Expert Opinion

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Gemcitabine-loaded innovative nanocarriers vs GEMZAR: Biodistribution, pharmacokinetic features and *in vivo* antitumor activity

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Introduction: Gemcitabine, an anticancer drug, is a nucleoside analog deoxycytidine antimetabolite, which acts against a wide range of solid tumors. The limitation of gemcitabine is its rapid inactivation by the deoxycytidine deaminase enzyme following its *in vivo* administration.
Areas covered: One of the most promising new approaches for improving the biopharmaceutical properties of gemcitabine is the use of innovative drug delivery devices. This review explains the current status of gemcitabine drug delivery, which has been under development over the past 5 years, with particular emphasis on liposomal delivery. In addition, the use of novel supramolecular vesicular aggregates (SVAs), polymeric nanoparticles and squalenoylation were treated as interesting innovative approaches for the administration of the nucleoside analog.
Expert opinion: Different colloidal systems containing gemcitabine have been realized, with the aim of providing important potential advancements through traditional ways of therapy. A possible future commercialization of modified gemcitabine is desirable, as was true in the case of liposomal doxorubicin (Doxil®, Caely®).

Keywords: gemcitabine, liposomes, polymeric nanoparticles, squalenoylation

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1. Introduction

Life without suffering and disease is one of the fundamental desires of every human being. Nevertheless, suffering and myriad pathologies afflict humanity in greater or lesser degrees. Medical research has been making a great effort to develop new therapeutic solutions in order to both improve the quality of life and reduce the incidence of illness, particularly when the latter is invalidating for the patient. Among the various pathologies, solid and liquid tumors represent the most invalidating disease of the new millennium. In fact, numerous psychological and pathological factors concur in the gradual advancement of the disease, thus progressing in many cases to death. Even though death represents an extreme outcome in this pathology, food contaminants [1], besides environmental and chemical factors [2], contribute to the development of new cases of cancer each year and an ever-increasing number of patients are necessarily subjected to therapeutic protocols. For this reason, researchers have been focusing their attention on the investigation of natural compounds [3,4], besides semi-synthetic [5,6] and synthetic anticancer drugs [7,8], in order to improve the quality of standard chemotherapy. Some antiproliferative,

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Article highlights.

- Gemcitabine represents the standard treatment of election for different types of cancer pathologies. GEMZAR®, a product commercially available, possesses a great degree of anticancer efficacy even though its brief plasmatic half-life requires multiple administrations in clinical therapy.
- The entrapment/encapsulation of gemcitabine in colloidal devices was generally observed to improve the biopharmaceutical properties of the drug. In particular, PEGylated liposomes assured the increase in the antitumoral action of the drug with respect to the free form, besides allowing a great reduction in the efficacious dosage.
- The coating of liposomes with innovative copolymers in order to obtain novel supramolecular vesicular aggregates able to be easily modified chemically could represent the next step in the development of colloidal formulations containing gemcitabine.
- Experimentation demonstrated that the conjugation of gemcitabine with squalenic acid favored the formation of novel colloidal aggregates in an aqueous environment, which increased the anticancer efficacy of the nucleoside analog.
- Different preclinical and clinical investigations are in progress in order to obtain further data on the possible use of the best described formulations on human beings.

This box summarizes key points contained in the article.

biological and antimetabolite drugs are already in use in the treatment of cancer while others are in Phases II and III of clinical trials.

Among the antimetabolite drugs, gemcitabine, the pyrimidine antimetabolite closely related to cytarabine, was approved by the Food and Drug Administration (FDA) in May 1996 for use against advanced or metastatic pancreatic cancer (PC) [9]; this fact generated immediate interest since gemcitabine shows chemotherapeutic activity against both RNA- and DNA-containing viruses [10] and represents an effective potential anticancer compound.

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC) is a deoxycytidine antimetabolite, which is active against a wide range of solid tumors, that is, pancreatic, non-small cell lung, breast, bladder, thyroid and multiple myeloma cancers as shown in **Table 1**. In order to exercise its anticancer activity, gemcitabine must first be activated by intracellular phosphorylation mediated by deoxycytidine kinases (dCKs); this process converts gemcitabine into its monophosphate, diphosphate and triphosphate derivates [11]. As a triphosphate derivate, gemcitabine competitively inhibits the effects of deoxycytidine on the synthesis of nucleic acid, thus terminating the elongation of the DNA polymerase chain (following the addition of another nucleotide) and blocking the replicating DNA [10]. However, after its systemic administration, most of any given dosage of gemcitabine is rapidly metabolized into its inactive difluorodeoxyuridine metabolite by the deoxycytidine deaminase present in the blood and liver and then quickly cleared by kidneys, thus demonstrating a half-life of about 15 min [12]. Diphosphate and triphosphate derivates are also reduced through deamination of this compound by a deoxycytidine monophosphate deaminase [13].

Due to its complex pharmacodynamic profile, the antitumoral effects of gemcitabine are highly dependent on the frequency of administration rather than the dosage, and in the case of some tumors a suitable therapeutic response is obtained following daily administration or a prolonged infusion [14,15]. As do a number of other anticancer drugs, gemcitabine shows serious side effects; in fact myelosuppression, mild and transient neutropenia, thrombocytopenia and anemia have been observed during clinical trials [10,16].

Moreover, the appearance of resistance phenomena to gemcitabine compromises the efficacy of the treatment and therapy. Like other nucleoside analogs, gemcitabine is hydrophilic and cannot traverse cell membranes by passive diffusion; in fact, gemcitabine is transported into cells by five nucleoside transporters, two equilibrative nucleoside transporters (ENTs; ENT1) (SLC29A1) and ENT2 (SLC29A2) and three concentrative nucleoside transporters (CNTs; CNT1) (SLC28A1), CNT2 (SLC28A2) and CNT3 (SLC28A3) [17]. Kinetic studies of human cell lines have shown that gemcitabine intracellular uptake is mediated mainly by ENT1 [18].

