Journal of Controlled Release 334 (2021) 318–326

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Magnum Opus

# (Poly-cyanoacrylate) nanomedicines for cancer and beyond: Lessons learned

P. Couvreur

*Institut Galien Paris-Sud, UMR 8612, CNRS, Univ Paris-Sud, Université Paris-Saclay, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, F-92296 Châtenay-Malabry, Cedex, France.*

ARTICLE INFO

*Keywords:*
Intracellular delivery
Biodegradable nanoparticles
Polyalkylcyanoacrylate
Multidrug resistance
Hepatocarcinoma
Squalene
Cancer
Stroke
Pain relief
Cytokine storm

ABSTRACT

This « Magnum Opus » emphasizes that serendipity is a corner stone in research. The paths of discovery and innovation often result from the interdisciplinarity of scientific areas that are a priori disconnected from each other. In the 1970s, fundamental discoveries in cell biology led to unexpected advances in galenic pharmacy with the emergence of nanotechnologies for the intracellular delivery of non diffusing molecules. As well, fluorescein-loaded polyacrylamide nanocapsules were shown to deliver this fluorescent agent precisely into cellular lysosomes which represented a seminal observation. However, due to the lack of biodegradability of this carrier polymer, this approach was still far from therapeutic application. The use of cyanoacrylates as surgical glue inspired us to use this material in the design of the first biodegradable nanoparticles for human use. Capable of transporting compounds with anti-tumor activity, these polyalkylcyanoacrylate nanoparticles demonstrated the unexpected property of overcoming multi-drug resistance. This discovery led to the development of a nanomedicine that has completed phase III clinical trials for the treatment of resistant hepatocarcinoma. Going beyond the state-of-the art, a step ahead in the nanomedicine field was the drug « squalenoylation » technology, which represents a shift from the « physical » to the « chemical » encapsulation paradigm. The bioconjugation of anticancer and other drugs to squalene, a natural and biocompatible lipid, enabled a dramatic increase in drug payload, and eliminated the so-called « burst release » of drug: Two major drawbacks commonly associated with drug nanoencapsulation. The drug « squalenoylation » approach resulted in a generic nanomedicine platform with broad pharmacological applications.

## 1. Polymer nanocapsules for the intracellular delivery of drugs

In the middle of the seventies, at the Catholic University of Louvain (Belgium), I had the chance to engage in regular scientific discussions with researchers from de Duve's laboratory. This laboratory was located very close to the laboratory of Galenical Pharmacy where I performed my PhD thesis on tabletting under the supervision of Prof Michel Roland. In 1974, Christian de Duve was awarded the Nobel Prize of Physiology and Medicine together with Albert Claude and George E. Palade "for their discoveries concerning the structural and functional organization of the cell", in particular the discovery of lysosomes. Being influenced by this unique environment, I became convinced that the pharmaceutical technology, although considered at that time as a second-class discipline, should play a key role in the intracellular delivery of drugs, eventually through the endocytosis and lysosomal cell pathway. Later, in 1976, I read an amazing and pioneering article by Birrenbach and Speiser (1) which described « nanoparts » which were polymerized micelles obtained by gamma irradiation of acrylamide with *N,N′*-methylene-bis-acrylamide as cross-linking agent. The reaction occurred in an organic phase containing an anionic surfactant (ie. bis-(2-ethylhexyl)-sodium succinate). These nanoparts were intended to be used as immunological adjuvants, due to the long-term liberation of encapsulated antigens. In 1976, I submitted a post-doctoral project aiming to encapsulate various anticancer compounds into these polyacrylamide nanocapsules, and testing their ability to release their drug payload intracellularly. This gave me the opportunity to spend one year in the laboratory of Peter Speiser (ETH Zürich) which was, at the time, at the forefront of pharmaceutical technology and formulation in Europe. Unfortunately, the first experiments were discouraging because neither doxorubicin, actinomycin D, or any other anticancer compounds could be encapsulated into the polyacrylamide nanocapsules due to their susceptibility to chemical degradation by gamma irradiation. Looking to the few chemical compounds unaffected by gamma irradiation, it was decided to encapsulate fluorescein. This molecule possessed a two-fold

*E-mail address:* patrick.couvreur@universite-paris-saclay.fr.

https://doi.org/10.1016/j.jconrel.2021.04.028

Available online 27 April 2021

benefit of an inability to diffuse intracellularly as a free compound (ie., non-encapsulated) and ability to track inside cells. Following incubation of the fluorescent nanocapsules with rat fibroblasts, we observed the cells becoming strongly fluorescent (Fig. 1, bottom). Whereas incubation with free probe left the cells unaffected. The concentration of the fluorescein in the lysosomal fraction was calculated to be 15-fold higher than in the extracellular medium. Thus, a seminal paper was published in FEBS Letters as early as 1977 (2), and included the first proof of concept that polymer nanoparticles could induce cell capture of a non-diffusible compound via the endocytotic pathway (Fig. 1, top).

In addition to the detrimental effects of gamma irradiation on most drugs, the clinical application of the polyacrylamide nanocapsules was hampered by three important limitations: (i) the polymer used was not biodegradable, (ii) the surfactant was not allowed for intravenous administration and (iii) the organic solvent employed during nanocapsule preparation represented a toxicological concern. Identification of a biodegradable polymer and a green process for nanoparticle preparation then became a challenging and urgent objective.

**Lesson learned**: Pluridisciplinarity and a motivating scientific environment are key to performing ambitious research that extends beyond the state-of-the art, especially at the starting point of a young research career. Mixing the chemistry of colloids (ie. nanotechnology) with cellular biology (ie. lysosomal pathway after endocytosis) resulted in the exciting observation that polymer nanocapsules loaded with fluorescein were capable of cell illumination, but still far from any clinical application.

## 2. From non-biodegradable to biodegradable material for nanoparticle design

The search for a monomer susceptible to polymerization in aqueous media resulted in the identification of a family of alkylcyanoacrylates, a surgical glue with excellent adhesion to tissues and in situ biodegradability. Through a unique anionic emulsion polymerization process in water containing a protectant of colloids (ie. dextran or an injectable non-ionic surfactant), these monomers were discovered to be able to grow as nanoparticles with a size of 50–300 nm (Fig. 2, C) (3). In fact, the two electro-attractive groups on the α-carbon of the double bond conferred a very high susceptibility of the β-carbon to nucleophilic attack by anions such as the hydroxyl ions of water or any other anion, which represented a unique chemical reaction in polymer chemistry (Fig. 2, A). Thus, after adding the cyanoacrylic monomer to water, the emulsion polymerization process occurred through the diffusion of the monomers into pseudo-micelles. The polymer molecules are synthesized and assembled as nanoparticles displaying a matrix structure. Interestingly, the length of the alkyl side chain of the cyanoacrylic monomer (ie. methyl, ethyl, isobutyl, hexadecyl etc.) enabled control over the biodegradability of the polymer and consequently the drug release rate. The longer the alkyl chain of the monomer, the slower the biodegradability and drug release and vice-versa. In vivo, the biodegradation was found to occur enzymatically (via esterases), inducing the hydrolysis of the polyalkykcyanoacrylate ester side chain. This produced the corresponding alkyl alcohol and the water soluble poly(cyanoacrylic acid) as metabolites (Fig. 2, B), the latter being eliminated by urinary excretion (4). In this context, it was mandatory to control the molecular weight of the polymer such that it was low enough to allow for renal filtration of the polycyanoacrylic acid metabolite, which could be successfully achieved by decreasing the pH of the polymerization medium to pH = 1.

