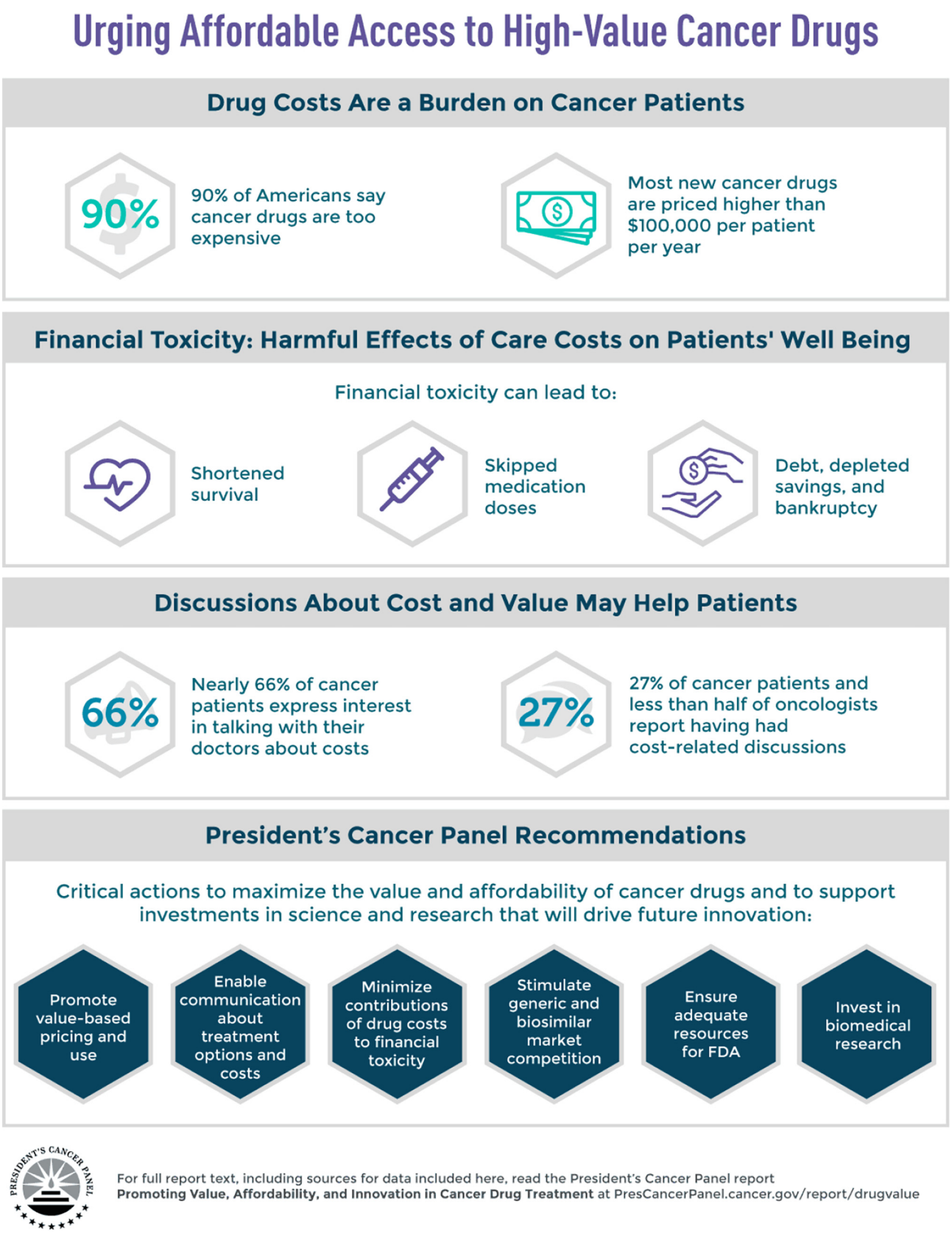

Fig. A11. Infographic from the National Cancer Institute in their article, *The Imperative of Addressing Cancer Drug Costs and Value* (Rimmer 2018).

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