Deficiency in nucleoside transport activity is a mechanism of drug resistance to a nucleoside analog as it has been initially described for Ara-C in leukemia cells [19-22]. Another important mechanism of resistance to gemcitabine and to nucleoside analogs is associated with an overexpression of the plasma membrane drug efflux pumps P-glycoprotein (P-gP), the product of the *mdr1* gene and multidrug resistance-associated protein [23,24]. On the other hand, the most frequently described form of acquired resistance to gemcitabine *in vitro* is dCK deficiency [25,26]. Moreover, down-expression of cytidine deaminase, amplification of the genes coding for the specific target enzymes of purine and pyrimidine biosynthesis and the inability of a cell to undergo apoptosis (by deregulation of p53 gene) have been proposed as further mechanisms of the drug resistance [25]. So an efficient and increased delivery of gemcitabine inside the tumor cells could allow to bypass these resistance phenomena as a consequence of a higher drug concentration, thus favoring the saturation of the efflux pumps and of the overexpressed enzymes associated to resistance.

The improvement of the therapeutic index, as well as a decrease in its side effects, is critical point that must be considered for anticancer treatment with gemcitabine. This is why a promising clinical approach up to now has been the repeated infusion of the maximum tolerated dose of the anticancer drug. An alternative approach is to increase the effectiveness of chemotherapy by using novel agents, combination therapy or by enhancing drug delivery inside the tumor tissue itself. In this review, we describe the state of the art of gemcitabine delivery, emphasizing the approaches based on the encapsulation of the drug in liposomes and polymeric nanoparticles.

Table 1. Therapeutic activity of gemcitabine alone or combined with other anticancer drugs in solid and liquid tumors.

Single agent	Combination therapy	Carcinoma	Ref.
Gemcitabine	–	Advanced pancreatic cancer	[104]
–	Gemcitabine/Sorafenib	Pancreatic cancer cells	[105]
Gemcitabine	–	Non-small cell lung cancer	[106]
–	Gemcitabine/Bevacizumab/Cisplatin	Advanced non-squamous non-small cell lung cancer (NSCLC)	[107]
Gemcitabine	–	Metastatic breast cancer	[108]
–	Gemcitabine/Cisplatin	Metastatic breast cancer	[109]
	Gemcitabine/Paclitaxel	Metastatic breast cancer	[110]
Gemcitabine	–	Non-muscle-invasive bladder cancer (NMIBC)	[111]
–	Gemcitabine/Oxaliplatin/Vinorelbine	Metastatic bladder cancer	[112]

Moreover, a new strategy, characterized by the 'squalenoylation' of the nucleoside analog, will be focused on and discussed in order to illustrate the features of this innovative concept.

2. Liposomes

Valid approaches in the clinical use of gemcitabine, employed to improve the effectiveness of chemotherapy, are based on different strategies such as the combination of this drug together with other chemotherapies or the use of drug delivery systems. The most promising therapeutic strategy in anticancer therapy with gemcitabine seems to be the use of colloidal devices, particularly liposomes [27,28].

Specifically, liposomes can improve the therapeutic index of gemcitabine by reducing its side effects and/or increasing its anticancer activity [29], protecting the drug against metabolic inactivation and improving its biopharmaceutical features (such as blood circulation longevity) [30]. In fact, following systemic administration, liposomes, and particularly PEGylated systems, are able to protect anticancer drugs from metabolic and plasmatic inactivation besides significantly reducing the degree of anticancer drug accumulation in healthy tissues [31].

Combination therapy is also extensively used in the therapeutic treatment of cancer. Controls carried out on patients enrolled in Phase II and III clinical trials showed that tumor masses are generally reduced and a certain degree of success is obtained when gemcitabine is administered together with other anticancer drugs [32,33].

A very interesting novelty in liposomal technology consists of the co-encapsulation of two anticancer drugs inside a single colloidal device, done in order to obtain a synergistic effect that could improve the resulting pharmacological action, thus achieving an intelligent multidrug therapy. This innovative aspect was proposed in the last years by different research teams [34-37]. For example, Tardi and coworkers co-encapsulated two hydrophilic compounds, namely irinotecan and floxuridine in liposomal formulations, which demonstrated that the contemporary presence of these compounds in the hydrophilic compartment of liposomes did not influence the stability of the vesicular device [38]. These findings opened a new frontier in combination drug therapy, demonstrating that the co-encapsulation of anticancer drugs allowed the achievement of a synergistic effect of the single agents in *in vitro* and *in vivo* carcinoma models [39,40] besides improving their biopharmaceutical and pharmacokinetic profiles [41]. These features, as well as *in vitro* and *in vivo* biological evaluations, could represent a milestone to speed up the therapeutic application of multidrug nanocarriers, thus allowing an upcoming application of these nanoformulations in cancer therapy [42].

Our research group has developed an innovative multidrug carrier (MDC), in which gemcitabine and paclitaxel have been co-encapsulated in PEGylated liposomes [43,44]. The rationality of this approach was based on the knowledge of the differing mechanisms of action of the two antitumoral compounds, the partially nonoverlapping toxicities of the two drugs and the possibility of reducing the effective drug dosage, thus decreasing the side effects provoked by both active compounds. The obtained results showed that an MDC containing co-encapsulated gemcitabine and paclitaxel evidenced physicochemical and technological parameters suitable for therapeutic application in cancer treatment [29]. *In vitro* experiments on MCF-7 cells showed that cell viability was drastically decreased by using the MDC formulation with respect to free single agents or separate gemcitabine and paclitaxel liposomal formulations, and that the obtained effect depended on both the dosage concentration and the exposition time [44]. The obtained results also showed that liposomal MDC induced a predominant G_2 cell arrest with a reappearance of G_0/G_1 and S phase processes. These results are the consequence of the combined anticancer action of gemcitabine and paclitaxel, that is, DNA interaction and microtubule polymerization, respectively [44].

Liposomal delivery of gemcitabine, as a single agent or in combination with other anticancer drugs, could enhance its anticancer effect. Gemcitabine liposomal formulations not only offer effective advantages at the cellular level, as shown by *in vitro* experimentation, but may also offer advantages in terms of both gemcitabine pharmacokinetics and biodistribution profiles. The betterment of these two aspects can further contribute to the improvement of its therapeutic index and hence its possible use in clinical practice for the treatment of cancerous diseases, by increasing the anticancer activity and reducing the drawbacks of the drug. Therefore, liposomal gemcitabine could represent both a new form of nanomedicine and a potential innovative formulation suitable for clinical trial.