Of note, this unique emulsion polymerization process for the preparation of polyalkylcyanoacrylate (PACA) nanoparticles was broadly applicable and highly flexible since it permitted the encapsulation of many drug molecules with various physico-chemical properties, including anticancer compounds (ie., actinomycin D, doxorubicin, vincristine, vinblastine, metotherexate etc.), antimicrobials (penicillin, ampicillin, ciprofloxacin, acyclovir etc.), peptides (insulin, calcitonin, GCSF, octreotide) (5) and antisense oligonucleotides or siRNA. Fine

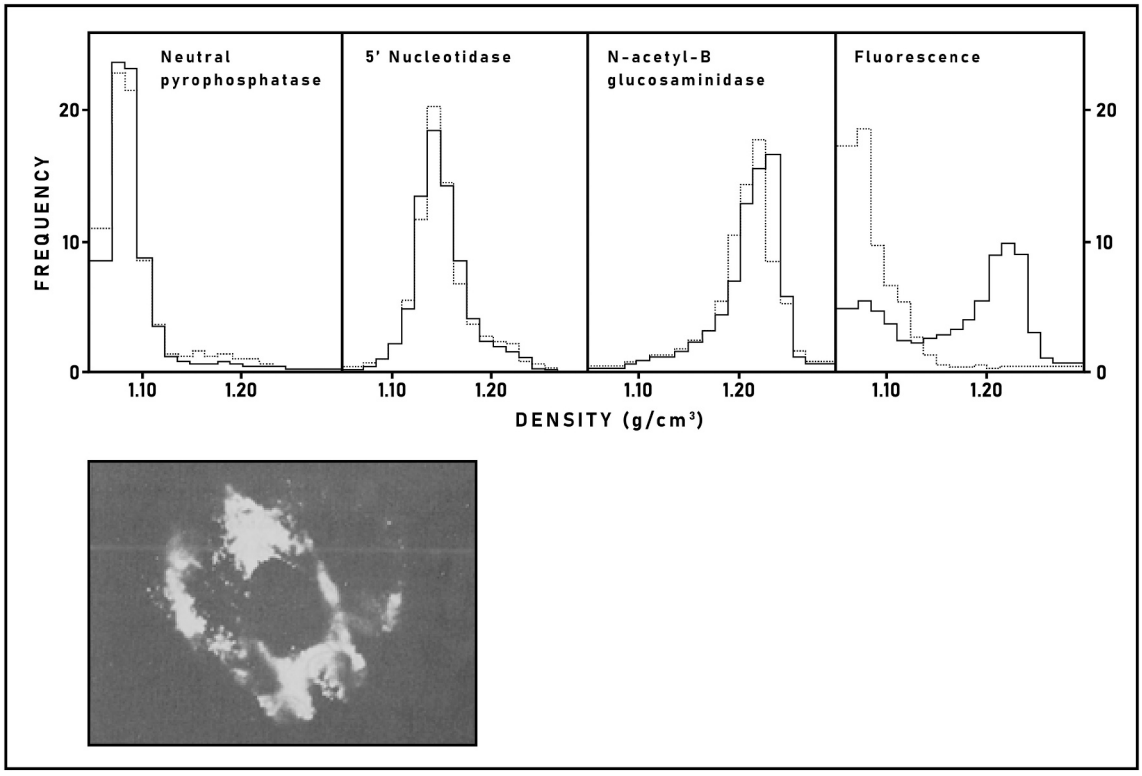


**Fig. 1.** Fluorescein-loaded polyacrylamide nanocapsules are captured intracellularly and distribute into lysosomes. (top) Isopycnic centrifugation showing that the fluorescence distributes similarly to *N*-acetyl-b-glucosaminidase, a lysosomal marker; (bottom) after incubation of rat fibroblasts with fluorescent polyacrylamide nanoparticles, the cells are becoming fluorescent. (Adapted from FEBS Letters, 1977).

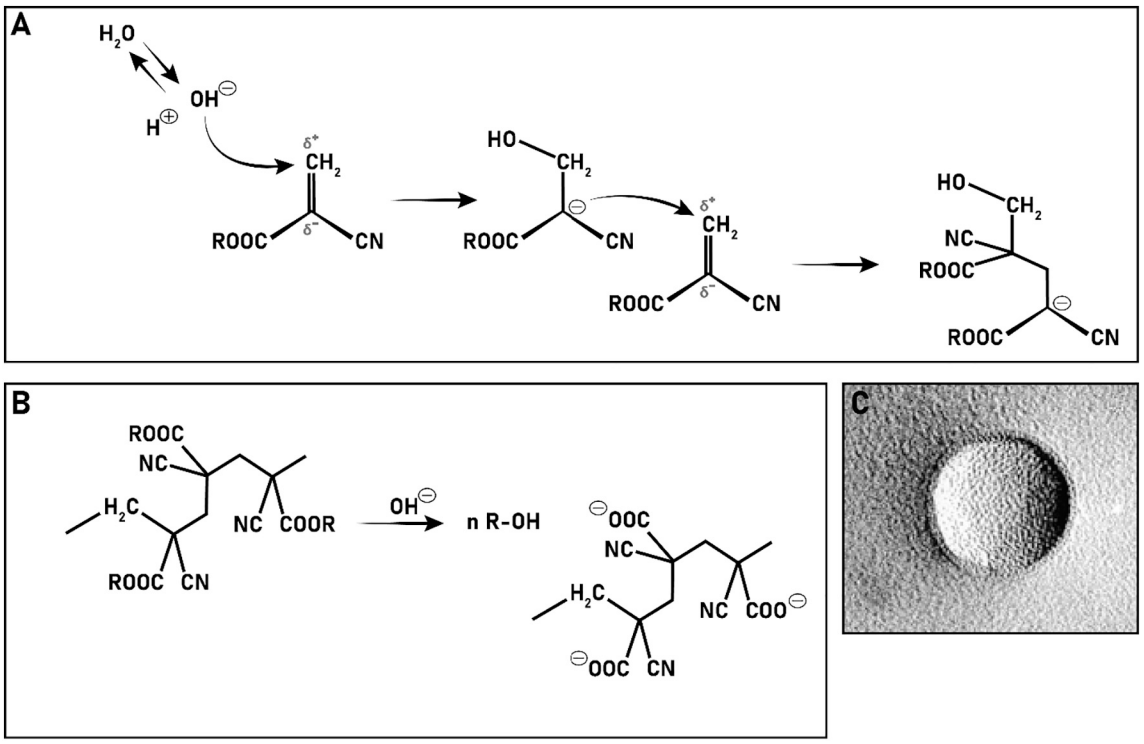


**Fig. 2.** Polyalkylcyanoacrylate nanoparticles. (A) Anionic polymerization of alkylcyanoacrylate monomer in water; (B) the bioerosion of the resulting polyalkylcyanoacrylate polymer occurs through hydrolysis of the ester side chain (chemically or enzymatically), leading to soluble polycyanoacrylic acid; (C) the emulsion polymerization of the cyanoacrylic monomer allows the formation of spherical nanoparticles of 150 nm with a matrix structure as seen by Transmission Electron Microscopy after freeze-fracture.

tuning the polymerization process provided control over the molecular weight of the polymer, as well as, the size and zeta potential of the nanoparticles. Synthesis of amphiphilic block copolymers containing PACA segments linked to polyethylene glycols, polysaccharides or other hydrophilic moieties was possible for specific targeting purposes (6).

**Lesson learned:** Inspiration may arise from reading old scientific articles which were either left behind, if not completely forgotten as they are not easily available via an internet search of the literature. Cyanoacrylate was, an unanticipated discovery by Dr. Harry Wesley Coover during World War II, when he was in search of materials for making clear plastic gun sights (7). Conceptually, the polycyanoacrylate nanostory originates from the discovery during the Vietnam war that cyanoacrylic monomers could be used as surgical glue, but the synthesis of alkyl cyanoacrylate monomers was already first described in 1949 (8).