2.1 Effects of PEGylation on *in vivo* anticancer treatment with gemcitabine-loaded liposomes

The physicochemical properties of liposomes are, in general, important prerequisites to be considered for *in vivo* administration. Generally, liposomes having large diameters (≥ 500 nm) and positive surface charges are rapidly taken up by the macrophages of the reticuloendothelial system (RES), cleared by systemic circulation and then eliminated in the liver, spleen, lungs and bone marrow tissue [45]. This rapid disappearance of colloidal devices from the blood stream drastically affects the biopharmaceutical properties of the entrapped anticancer drug and modifies its biodistribution and pharmacokinetic features. To overcome this limitation, a valid approach consists of a decrease in the mean sizes and positive surface charges of liposomal systems and the decoration of their surfaces with hydrophilic polymeric chains. Colloidal drug delivery systems can be coated with a hydrophilic polymer in order to bring about both the inhibition of opsonization and the enhancement of water solubility. Poly(ethylene glycol) (PEG) is the most commonly applied nonionic hydrophilic polymer exhibiting stealth behavior. Furthermore, PEG reduces the tendency of particles to aggregate, thus providing steric stabilization, and, therefore, formulations endowed with increased stability during storage and therapeutic application [46].

PEGylation, originally defined as the attachment of PEG to drugs, proteins and macromolecules, is a procedure capable of changing both the basic structure of the drug compound and its systemic metabolism, in such a way as to alter the pharmacokinetic parameters of the drugs used [47]. The ability of PEG to influence the pharmacokinetic properties of drugs and drug carriers is currently used in a wide variety of applications in pharmaceutics. Pharmacokinetic modifications of drugs to be administered lead to a protraction of their presence in the circulatory system. This effect generally depends on the degree of shielding effect PEG has on drugs or colloidal devices following its direct conjugation to the various compounds or the decoration of the drug delivery systems. Another benefit of this effect is generally the increase in the probability that a drug can reach its site of action before being recognized as foreign and consequently cleared from the body [46].

The dual development of the process of PEGylation and liposomal technology opened a new frontier in the field of drug delivery systems. Even though liposomes have been known as versatile drug delivery devices [48] since 1960, only the combination of these two technologies and the subsequent attachment of PEG onto the surfaces of liposomes drastically increased the blood circulation time of this carrier and modified the pharmacokinetic and biodistribution features of liposomes [49]. Klibanov *et al.* demonstrated that 49% of sterically stabilized PEGylated liposomes are still present in blood circulation 5 h after administration with respect to conventional vesicles [50]. PEGylated carriers also show slower uptake by the organs of the RES system (liver and spleen) [47]. Reduced renal filtration and a diminished degree of enzymatic degradation generally occur when PEG moieties coat the surfaces of liposomes. This is why a prolonged half-life and enhanced bioavailability are generally obtained after administration of PEGylated devices. As a consequence, PEG-coated liposomes can diminish the frequency and the amount of drugs administered, improve the quality of life of patients and reduce the clinical cost of therapeutic treatment [51], as evidenced in the case of the marketed PEGylated liposomal formulation encapsulating doxorubicin, Doxil®/Caelyx® [52].

The physicochemical characteristics of PEG are important factors in the determination of its biocompatibility and stealth behavior. As previously reported, the conjugation of PEG to lipid materials assembled into liposomes forms a steric hindrance and/or masking of charges through the formation of a conformational cloud of highly flexible polymer chains able to organize into different possible conformations. This surface distribution prevents interaction with blood components and proteins and reduces the opsonization effect mediated by RES components [49]. The steric hindrance exerted by PEG polymers, as well as other physicochemical parameters of liposomes [53], drastically reduces their recognition by the immune system through the opsonization process and the obtained stealth effect is the basis of PEG's favorable properties in pharmacokinetics [46].

Not only the shielding effect of PEG but also the mean sizes and surface charges of liposomes could influence their biodistribution and pharmacokinetic features after *in vivo* administration [54]. The potential use of liposomes as drug delivery systems in therapeutic practice is also correlated to and depends on different physicochemical and technological parameters of vesicular colloidal carriers [30,31]. In particular, vesicular drug delivery systems with mean sizes inferior to 200 nm are suitable for *in vivo* delivery of anticancer drugs [30,55]. Small-size liposomes evince a long circulating stability and intracellular targeting as compared with large multilamellar ones [28,53]. The reduced size of the colloidal devices allows them to avoid the rapid clearance effects by the macrophages of RES; moreover, 100- to 200-nm liposomes have the ability to pass through

the 'leaky' vasculature of the fenestrated endothelium of tumors [45]. The presence of a negative charge on the surfaces of the liposomes also allows them to avoid the interaction of opsonins, thus reducing their RES uptake [45]. Of course, these features are not sufficient to completely prevent the RES uptake of liposomes; this is why it is always a good thing to decorate the liposomal surfaces with PEG moieties, which reduce the negative zeta potential value of systems due to their effects on the polar head groups of the phospholipids [28,44]. In fact, the nearly uncharged, highly hydrophilic liposomal surface is the fundamental reason why they escape uptake by the RES system besides obtaining a long blood circulation time. These features are indeed able to confer a long circulation half-life to the entrapped drug, which allows a sustained therapeutic regimen [56].

2.2 Effect of liposomal gemcitabine entrapment efficiency for *in vivo* anticancer treatment

Another important parameter to be considered for *in vivo* application of liposomes in anticancer therapy is the entrapment efficiency of molecules. In fact, anticancer drugs could be delivered inside tumor tissues at an efficacious dosage similar to those administered in animals and humans in therapeutic protocols.

Liposomes are extremely versatile drug carriers, which are able to entrap both hydrophilic and hydrophobic drugs [57]. Hydrophobic compounds are almost completely entrapped within the liposomal lipid bilayer and a high degree of encapsulation efficiency is generally obtained [58]. A drastic limitation in the entrapment efficiency of anticancer drugs occurs when hydrophilic compounds and certain high-molecular-weight derivates such as gemcitabine and cytarabine are entrapped in the liposomes [59,60]. This limitation is particularly exasperated when PEGylated or non-PEGylated small unilamellar liposomes (SUVs) are prepared for *in vivo* administration. In fact, gemcitabine becomes exclusively localized in the reduced aqueous compartment of the SUVs and its uncharged form (at physiological pH) allows its rapid diffusion through the liposomal bilayer. This leakage of gemcitabine from the SUVs negatively modifies the dosage of drug that is delivered and accumulated in the tumor tissue, thus eliminating the therapeutic advantages of this compound in the use of liposomal technology in an attempt to ameliorate the pharmacokinetic and biodistribution features of anticancer drugs [30].