## 3. Doxorubicin loaded polyalkylcyanoacrylate nanoparticles overcome multidrug resistance

Among the different drugs which were loaded into PACA nanoparticles, special attention has been given to doxorubicin. Doxorubicin is a compound with a broad spectrum of anticancer activity but with detrimental cardiotoxicity, limiting its treatment efficacy. Pharmacokinetic and biodistribution studies on PACA nanoparticles loaded with doxorubicin revealed liver capture of the drug, following intravenous administration, which was paralleled with a dramatic decrease in drug concentration in the heart. As a result, we considered this nanomedicine as a potential treatment for hepatocellular carcinoma with reduced drug cardiotoxicity. Hepatocellular carcinoma is a severe disease with a poor prognosis and this is in large part due to the hyper-expression of efflux proteins (ie. P-glycoprotein, MRP etc.). The efflux proteins allow the cancer cells to pump anticancer chemotherapies out of the cells resuting in so-called multidrug resistance (mdr). To overcome the detrimental action of these membrane transporters, it was hypothesized that when encapsulated into nanoparticles, doxorubicin could be masked from recognition by these transporters, thus overcoming the mdr phenotype. We first determined in vitro the 50% inhibition concentration (IC50) of drug in different human hepatoma cell lines. When compared to the free drug, doxorubicin-loaded nanoparticles displayed a higher cytotoxic activity against resistant hepatocellular carcinoma cell lines. The IC50 was reduced with polyisohexylcyanoacrylate-doxorubicin (PIHCA-Dox) nanoparticles relative to free doxorubicin in Huh7 (1.7-fold reduction), HepaRG (4.5-fold reduction), HepG2 (1.5-fold reduction), and HepG2.2.15 (1.5-fold reduction) cell lines (9) (Fig. 3, A and B). In vivo, it was shown that after intravenous administration, PACA-Dox nanoparticles accumulated in liver Kupffer cells. They then served as as a reservoir for doxorubicin and created a gradient in drug concentration that resulted in a massive and prolonged diffusion of free drug towards the cancer tissue (10). In X/myc transgenic mice used as an in vivo model of hepatocellular carcinoma, it was found that PIHCA-Dox nanoparticles (and not free doxorubicin) induced impressive cell apoptosis. The apoptosis was found to be specific and restricted to hepatocellular carcinoma cells (Fig. 3, C, D and E). Indeed, the rate of apoptosis in non-cancer hepatocytes in the peritumor areas was not affected by the treatment. Noteworthy, at the time of this experiment, it was the first report on an efficient anti-cancer chemotherapy in a transgenic murine model of hepatocellular carcinoma (9). In addition to other data (11), these observations supported initiation of a Phase I–II clinical trial in humans with cyanoacrylate doxorubicin nanotechnologies. The development of formulation that complied with Good Manufacturing Practice (GMP) and the need to perform regulatory toxicological studies in two animal species could not be achieved in an academic environment. Therefore, the start-up company Bioalliance was created in 1998 and entered the stock market in 2005.

**Lesson learned**: since the beginning of this nano story and due to my education as a pharmacist, I was committed to performing the best academic research with the aim of designing a nanomedicine for patients

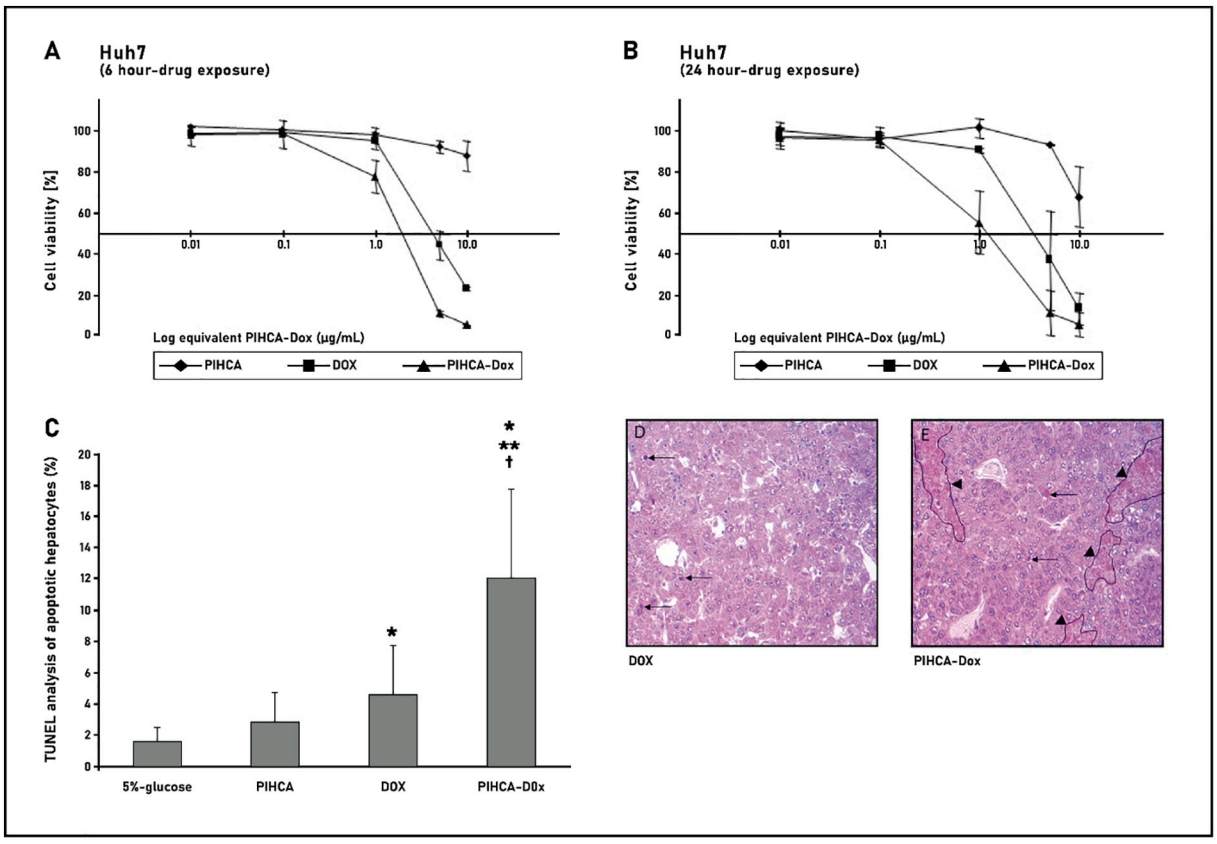


**Fig. 3.** Doxorubicin-loaded Polyisohexylcyanoacrylate nanoparticles overcome multidrug resistance of hepatocellular carcinoma. (A) and (B) In vitro cytotoxicity of doxorubicin-loaded Polyisohexylcyanoacrylate nanoparticles (PIHCA-Dox) versus free doxorubicin (Dox), in Huh7 cell lines, after 6 h (A) or 24 h (B) drug exposure; (C) In vivo rate of apoptosis in cancerous hepatocellular carcinoma cells in X/myc transgenic mice after treatment with PIHCA-Dox or Dox (controls: 5% glucose and PIHCA drug free nanoparticles); (D-E) Histological examination of hepatocellular carcinoma in X/myc transgenic mice treated with Dox (D) or PIHCA-Dox (E). Arrows show apoptotic bodies and arrowheads the limits of the large necrotic tumor area observed in PIHCA-Dox treated mice (Adapted from J Hepatology, 2005).

with severe disease and without efficacious treatment options. To reach that goal, the creation of a start-up company became necessary; this represented an exciting challenge, going beyond the usual pure research and teaching tasks of an academic professor.

## 4. Towards clinical trials

A Phase I clinical trial of PIHCA-Dox nanoparticles was first conducted in 21 patients with refractory solid tumors; the nanoparticles were administered as a 10 min infusion (12). Aside from grade 2 allergic reactions during infusion, which was rapidly reversible once drug administration was discontinued, no cardiotoxicity was observed among the 18 evaluable patients. At a drug dose of 75 and 90 mg/m2, grade 3 hematologic toxicity was observed, the limiting toxicity being neutropenia.

In a Phase II clinical trial, among the 28 participants, 17 patients were randomized to Dox nanoparticles (Transdrug™) and received treatment (30 mg/m$^2$) via intra-arterial administration and 11 patients received standard of care (ie. chemoembolization) (13). However, the trial was terminated in July 2008 because of severe respiratory distress that resulted in death in 2 patients. Slow intravenous infusion of the Dox nanoparticles had been recommended by us for two reasons: this mode of administration had been well tolerated during the Phase I trial, and it resulted in an important liver capture as observed in many pre-clinical studies in rodents. Nonetheless, the clinicians did not follow this advice and used intra-arterial injection which was generally preferred by hepatologists as it provided better access to the liver. At that time, the C-related pseudo-allergic reaction (CARPA) was already well identified as the major side effect associated with nanomedicine treatment. This reaction is attributed to the activation of complement at the surface of the injected nanoparticles, triggering cytokine storm in some patients. The occurrence of the CARPA syndrome was rather difficult to predict at an individual level, due to genetic background variability. However, it was well documented that the event directly correlated with the number of nanoparticles per ml of blood during injection. As a result there is a need to administer the nanoparticles slowly intravenously using a catheter. This was not the case in the Phase II trial where intra-arterial administration was performed with the Transdrug™ nanoparticles. This explained the toxicity observed in the two patients and additional investigations have confirmed this issue. The good news was that analysis of the Phase II trial revealed that the duration of the overall patient survival was substantially increased in the doxorubicin nanoparticle group (31.7 months), compared to standard of care (15 months).

This allowed us to start a multicentric, randomized, open-label study (ie. the ReLive Study), comparing the efficacy and safety of slow repeated intravenous infusion of Transdrug™ (20 mg/m$^2$ or 30 mg/m$^2$) in comparison to the standard of care (tri-therapy). All enrolled patients suffered from advanced hepatocellular carcinoma after failure or intolerance to Sorafenib (14). At the time of the trial (2015–2019), patients randomized to the control group were treated and monitored according to the usual practice at the center and according to their physician's judgment, generally a cocktail of three anticancer compounds. At the end of the trial, the overall survival and survival curves were equivalent for patients treated with the Transdrug™ nanomedicine and patients treated with standard of care. This has hindered further development of the Transdrug™ nanoparticles.

**Lesson learned**: The development of a nanomedicine through to the end of a Phase III clinical trial requires close collaboration between

researchers and clinicians with intense exchange of scientific and clinical information. The scientific experience of researchers who have spent years on the pre-clinical development of the nanomedicine should be taken into consideration by the clinicians and adapted into clinical evaluation and application. And vice-versa, the translational ability of basic research can be significantly improved if conducted in view of clinical practice. This failed during the Phase II clinical trial of Transdrug™ when intra-arterial injection, which was the usual route of administration employed in clinical hepatology, was not adapted to the case of nanomedicine delivery to the liver. Concerning the Phase III, it was conducted by Bioalliance (now ONXEO), a start-up company which despite being listed on the stock market didn't have the experience or the financial support of a big pharma. And this resulted in clinical trials that lasted too long. The standard of care for treatment of hepatocellular carcinoma improved over this extended period of time.The standard of care during the Phase II clinical trial(i.e. chemoembolization) was not the same as the standard of care in the Phase III (ie. multiple chemotherapies) trial. As a result, the Transdrug™ nanoparticles came too late and missed their opportunity to become best in the class for this indication. The author of this paper is convinced that Phase III clinical trials need to be conducted in collaboration with big pharmaceutical companies.

## 5. From cyanoacrylate nanoparticles to "squalenoylation": a serendipitous research story

In an attempt to improve the targeting ability of PACA nanoparticles towards cancer cells, poly(ethylene glycol) (PEG)-coated nanoparticles were coupled to folic acid at the end of the PEG chains. The resulting folate-decorated nanoparticles were found to be capable of specific recognition for the folate-binding protein, a recognized tumor marker (15). The chemical functionalization of the nanoparticles and their fluorescent tagging, allowed us to follow their cellular recognition and intracellular trafficking. These studies were performed by Barbara Stella, a student from the University of Turino (Italy), who pursued her PhD thesis under my supervision. To test the anticancer activity of the folate PACA nanoparticles in vivo, we considered gemcitabine as a good drug candidate for encapsulation. Gemcitabine has broad anticancer activity with, nevertheless, a short biological half-life resulting from rapid metabolism into difluorouracile, an inactive metabolite. Unfortunately, due to gemcitabine's hydrophilicity, its loading into the nanoparticles was inefficient, as observed following ultracentrifugation of the nanoparticle suspension and measurement of the concentration of drug in the supernatant. In contrast to Dox, the preparation of gemcitabine PACA nanoparticles was not possible, due to the lack of hydrophobic interactions between the nucleoside analogue and the polymer. This was a very disappointing result.

Rather than giving up by changing the drug molecule to be encapsulated, the idea came to use a lipophilic derivative of gemcitabine. We began with the synthesis of stearoyl-gemcitabine and other fatty acid-gemcitabine derivatives. However, despite our synthetic efforts, these prodrugs were not soluble in water and therefore not amenable to the preparation process for the PACA nanoparticles which occurred in a water medium. These discouraging results gave us the idea to use a lipid with a more condensed molecular conformation than the fatty acids that we had tried to conjugate to gemcitabine. As it was not advisable to inject a cholesterol derivative, we focused our attention on squalene, a biocompatible lipid precursor of cholesterol biosynthesis. Remarkably, this acyclic triterpene widely distributed in nature, contains two farnesol moieties joined in a tail-to-tail fashion and adopts a dynamically folded molecular conformation in water. Thus we chemically conjugated squalene (via the squalenic acid obtained through the Van Tamelen reaction) to the amino group of gemcitabine. After performing the preparation of the PACA nanoparticles with gemcitabine-squalene as the prodrug to be encapsulated, we unexpectedly observed that after ultracentrifugation, and irrespective of the quantity of gemcitabine-squalene added versus the amount of PACA polymer, all of the gemcitabine-squalene was found in the sediment. This was also found to be the case following ultracentrifugation in the absence of the PACA polymer.

In fact, we discovered that chemical linkage of gemcitabine to squalene resulted in amphiphilic molecules capable of assembling in water to form nanoparticles of 100-300 nm (Fig. 4). This was found to be related to the unique conformation of squalene under aqueous conditions, triggering the spontaneous formation of gemcitabine-squalene nanoassemblies. The same was observed with other nucleoside analogues with antiviral activity (16). From the respective contribution of the molecular weight of gemcitabine and of the squalene, it was possible to calculate a drug loading of 41 wt%, whereas the best reported result with other nanocarriers was 8.3 wt%. By moving from the "physical" encapsulation to the "chemical" encapsulation paradigm, the so-called "squalenoylation" technology represented a step forward in the nanomedicine field. It resolved the low drug loading and avoided the unsuitable "burst release" that is often associated with physical entrapment of drug in nanocarriers.

In comparison to free gemcitabine, the nanoparticles of gemcitabine-squalene displayed an increased anticancer activity in various pre-clinical models, including aggressive metastatic leukemia (ie. L1210 and P388) (17) (Fig. 4) and orthotopic pancreatic cancers Panc 1 and Capan 1 (18). Interestingly, it was further observed that after intravenous administration, nanoparticles made of the squalene derivative of gemcitabine strongly interacted with blood plasma lipoproteins. The lipoproteins then becoming somewhat of an endogenous carrier of the gemcitabine squalenoyl prodrug in the blood stream (19) (Fig. 5). This indirectly confers targeting capacity towards cells with hyper-expression of the LDL receptor, a typical attribute of cancer cells. This also explained the long circulating properties of gemcitabine in blood following iv administration of the gemcitabine-squalene nanoparticles. The administration of gemcitabine in this formulation approach also hindered rapid deamination of the drug. Interestingly, in addition to gemcitabine-squalene other squalene derivatives were found to interact with the blood lipoproteins in a similar manner. Therefore, « squalenoylation » represented a new concept in the drug delivery field, exploiting endogenous lipoproteins without the need for complex nanoparticles with surface functionalization or production of artificial lipoproteins for drug targeting purposes.