Three different potential solutions have recently been proposed to overcome this problem. The first option consists of encapsulating the gemcitabine into vesicular phospholipid gels (VPG), since the encapsulation is complete (~100%) of anticancer drugs in gel-like formulation as free and entrapped gemcitabine [61]; the second strategy involves the synthesis of lipophilic pro-drugs of gemcitabine dissolved in the lipid bilayers of the liposomes [62]; the third strategy is based on the entrapment of gemcitabine by using the pH gradient method (**Figure 1**) [28], a procedure that is normally proposed for doxorubicin [63].

Although the efficiency of these three methods is extensively discussed in literature [62], the pH gradient method remains the best option, both to enhance the entrapment efficiency of gemcitabine and to obtain suitable liposomes for *in vivo* administration. In fact, the amount of drug that becomes encapsulated in the VPG liposomes is generally a mixture of the free gemcitabine present in the outer, extremely dense gel–lipid matrix surrounding the liposomal surface (67%) and the gemcitabine entrapped in the liposomal aqueous compartment (33%). The intrinsic features of VPG formulations (i.e., very densely packed liposomes, semisolid or gel-like consistency, and the same magnitude between volumes of the aqueous phases inside and outside the vesicles) apparently result in an increase in shelf-life and pharmacokinetic properties for gemcitabine with respect to conventional liposomes [61]. Unfortunately, VPG liposomes are very viscous and cannot be administered in this gel-like form in *in vivo* models. This formulation must be diluted before i.v. administration and after dilution the aliquot of gemcitabine outside the liposomes is rapidly leaked, the shelf-life and entrapping efficiency of the formulation become unstable and the formulation has to be administered immediately [61]. During experimentation, this mechanical modification of the original liposomal formulation drastically modified the pharmacokinetic properties and the anticancer effectiveness of gemcitabine both in human tumor xenograft models of the soft tissue sarcoma SXF 1301 (growing subcutaneously) and in the human bladder tumor xenograft BXF 1299T (growing orthotopically) [61].

Secondary approaches involve the synthetic production of gemcitabine pro-drugs by conjugating 4-N-acyl derivates with differing molecular weights (4-(N)-valeroyl (C5), 4-(N)-heptanoyl (C7), 4-(N)-lauroyl (C12) and 4-(N)-stearoyl (C18)) to the anticancer drug [62]. Liposomal formulations containing these lipophilic pro-drugs of gemcitabine increased the drug entrapment efficacy with respect to conventional liposomes. A two-step synthetic approach is generally required to obtain these products, and among the different derivates, only lauroyl (C12) and stearoyl (C18) derivates are stable in both plasma and pH buffer solutions, thus showing suitable physicochemical features for liposomal encapsulation and *in vitro* and *in vivo* administration [62]. Nevertheless, pharmacokinetic and plasmatic stability studies of C12 to C18 pro-drugs of gemcitabine also clearly show that the liposomal gemcitabine formulation offers optimal conditions for the passive targeting of gemcitabine to tumor cells (obtained derivates are stable in plasma, show a long half-life and high area under the curve (AUC), and release gemcitabine inside the cells) [62] and could represent promising formulations for further *in vivo* administration in anticancer therapy [64]. However, the synthetic approach appears expensive and, more importantly, the technology fails to increase the entrapment efficiency of gemcitabine.

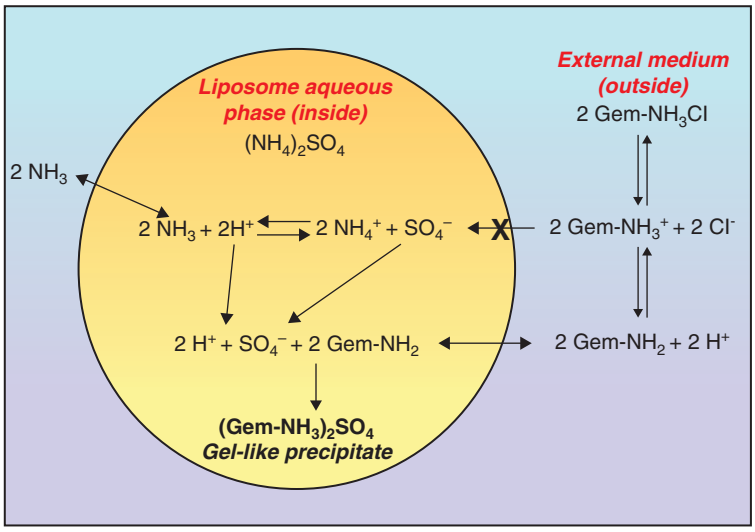

Figure 1. Schematic representation of the gemcitabine encapsulation process within liposomes by means of the presence of a pH gradient produced by the co-encapsulation of a 250 mM ammonium sulfate solution [29].

The last reported option remains the best of the three, which could be used to increase the entrapment efficiency of gemcitabine inside liposomes. As reported in literature by Fritze and coworkers, the transmembrane pH gradient, obtained by using an ammonium sulfate solution (250 mM), showed the most promising physicochemical features for the efficient entrapment of doxorubicin [63]. In a manner similar to anthracycline anticancer drugs, the possibility of protonating the gemcitabine molecule led our research group to use a pH gradient method in order to increase the entrapment efficacy of liposomes [29]. In fact, this procedure allowed a ~90% entrapment of the drug [27,28,44,53]. In the case of the transmembrane pH gradient, the interior of the liposome is acidic, whereas the exterior pH value is adjusted to physiological conditions. The uncharged gemcitabine, which is incubated together with these liposomes, diffuses into the vesicles and the obtained acidic environment in the liposomal core causes the protonation of the gemcitabine amino group, thus forming a gel-like precipitate with anionic sulfate as the counterion of the positively charged gemcitabine and determining the complete entrapment of this drug in the aqueous compartment of the liposomes. The drug precipitate together with its positive charge reduces the leakage of gemcitabine through the liposomal bilayers and the gemcitabine remains efficiently entrapped (**Figure 1**), thus obtaining an effectively high dosage of the anticancer drug in the liposomal formulations [63]. The use of the pH gradient method does not cause any mechanical stress or disrupt the integrity of the vesicular structure; at the same time, the pH gradient method does not require multistep chemical conjugation processes to synthesize gemcitabine derivates and it is easy to apply during the preparation procedure of the liposomes.