At the cellular level, it was also observed that gemcitabine-squalene could overcome drug resistance (due to nucleoside transporter deficiency), enabling slow and progressive intracellular cleavage of the bioconjugate into active gemcitabine (20). Interestingly, further inclusion of magnetite nanocrystals into squalene-gemcitabine nanoparticles resulted in one of the first nanotheranostic approaches: The inclusion of the magnetite nanocrystals enabled targeting of an experimental solid tumor under the influence of a magnetic field and imaging of the targeted tumor nodule (21).

The conjugation of various other anticancer compounds to squalene resulted in the formation of nanoparticles of similar size but quite different supramolecular organizations. Whereas gemcitabine-squalene displayed inverted hexagonal phases (17), cisplatin-squalene bioconjugates self-assembled to form lamellar structures (22). Doxorubicin-squalene molecules formed elongated structures with their shape and stiffness being modulated by the ionic strength of the medium (23). Due to their elongated shape, doxorubicin-squalene filomicelles extended along streamlined blood flow after intravenous administration, which hindered their capture by the mononuclear phagocyte system (MPS). This resulted in a prolonged circulation of the filomicelles without the use of PEG. However, these nanoparticles could enter cancer cells under static conditions (24). This new doxorubicin nanomedicine dramatically decreased the cardiac and intestinal toxicity of the drug, and lead to considerable anticancer efficacy in pre-clinical murine and human experimental models of pulmonary and pancreatic cancers (24). Surprisingly, it was also discovered that when linked to the sense

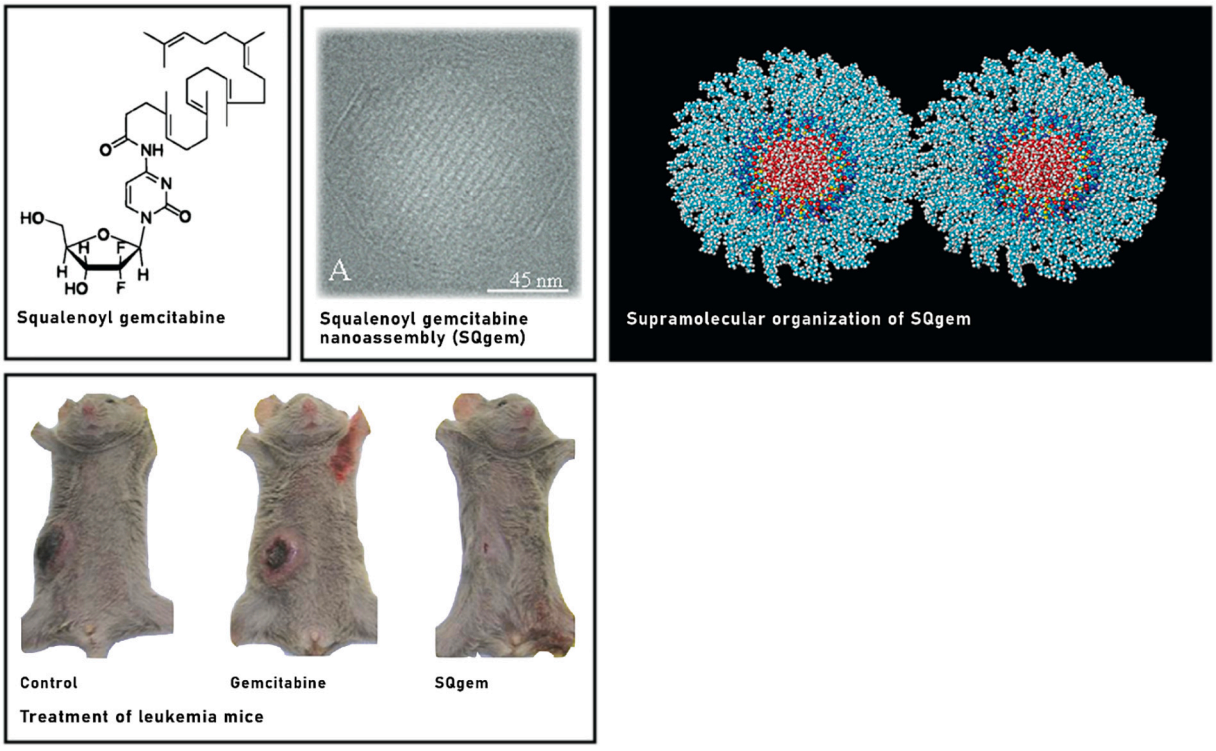


**Fig. 4.** The conjugation of gemcitabine with squalene resulted in gemcitabine-squalene bioconjugates which self-assembled into nanoparticles of 100 nm in size and displayed an inverted hexagonal phase supramolecular structure (adapted from Small 2008).

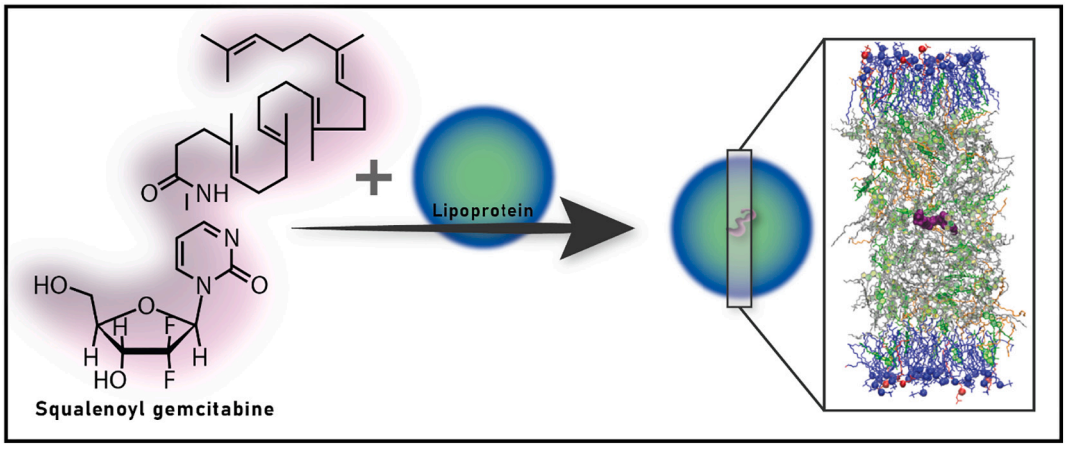


**Fig. 5.** Schematic representation of the interaction between gemcitabine-squalene and cholesterol-rich lipoproteins. After intravenous administration, nanoparticles disassemble in the blood stream. Due to molecular similarity with cholesterol, the squalene moiety of the bioconjugate inserts into low density lipoproteins (LDLs), which indirectly confers targeting capacity towards LDL receptor expressing cells (adapted from Nature Comm. 2017).

oligonucleotide strand of an anticancer siRNA, the small lipophilic squalenoylated moiety induced the formation of nanoparticles, despite the much higher molecular weight of the siRNA-based hydrophilic fraction (25). This demonstrates the high versatility of the "squalenoylation" approach, which applies not only to small anticancer drugs, but also to biomacromolecules for oncogene inhibition.

**Lesson learned**: The discovery that squalene can induce the formation of nanoparticles by supramolecular chemistry is another example of serendipity which has played an important role in my scientific career. Serendipity, even if accidental, may result in unexpected observations and discoveries. Here, we failed to encapsulate gemcitabine into polyalkylcyanoacrylate nanoparticles but we didn't give up and our continued effort led to the coupling of this molecule to squalene resulting in an entirely new nanomedicine concept. A failure or an error may have a significant effect on the outcome of research but the mind must be open and prepared to seize the opportunity of an unexpected observation.

## 6. Squalenoylation, a broad platform with applications in neurological diseases

To further broaden the squalenoylation concept, we explored its applicability in the treatment of neurological diseases which represent another important medical challenge. It's likely that more than 99% of the research efforts in this field are focused on drug discovery and less than 1% on drug delivery. Against the tide of research aimed at

developing nanodevices with the ability to translocate through the Blood Brain Barrier (BBB), we considered the possibility of designing nanomedicines capable of acting on peripherical rather than central receptor targets. Indeed, delivering particles directly into the brain parenchyma raises toxicological issues related to the fate and elimination of nanoparticles from this tissue. In this line of research, we considered adenosine as an interesting drug candidate. In fact, through the interaction with four different G protein-coupled receptors (GPCRs) identified as A1, A2A, A2B and A3, adenosine displays various pathophysiological effects, including regulation of the cardiovascular, nervous and immune systems. It was discovered that the linkage of adenosine to squalene and its subsequent formation into nanoassemblies could be used to trigger impressive neuroprotective activity in preclinical models of brain ischemia (Fig. 6) and spinal cord injury in rodents (26). Findings from an acute stroke study demonstrated that adenosine released from nanoparticles could interact with the adenosine receptors located at the surface of the brain endothelial cells, leading to a diminution of the thrombo-embolic events in the brain microcirculation (Fig. 6, D, E), due to microvessel relaxation and a cytoprotective effect on the cells of the neurovascular unit. This was not observed with free adenosine as this molecule is quickly metabolized after administration ($t1/2 = 10$ s). In the subacute spinal cord injury model, suppression of peripheral inflammation may have also contributed to recovery in addition to microvascular protection (26).

Along the same lines of reasoning, we recently focused on the development of a new endorphin pain killer nanomedicine, for overcoming the severe side effects associated with treatment with morphine and morphine derivatives. Opioid addiction represents a major public health concern, the United States being particularly affected with 11 million people with opioid dependence and about 175 deaths per day resulting from opioid overdose. Since endorphin neuropeptides do not cause tolerance and addiction, they may represent an alternative to the misuse of opioids. Unfortunately, after administration, these molecules degrade in a few minutes. Thus, Leu-enkephalin, an endogenous opioid neuropeptide, was conjugated to squalene to design nanoparticles for alleviation of pain. It was observed that Leu-enkephalin nanoparticles triggered a significant and prolonged pain-relief effect in rats, with prolonged efficacy in comparison to morphine (Fig. 7 E). Noteworthy, unlike morphine, the nanoparticles spared the brain tissue, acting selectively on peripheral opioid receptors. Imaging provided evidence that the neuropeptide was specifically delivered into painful inflammatory tissue, thus avoiding the central effects responsible for addiction and tolerance (Fig. 7 C). In addition, biochemical and histological investigations conducted on the treated animals, demonstrated that the nanoparticles did not induce toxicity or side effects (27).

Very recently, the squalenoylation of a siRNA for reducing PMP22, a protein for which overexpression was associated with type 1A of Charcot-Marie Tooth disease, has impressively restored mouse locomotor activity (31).

**Lesson learned**: In science, there are often major trends that shape the research grants at the national and international level. In the nanomedicine field, the development of nanoparticles for blood brain barrier translocation or their PEGylation to obtain "stealthy" nanoparticles have attracted a lot of interest. These objectives are, of course, respectable but it remains difficult to innovate by just following the current trends, where most of the researchers are focused. I have been fascinated by the work of Pardridge (28) and others for the delivery of nanodrugs at the brain level and I have myself spent a lot of effort to develop functionalized nanoparticles for brain targeting. But our own experiments led to the conclusion that, in general, the amount of nanodrugs able to translocate into the brain remained very limited and that the elimination of polymers or other material from the brain parenchyma should, on the other hand, hamper pharmaceutical development of this approach. This is why we decided to investigate the targeting of peripheral rather than central receptors for the safe treatment of neurological disorders, including brain ischemia, spinal cord injury or pain. And the strong interaction of the squalenoylated nanoparticles with circulating LDL resulted in nanoparticles with long circulating properties, without use of PEG. Out of the box thinking is necessary in the pursuit of ambitious research that goes beyond the state-of-the-art.

## 7. Multidrug nanomedicine and Covid-19

By delivering drugs in tandem to targeted sites, multidrug nanoparticles may boost the drugs' effectiveness, and have the potential to

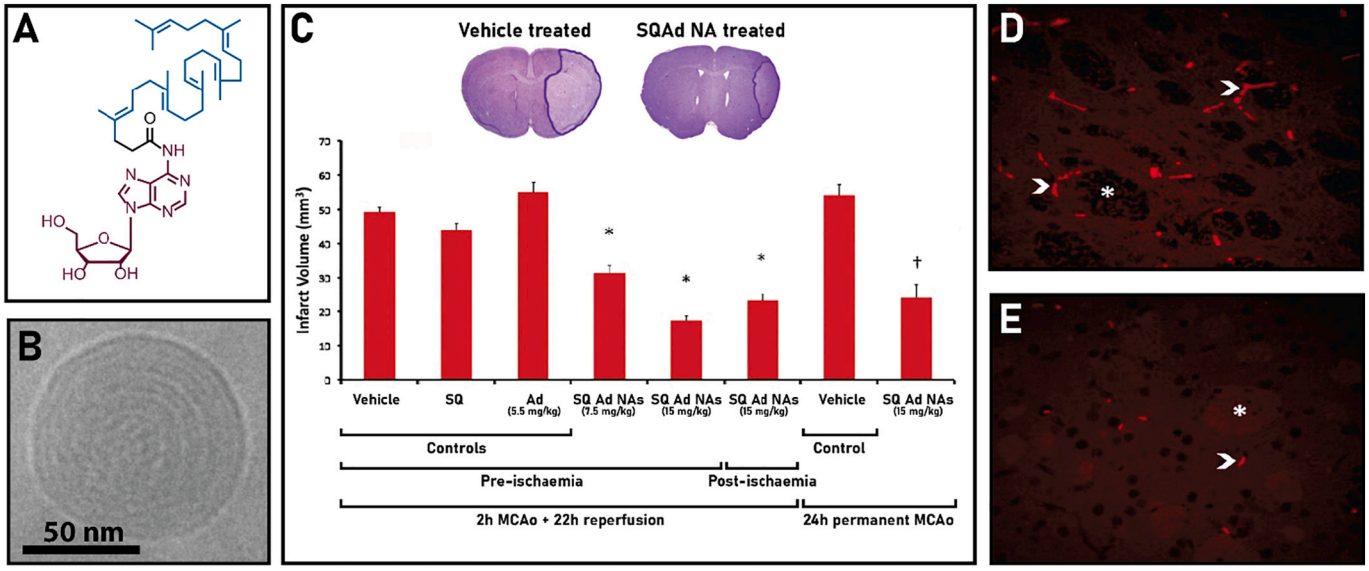


**Fig. 6.** Adenosine-squalene nanoparticles (AdSQ) provide neuroprotection in stroke. (A) Adenosine-squalene bioconjugate (adenosine, red; squalene, blue); (B) Representative Cryo Transmission Electron Microscopy (CryoTEM) image of adenosine-squalene nanoparticles; (C) Ischaemic volumes in control and AdSQ treated mice subjected to transient and permanent (24 h MCAo) focal cerebral ischaemia; (D-E) In untreated mice, capillaries in the ischaemic brain were filled with trapped erythrocytes (D, red fluorescence, arrowheads), whereas the majority of capillaries were not clogged in AdSQ-treated mice (E) (Adapted from Nature Nanotechnology, 2014).