All these considerations suggest that the pH gradient method offers effective advantages in the attempt to increase the encapsulation efficiency of gemcitabine in liposomes with respect to gelling inclusion or prodrug derivates. It may also offer some advantages in terms of both gemcitabine pharmacokinetics and biodistribution in *in vivo* models.

2.3 Pharmacokinetic and biodistribution features of gemcitabine-loaded liposomes

Different approaches have been proposed to overcome the short half-life, to increase the bioavailability and biopharmaceutical features of gemcitabine, such as its conjugation with biocompatible materials [60,65] and its encapsulation within innovative colloidal drug delivery systems [66]. In spite of all these efforts, liposomes remained the most reliable option and presented the best characteristics for a rapid translation into *in vivo* models and then into clinical practice.

The design of formulation with physicochemical features, available to escape from macrophage RES uptake and to increase their circulation in the blood stream [49], provides some advantages in anticancer drug delivery. The PEGylation of liposomes generally plays a crucial role in this process due to its efficiency to modulate biodistribution and pharmacokinetic features of the colloidal vesicles and also to influence the biopharmaceutical properties and metabolic fate of the entrapped drug [47].

After systemic administration, gemcitabine is generally rapidly metabolized by deoxycytidine deaminase into its inactive derivate 2',2'-difluorodeoxyuridine and eliminated through urine [10]. Its pharmacokinetic behavior generally imposed different daily administrations of gemcitabine to maintain the therapeutic dosage, thus reducing the compliance of patients during the treatment and increasing their suffering. In fact, free gemcitabine rapidly disappears from plasma after i.v. administration in rat models [30]. Experimental data showed that the plasmatic concentration profile of gemcitabine can

be generally modified after its entrapment in liposomes [30]. In fact, the drug entrapment increased its blood circulation time and a gemcitabine is still detectable in the plasma up to 16 h after *in vivo* administration. The modification of long circulating and biopharmaceutical features of liposomal gemcitabine versus naked drug is also enforced by other principal pharmacokinetic parameters, that is, AUCs and volume of distribution (V_d) [30]. At the same time, the kinetic profile of gemcitabine metabolite is also modified for liposomal formulations. In fact, if comparing the pharmacokinetic data of gemcitabine metabolite, 2',2'-difluorodeoxyuridine, of free drug versus those of liposomal formulation, its amount in plasma concentration is increased and all parameters seemed to suggest that a major amount of gemcitabine is still available for a prolonged period in plasma for metabolic modification by deoxycytidine deaminase enzyme [30].

Moreover, the pharmacokinetic differences between free and liposomal gemcitabine and their respective metabolites can be explained by a protective effect of liposomes against metabolic inactivation of the drug (**Figure 2**) [30]. This protective effect is also evinced by the modification of gemcitabine pharmacokinetic parameters after *in vivo* administration in immunodeficient NOD-SCID mice [31]. *In vivo* mice pharmacokinetic data fit with rat experiments and differences obtained for C_{max} and V_d probably depended on metabolic variations of the two animal models [61]. Also these data agreed with the liposomal protection of gemcitabine in *in vivo* models as previously demonstrated by Moog *et al.* [61]. In fact, gemcitabine-loaded liposomes show a value of AUC that was 35-fold higher than that observed for the free drug and different kinetic orders are carried out for half-lives of free and liposomal drugs in serum model. These differences probably depended on the gel-like consistency of liposomes, which after dilution, but before *in vivo* administration, rapidly leak most of the entrapped drug into the external viscous matrix gel of the system [67].

The protective effect of PEGylated liposomes against plasmatic elimination of gemcitabine is clearly evidenced by clearance (Cl) data. In fact, a decrease of almost twice the amount of Cl is carried out by comparing free drug and liposomal formulation, 69.02 ml/h versus 39.15 ml/h (unpublished data). These Cl values show that gemcitabine prolonged its blood stream circulation in the liposomal form.

The modification of the pharmacokinetic parameters of liposomal gemcitabine and the effect of PEGylation on the enhanced permeability and retention (EPR) passive targeting intra-tumor accumulation generally decreases the drug distribution in healthy tissue, thus preventing the appearance of anticancer drug side effects. To demonstrate this hypothesis, the biodistribution profile of gemcitabine-loaded liposomes was investigated in rats. PEGylated liposomes, prepared by including [^{3}H]cholesteryl hexadecyl ether in the liposomal composition to act as a radiolabeled marker, have an incidence of distribution in the blood, which is higher than that observed for the liver and spleen, thus showing that these liposomes remain in circulation for a prolonged time and the entrapment of gemcitabine in the vesicular carrier is able to modify the resultant biopharmaceutical features. At the same time, the degree of localization of liposomes inside the tumor remained higher than that observed in the liver and spleen and was detectable for up to 24 h following administration [31].

The information resulting from the use of radiolabeled liposomes was similar to that obtained by entrapping the radiolabeled drug [5-^{3}H]gemcitabine. These findings evidence how closely the biopharmaceutical features of gemcitabine are related to those of the liposomes in which it is enclosed. In fact, the degree of accumulation of [5-^{3}H]gemcitabine inside tumor tissue is increased after its entrapment in liposomes. In particular, experimental findings showed that the amount of liposomal gemcitabine detected in the tumor mass is five-fold greater than that measured when using the un-entrapped form of the drug [31].

The accumulation of gemcitabine inside tumor tissues, after liposomal entrapment, was also investigated by Moog *et al.* [61]. ^{14}C-radiolabeled liposomes showed that liposomal gemcitabine is still present in the blood stream after 72 h, while conventional gemcitabine reaches the maximum peak concentration in a very short time following administration and a complete clearance of the anticancer drug is obtained after 24 h [61]. This enhancement of the staying power of encapsulated gemcitabine can be attributed to a significant degree of extravasation and accumulation of the liposomes in the primary tumor and this evidence is in agreement with the hypothesis of Matsumura and Maeda that solid tumors exhibit a heightened permeability of their vasculature, which allows macromolecules as well as liposomes [68] to passively target the tumor. In fact, this elevated concentration of colloidal vesicles administered in *in vivo* models could quickly saturate liver, spleen and circulating macrophages and allow the remaining circulating vesicles to accumulate in the tumor by way of the EPR effect [61]. This hypothesis is further supported by the elimination kinetic of liposomal gemcitabine.