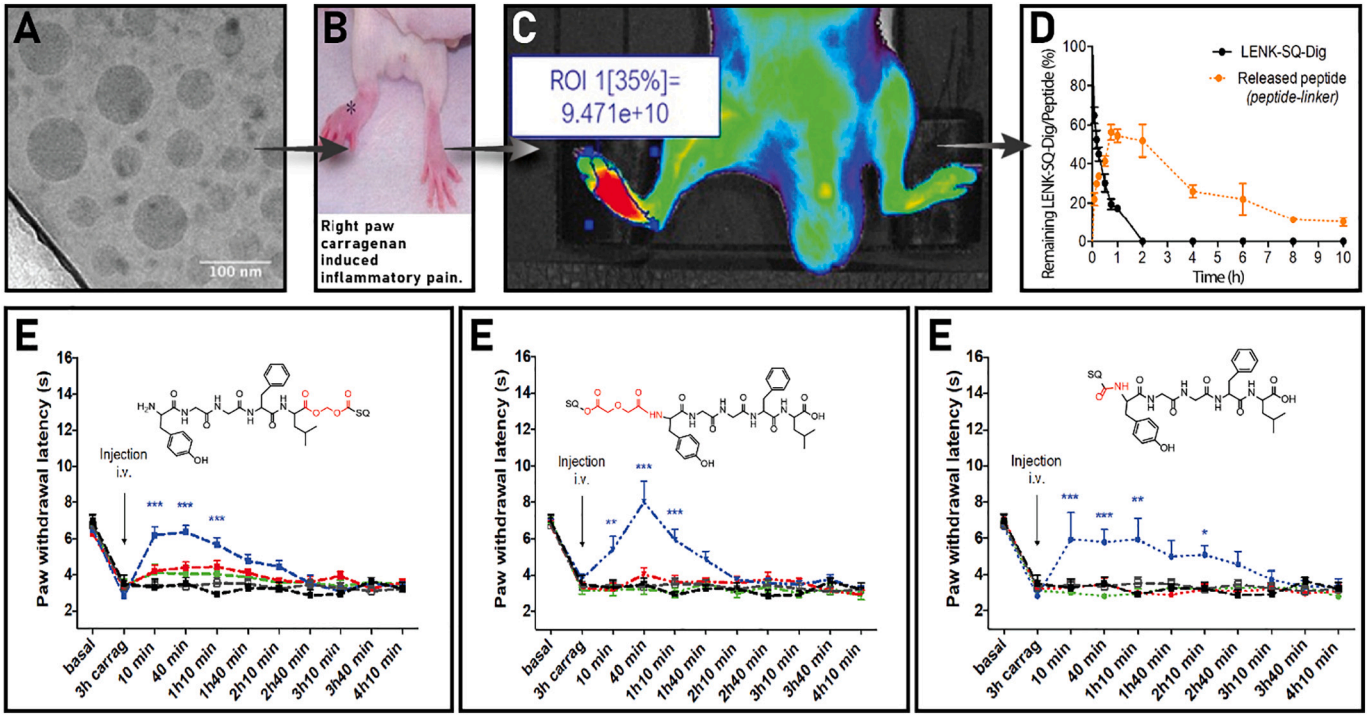


**Fig. 7.** Anti-nociceptive pharmacological activity of Leu-Enkephalin-Squalene nanoparticles (LENK-SQ NPs). (A) Representative cryo transmission electron microscopy image of LENK-SQ NPs revealing their spherical structure with a size of ca. 70 nm. (B) When injected iv. to a rat with right paw induced inflammatory pain, (C) LENK-SQ NPs concentrate in the inflamed right paw (not in the control healthy left paw), (D) where LENK-SQ (black curve) is enzymatically activated to release free LENK neuropeptide (orange curve). (E) The pain relief effect of LENK-SQ NPs (blue curve) with different chemical linkers is abolished after treatment with both naloxone methiodide antagonist (green curve) and naloxone antagonist (red curve), demonstrating local anti-nociceptive activity, since naloxone methiodide doesn't diffuse through the blood brain barrier. Treatment with LENK free or SQ alone has no effect (black and grey curve) (Adapted from Science Advances, 2019).

treat uncontrolled inflammation associated with many diseases, including severe medical issues that result due to COVID-19. Growing evidence suggests that paradoxal inflammation responsible for serious health effects is often caused by feedback loops between pro-inflammatory signaling and an imbalance of free radicals and antioxidants (29). However, efforts to curb these vicious cycles through powerful anti-inflammatory drugs are not always effective in inflammatory conditions such as sepsis, because they impede tissue repair (30). As mentioned previously, adenosine possesses anti-inflammatory pharmacological activity when the nucleoside is protected from rapid metabolism. Thus, we created multidrug nanoparticles to include both adenosine's anti-inflammatory potential with the strong anti-oxidative properties of tocopherol (Vit E). The preparation of adenosine-squalene/Vit E nanoparticles was rather easy due to the strong lipophilic character of both components (30). The efficacy of the adenosine/Vit E nanoparticles was tested in mice by injecting them with lipopolysaccharide, which triggered a cascade of inflammation resulting in a lethal "cytokine storm". Cytokine levels were then measured at various time points after treatment. Mice that received the multidrug nanoparticle injections displayed 100% survival together with a significant decrease in inflammatory tumor necrosis factor alpha and an increase in anti-inflammatory interleukin-10 (30). Pro-inflammatory cytokines in the mice's lungs, liver and kidneys showed significantly reduced levels after adenosine/Vit E multidrug nanoparticle treatment, effects that were not observed following treatment with free drugs.

## 8. Conclusion

As illustrated in this Magnum Opus, I had a chance to start my career at the very beginning of the nano era, when nanomedicine did not yet exist, except the few pioneering articles on liposomes by Bangham and Gregoriadis. I confess that it is always easier to discover in a new field rather than a crowded field. To pursue a new direction is more exciting when there is plenty of room for research. In comparison, continuing in a mature discipline is more difficult and more challenging to develop breakthrough ideas and concepts. But, on the other hand, starting a new research horizon is more of a risk than making incremental findings along a clear path. Therefore, passion and ambition are important drivers in the pursuit of innovative and successful research in still unexplored fields. Noteworthy, negative results may also open new and unexpected perspectives, if the mind is prepared to seize these opportunities by performing additional experiments to better understand the reasons for the unexpected disappointing result.

The scientific environment, preferably with strong interdisciplinarity, is very important to trigger scientific discussions and constructive criticism of experimental data. When growing as a principal investigator, fair management is also mandatory to allow the younger researchers to express their vision. Even if sometimes naïve, the input of PhD students is always very important and often the source of new experimental or even conceptual ideas. And since discovery is not the matter of a single man or woman but the result of a team of individuals working together, an open and friendly environment with diverse scientific culture where everybody can find its place and has a chance to be promoted is also a key for success.

## Acknowledgements

The author is thankful to Profs Christine Allen and Stefaan De Smedt for corrections and careful editing of this manuscript and Maximilian Regenold for improving the figures.

## References

[1] G. Birrenbach, P.P. Speiser, Polymerized micelles and their use as adjuvants in immunology, J. Pharm. Sci. 65 (1976) 1763–1766.

[2] P. Couvreur, P. Tulkens, M. Roland, A. Trouet, P. Speiser, Nanocapsules a new type of lysosomotropic carrier, FEBS Lett. 84 (1977) 323–326.
[3] P. Couvreur, B. Kante, M. Roland, P. Guiot, P. Bauduin, P. Speiser, Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties, J. Pharm. Pharmacol. 31 (1979) 331–332.
[4] V. Lenaerts, P. Couvreur, D. Christiaens-Leyh, E. Joiris, M. Roland, B. Rollman, P. Speiser, Identification and study of a degradation way for polyisobutylcyanoacrylate nanoparticles, Biomaterials 5 (1984) 65–68.
[5] C. Damgé, C. Michel, M. Aprahamian, P. Couvreur, A new approach for oral administration of insulin using polyalkylcyanoacrylate nanocapsules as a drug carrier, Diabetes 37 (1988) 246–251.
[6] M.T. Perrachia, D. Desmaële, P. Couvreur, J. d'Angelo, Synthesis of a novel poly (MePEG-co-alkyl)cyanoacrylate, amphiphilic copolymer for the development of «stealth» PEG-coated nanoparticles, Macromolecules 30 (1997) 846–851.
[7] https://www.warhistoryonline.com/history/history-of-the-super-glue.html.
[8] Ardis, AE, Preparation of monomeric alkyl alpha-cyano-acrylates, Us Patent No. 2467927 (1949).
[9] L. Barraud, P. Merle, E. Soma, L. Lefrançois, S. Guerret, M. Chevallier, C. Dubernet, P. Couvreur, C. Trépo, L. Vitvitski, Increase of doxorubicin sensitivity by doxorubicin loading into nanoparticles for hepatocellular carcinoma cells in vitro and in vivo, J. Hepathology 42 (2005) 736–743.
[10] N. Chiannilkulchai, N. Ammoury, B. Caillou, J.P. Devissaguet, P. Couvreur, Hepatic tissue distribution of doxorubicin-loaded nanoparticles after i.v. administration in reticulosarcoma M5076 metastasis-bearing mice, Cancer Chemother. and Pharmacol. 26 (1990) 122–126.
[11] A. Colin de Verdière, C. Dubernet, F. Némati, M.F. Poupon, F. Puisieux, P. Couvreur, Uptake of doxorubicin from loaded nanoparticles in multidrug resistant leukemic murine cells, Cancer Chemother. Pharmacol. 33 (1994) 504–508.
[12] J. Kattan, J.P. Droz, P. Couvreur, J.P. Marino, A. Boutan-Laroze, P. Rougier, P. Brault, H. Vranckx, J.M. Grognet, X. Morge, H. Sancho-Garnier, Phase I clinical trial and pharmacokinetic evaluation of doxorubicin carried by polyisohexylcyanoacrylate nanoparticles, Investig. New Drugs 10 (1992) 191–199.
[13] National Institute for Health Research, Intravenous Doxorubicin Nanoparticles for Advanced Hepatocellular Carcinoma 8621, NIHR HSRIC ID: 10394 NICE ID, 2017.
[14] ClinicalTrials.gov, Efficacy and Safety Doxorubicin Transdrug Study in Patients Suffering From Advanced Hepatocellular Carcinoma (ReLive), Available from: https://clinicaltrials.gov/ct2/show/NCT01655693 (Accessed 27-03-2017).
[15] B. Stella, S. Arpicco, M.T. Peracchia, D. Desmaele, J. Hoebeke, M. Renoir, J. d'Angelo, L. Cattel, P. Couvreur, Design of folic acid-conjugated nanoparticles for drug targeting, J. Pharm. Sci. 89 (2000) 1452–1464.
[16] P. Couvreur, B. Stella, L. Harivardhan Reddy, H. Hillaireau, C. Dubernet, D. Desmaële, S. Lepêtre-Mouelhi, F. Rocco, N. Dereuddre-Bosquet, P. Clayette, V. Rosilio, V. Marsaud, M. Renoir, L. Cattel, Squalenoyl nanomedicines as potential therapeutics, Nano Lett. 6 (2006) 2544–2548.
[17] L.H. Reddy, S. Mangenot, J.H. Poupaert, D. Desmaele, S. Lepetre-Mouelhi, B. Pili, C. Bourgaux, H. Amenitsch, M. Ollivon, P. Couvreur, Discovery of new hexagonal supramolecular nanostructures formed by squalenoylation of an anticancer nucleoside analogue, Small 4 (2008) 247–253.
[18] S. Réjiba, Reddy L. Harivardhan, C. Bigand, C. Parmentier, P. Couvreur, A. Hajiri, Squalenoyl gemcitabine nanomedicine overcomes the low efficacy of gemcitabine therapy in pancreatic cancer, Nanomedicine 7 (2011) 841–849.
[19] D. Sobot, S. Mura, S.O. Yesylevskyy, L. Dalbin, F. Cayre, G. Bort, J. Mougin, D. Desmaële, S. LepetreMouelhi, G. Pieters, B. Andreiuk, A.S. Klymchenko, J. L. Paul, C. Ramseyer, P. Couvreur, Conjugation of squalene to gemcitabine as unique approach exploiting endogenous lipoproteins for drug delivery, Nat. Commun. 8 (2017) 15678, https://doi.org/10.1038/ncomms15678.
[20] L. Bildstein, C. Dubernet, V. Marsaud, H. Chacun, V. Nicolas, C. Gueutin, A. Sarasin, H. Bénech, S. Lepêtre-Mouelhi, D. Desmaële, P. Couvreur, Transmembrane diffusion of gemcitabine by a nanoparticulate squalenoyl prodrug: an original drug delivery pathway, J. Control. Release 147 (2010) 163–170.
[21] J.L. Arias, L. Harivardhan Reddy, M. Othman, B. Gillet, D. Desmaële, F. Zouhiri, F. Dosio, R. Gref, Couvreur, squalene based nanocomposites: a new platform for the design of multifunctional pharmaceutical theragnostics, ACS Nano 22 (2011) 1513–1521.
[22] L. Kotelevets, E. Chastre, J. Caron, J. Mougin, G. Bastian, A. Pineau, F. Walker, T. Lehy, D. Desmaële, P. Couvreur, A squalene based nanomedicine for oral treatment of colon cancer, Cancer Res. 77 (2017) 2964–2975.
[23] J. Mougin, S. Yesylevskyy, C. Bourgaux, D. Chapron, J.P. Michel, F. Dosio, B. Stella, C. Ramseyer, P. Couvreur, Stacking as a key property for creating nanoparticles with tunable shape: the case of squalenoyl-doxorubicin, ACS Nano 13 (2019) 12870–12879.
[24] A. Maksimenko, F. Dosio, J. Mougin, A. Ferrero, S. Wack, L. Harivardhan Reddy, A. A. Weyn, E. Lepeltier, C. Bourgaux, B. Stella, L. Cattel, P. Couvreur, Squalenoylated doxorubicin: a new long circulating and non pegylated anticancer nanomedicine, Proceed. Natl. Acad. Sci. USA (2014) E217–E226.
[25] M. Raouane, D. Desmaele, M. Gilbert-Sirieix, C. Gueutin, F. Zouhiri, C. Bourgaux, E. Lepeltier, R. Gref, R. Ben Salah, G. Clayman, L. Massaad-Massade, P. Couvreur, Synthesis, characterization, and in vivo delivery of siRNA-Squalene nanoparticles targeting fusion oncogene in papillary thyroid carcinoma, J. Med. Chem. 54 (2011) 4067–4076.
[26] A. Gaudin, M. Yemisci, H. Eroglu, S. Lepêtre-Mouelhi, O.F. Turkoglu, B. Dönmez-Demir, S. Caban, M.F. Sargon, S. Garcia-Argote, G. Pieters, O. Loreau, B. Rousseau, O. Tagit, N. Hildebrandt, Y. Le Dantec, J. Mougin, S. Valetti, H. Chacun, V. Nicolas, D. Desmaële, K. Andrieux, Y. Capan, T. Dalkara, P. Couvreur, Squalenoyl adenosine nanoparticles provide Neuroprotection after stroke and spinal cord injury, Nat. Nanotechnol. 9 (2014) 1054–1063.
[27] J. Feng, S. Lepetre-Mouelhi, A. Gautier, S. Mura, C. Cailleau, F. Coudore, M. Hamon, P. Couvreur, A new painkiller nanomedicine to bypass the blood-brain barrier and the use of morphine, Science Advances 5 (2019) eaau5148.
[28] J. Huwyler, D. Wu, W.M. Pardridge, Brain drug delivery of small molecules using immunoliposomes, Proc Natl Acad Sci USA 93 (14) (1996) 169, 164–14.
[29] R. Brusini, M. Varna, P. Couvreur, Advanced nanomedicines for the treatment of inflammatory diseases, Advanced Drug Delivery Reviews (2020), https://doi.org/10.1016/j.addr.2020.07.010.
[30] F. Dormont, R. Brusini, C. Cailleau, F. Reynaud, A. Peramo, A. Gendron, J. Mougin, F. Gaudin, M. Varna, P. Couvreur, Squalene-based multidrug nanoparticles for improved mitigation of uncontrolled inflammation, Science Advances 6 (23) (2020) eaaz5466.
[31] S. Boutary, M. Caillaud, M. El Madani, J.M. Vallat, J. Loisel Duwattez, A. Rouyer, L. Richard, C. Gracia, G. Urbinati, D. Desmaële, A. Echaniz-Laguna, D. Adams, P. Couvreur, M. Schumacher, C. Massaad, L. Massaad-Massade, Squalenoyl siRNA PMP22 nanoparticles are effective in treating mouse models of Charcot MarieTooth disease type 1A, Communications Biology 4 (2021) 317.