The experimental investigations of Paolino *et al.* [31] and Moog *et al.* [61] confirmed the hypothesis that the entrapment of gemcitabine in liposomes increased the accumulation of this anticancer drug inside the tumor as compared with the native anticancer drug and that the biodistribution features are generally influenced both by the physicochemical properties of colloidal devices and by the concentration of the lipid formulation injected into *in vivo* models.

2.4 *In vivo* antitumor activity in xenograft animals and histological analysis

Both the pharmacokinetic and the biodistribution features of liposomal gemcitabine [30,31], as well as its antiproliferative efficiency exhibited in *in vitro* models [27,28,44,53] encouraged *in vivo* investigation in order to evaluate the real potential of liposomal gemcitabine as a valid formulation for the therapeutic treatment of solid cancer diseases.

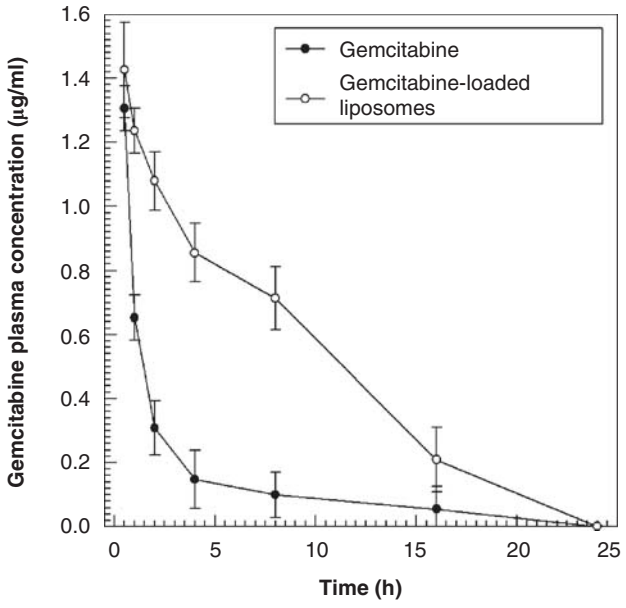

Figure 2. Pharmacokinetic profile of the free gemcitabine and gemcitabine-loaded liposomes after i.v. administration in NOD-SCID mice. Data are expressed as the mean of the drug plasma concentration (µg/ml) ± standard deviation of three independent experiments. Error bars if not visible are within the symbols. The pharmacokinetic values of gemcitabine-loaded liposomes are significantly different from those of the free drug, ANOVA $p < 0.001$ (unpublished data).

In our initial work, our research group investigated the *in vivo* anticancer activity of liposomal gemcitabine in two xenograft murine models of human pancreatic carcinoma cells. SCID mice bearing subcutaneous PSN-1 or BxPC-3 human xenografts were intraperitoneally (i.p.) treated for 35 days with free and liposomal gemcitabine [30]. Experiments showed that the treatment with liposome gemcitabine provided significant advantages in the xenograft murine models over the free drug (**Figure 3**) in terms of both survival rate and tumor growth inhibition, and a slight decrease in tumor mass proliferation was observed in the case of the PSN-1 xenograft animal model (**Figure 3**). These findings probably depended on the greater sensitivity of this PC cell line to treatment with gemcitabine. For both *in vivo* xenograft animal models, experimental findings demonstrated that liposomal gemcitabine exerted a stronger anticancer effect than the free drug, even though the liposomal formulation was administered at a threefold lower drug concentration than the free gemcitabine [30].

A similar effect was successively demonstrated in immunodeficient NOD-SCID mice subcutaneously bearing a human anaplastic thyroid carcinoma cell (ARO cell) xenograft [31]. The *in vivo* treatment with liposomal gemcitabine (5 mg/kg) and free gemcitabine (50 mg/kg) determined a similar decrease in the tumor volume with respect to the control. These findings were very interesting because they were obtained using a 10-fold lower concentration of gemcitabine in the case of the liposomal formulation [31]. The fact that a 10-fold lower drug concentration was effective in the case of the liposomal gemcitabine could represent an important advancement in anticancer treatment due to the possibility of reducing the most common side effects, such as the thrombotic and vascular toxicity of gemcitabine. This hypothesis was supported by investigations on the hematological parameters of mice treated with liposomal gemcitabine (unpublished data).

Histological analysis of the tumor masses was also carried out and the data obtained from animals subjected to various treatments showed no significant differences in tumor tissue organization. The pharmacodynamic profile of gemcitabine was not modified by its encapsulation in PEGylated liposomes. Micrographs highlighted the fact that undifferentiated carcinoma cells are multinucleated and alveolar and trabecular patterns are also evident [31]. Indeed, modified and large nuclei, often vesicular, were present in the morphology of carcinoma cells. Large amounts of hyalinized fibrous tissue and dense material were also present in undifferentiated thyroid carcinomas (**Figure 4**).

These experiments enforced the *in vivo* anticancer activity of gemcitabine-loaded liposomes with respect to the native drug as also demonstrated by Moog *et al.*, using two other xenograft models: a soft tissue sarcoma model and an orthotopic bladder carcinoma model [61].

Soft tissue sarcoma xenograft SXF 1301 showed that at gemcitabine equimolar dosage (6 mg/kg), a complete remission of the tumor mass was obtained in response to the liposomal formulation, while the free gemcitabine determined only a moderate inhibition of tumor growth. A significant decrease in the tumor volume in the soft tissue sarcoma xenograft SXF 1301 with respect to control was obtained when free gemcitabine was administered at a maximum tolerated dosage of 360 mg/kg, but even this high therapeutic dosage did not induce a complete regression of the tumor as was true in the case of the liposomal gemcitabine [61]. Considering liposomes as sustained release formulations, even in the case of a daily administration of free gemcitabine, significant advantages in terms of growth inhibition and remission of tumors are still maintained by the liposomal form of gemcitabine [61].

In an orthotopically growing bladder carcinoma BXF 1299T, the anticancer activity of gemcitabine liposomes was compared with that of vindesine and taxotere at their maximum tolerated dosages, and the chemotherapeutic efficacy was assessed in terms of growth inhibition of the primary tumors and their peritoneal metastases. The most effective treatment turned out to be that carried out using liposomal gemcitabine, which also induced tumor stasis during therapy [61].

Analysis of micro-metastasis in the lung, liver, peritoneum and regional lymph nodes of BXF 1299T rats using

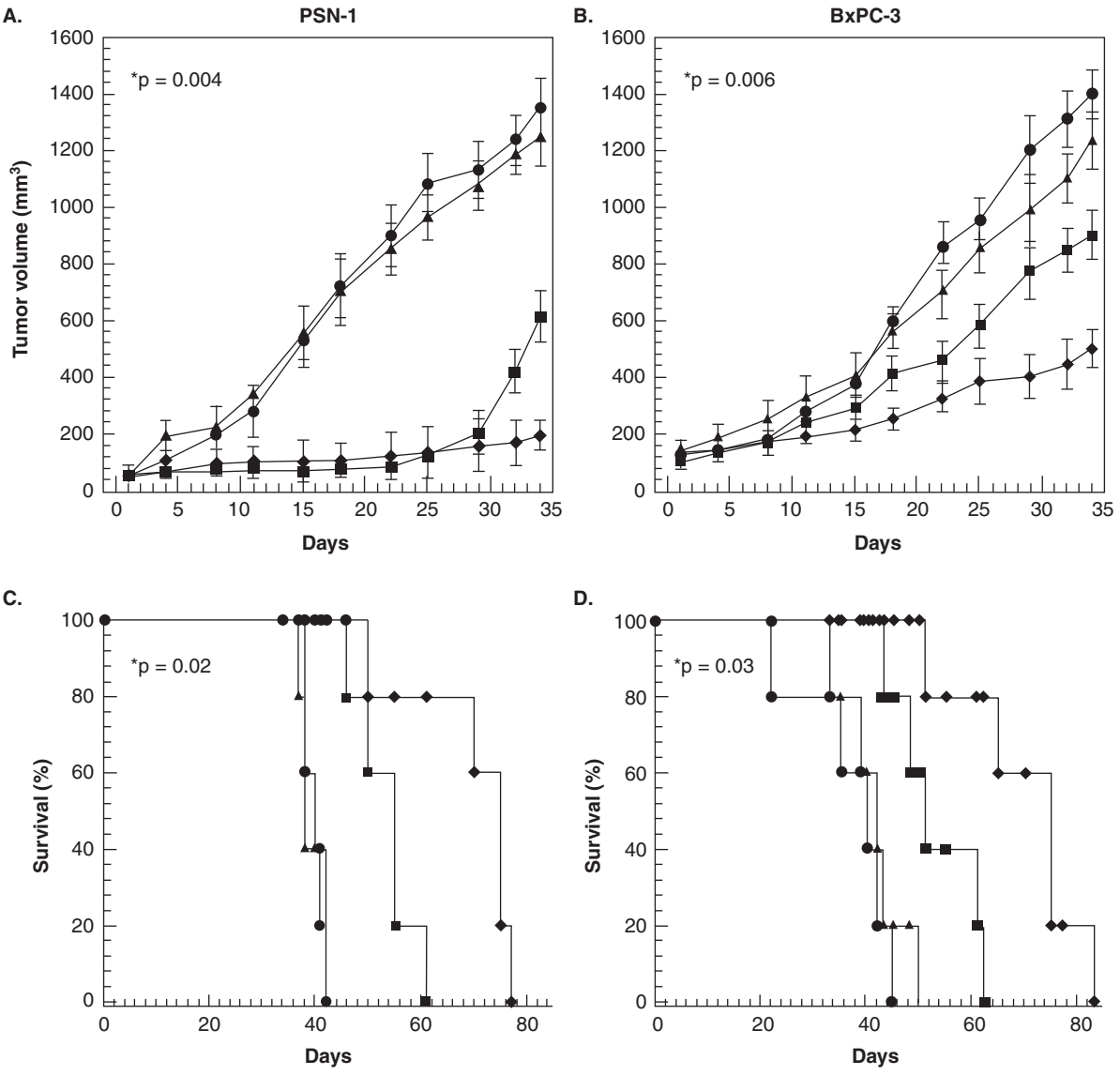

Figure 3. *In vivo* antitumor effects of gemcitabine (15 mg/kg) and L-gemcitabine (5 mg/kg) against PC xenografts (a, b). CB-17 SCID mice bearing PSN-1 and BxPC- 3 xenografts cancer were i.p. treated twice a week for 5 weeks. Mouse survival following treatments is shown in c and d. Symbols: ● control; ▲, treatment with unloaded liposomes; ■, treatment with gemcitabine; ◆, treatment with L-gemcitabine.

With kind permission from [30].

*p value refers to L-gemcitabine versus gemcitabine.

immunohistochemical staining of human cytokeratin also showed that none of the animals treated with liposomal gemcitabine presented detectable metastases after treatment; while seven, three and one detectable metastases were observed among six animals in the cases of the control-, vindesine- and taxotere-treated groups, respectively [61].

The chemotherapeutic effectiveness of gemcitabine was also evaluated in another *in vivo* xenograft model. The drug was entrapped in pH-sensitive epidermal growth factor receptor (EGFR)-targeted immunoliposomes. The analysis of tumor volumes after i.v. injections of gemcitabine entrapped in pH-sensitive EGFR-targeted immunoliposomes and PEGylated liposomes showed a tumor volume reduction two to three times greater in comparison with animals treated with gemcitabine-loaded PEGylated liposomes. The chemotherapeutic effect of gemcitabine-loaded pH-sensitive EGFR-targeted immunoliposomes showed a dose-dependent effect: the tumor volume decreased with increasing doses of the entrapped drug [69]. At the same time, the tumor volume remained the same for up to 1 week after the suspension of the treatment with gemcitabine-loaded pH-sensitive EGFR-targeted immunoliposomes, while a continuous increase

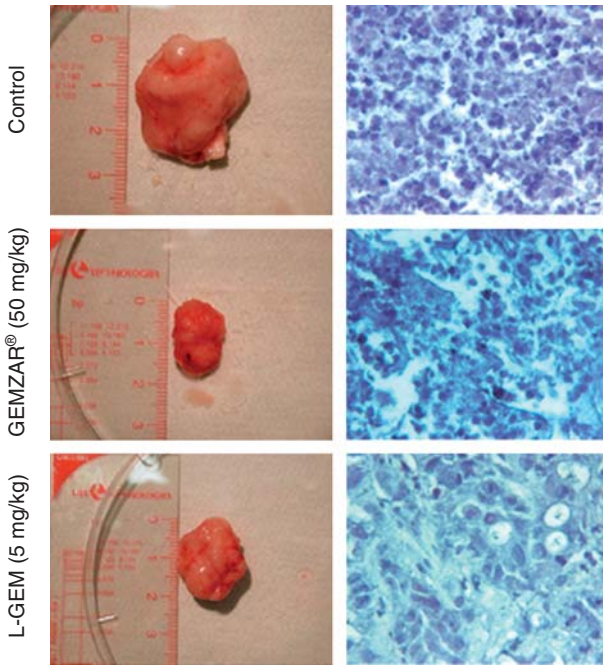

Figure 4. Histological analysis of neoplastic masses excised from immunodeficient NOD-SCID mice bearing human anaplastic thyroid xenograft tumors following their sacrifice at the end of the experiments. Sections with a thickness of 7 – 10 μm were sliced and stained with the eosin B/hematoxylin method. The photos of the analyzed tumor masses were reported close to the respective histological slices. Animals were treated with saline (control group), GEMZAR® (50 mg/kg) and L-gemcitabine (5 mg/kg).
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was observed during use of the other formulations [69]. This response to therapeutic treatment depends on the conjugation of EGFR in the pendent chains of the PEG surrounding the surfaces of the liposomes, which presumably enhances the delivery of the anticancer drug into A549 non-small lung cancer cells as a consequence of the binding of anti-EGFR antibodies to the EGFR receptors in non-small cell lung cancer [69]. Therefore, pH-sensitive EGFR-targeted immunoliposomes could be an effective drug delivery system employed to suppress tumor growth due to the targeting capability of the EGFR antibody as well as the pH sensitivity of the liposome [69].

Tumor proliferation and apoptosis in an A549 tumor nude mouse model were confirmed using immunohistochemical staining after treatment with various gemcitabine formulations. Tumor tissues were isolated and then analyzed using proliferation assay and TdT-mediated dUTp nick end labeling (TUNEL) assay. The proliferating cell nuclear antigen, a marker of cell proliferation, exhibited a markedly lower count of brown-colored tumor cells treated with pH-sensitive EGFR-targeted immunoliposomes with respect to the control and other groups, while TUNEL assay showed a marked increase in apoptotic foci when A549 cells were treated with pH-sensitive EGFR-targeted immunoliposomes [69].

In vivo investigations also showed that the anticancer activity of liposomal gemcitabine in xenograft animal models is generally more efficacious than treatment with the free form of gemcitabine and/or control [30,31]. Anticancer activity could generally depend on the formulation compositions of the liposomes and the entrapped drug concentrations [69]. In fact, an increase in the concentration of gemcitabine in the liposomal devices, their surface modification with targeting agents and the use of pH-sensitive liposomal carriers drastically inhibited tumor growth volume and allowed a significant amelioration of anticancer activity in *in vivo* therapy [69].

3. Supramolecular vesicular devices for gemcitabine anticancer therapy

Supramolecular devices have been extensively investigated in the last decade as potential drug delivery systems. The idea of developing these innovative formulations is based on the possibility of employing biocompatible and biodegradable materials having different physicochemical compositions, able to be self-assembled into complex structures without using chemical conjugation processes. Supramolecular aggregates have the advantage of combining the safe and biocompatible properties of phospholipid-based colloidal vesicular carriers with those of polymeric materials composed of different monomers and designed to receive the introduction of several functional groups into their side chains (grafting). Nevertheless, the first-generation supramolecular devices, such as polymersomes, have been extensively investigated by Discher and coworkers [70]. The heightened interest in this research field has recently increased the attraction of scientists for supramolecular aggregates and various complex systems have been proposed as drug delivery systems [71].

Innovative supramolecular vesicular aggregates (SVAs) have recently been proposed by our research group as devices suitable for anticancer therapy [66,72]. In particular, the SVAs designed in our laboratory combine the features of self-assembly of the phospholipid-based vesicles and polymeric materials without altering the vesicular structure and architecture of the bilayer structure.

The biodistribution and pharmacokinetic features of these SVAs were investigated by our research group [72]. When compared with PEGylated liposomes, SVAs show a similar biodistribution profile following their administration both in rats and in mice. In fact, the α,β-polyaspartyl[(hydrazide)-co(butyrylhydrazide)]copolymer and its folate derivate can self-assemble with liposomal vesicles thus acting in a manner similar to that of the PEG moieties [55].

The administration of [^{3}H]cholesteryl hexadecyl ether radiolabeled folate-targeted (FT) SVAs and untargeted SVAs showed a more rapid initial uptake of both formulations in the liver with respect to other organs. These findings may be due to a specific process of receptor endocytosis, which led

first to liver saturation and then to the biodistribution of SVAs and FT-SVAs to the other organs [73]. In particular, the FT-SVAs caused a more modest increase in liver uptake with respect to the SVAs. This difference disappeared after 4 h and it was inverted after 8 and 16 h from the administration of the two drug carriers. [72]. Overall biodistribution data showed that there was no substantial difference between SVAs and FT-SVAs after 1 h. No significant differences were observed in the blood biodistribution and residence time of both formulations, thus evidencing that the presence of folate moieties conjugated to the backbone of the α,β-polyaspartyl [(hydrazide)-co(butyrylhydrazide)]copolymer exerted no influence over the biodistribution features of SVAs in the circulatory system.

To evaluate whether the biodistribution patterns of SVAs and FT-SVAs are able to modify the biopharmaceutical features of the delivered anticancer drug, *in vivo* pharmacokinetic experiments were carried out. The pharmacokinetic profile of gemcitabine entrapped in SVAs and FT-SVAs showed that supramolecular devices modify the residence time of the entrapped drug in the bloodstream after systemic administration. As in the case of the liposomal devices [31], both the SVAs and the FT-SVAs protected the entrapped gemcitabine from rapid metabolic bioconversion into the inactive deoxyuridine metabolite. In fact, the pharmacokinetic parameters showed that the free gemcitabine rapidly disappeared from the blood due to its metabolic conversion into the 2',2'-difluorodeoxyuridine metabolite [74] and was also rapidly excreted as an inactive metabolite through the urinary pathway [10]. By contrast, the gemcitabine-loaded FT-SVAs were slowly removed from the bloodstream and a prolonged plasmatic concentration of anticancer drug was achieved for up to 16 h following i.v. administration. Other pharmacokinetic parameters, such as AUC, V_d and $t_{1/2}$, were generally modified when gemcitabine was entrapped in FT-SVAs, thus obtaining an overall improvement in the biopharmaceutical features of gemcitabine-loaded FT-SVAs [75].

4. Squalenoylation

An innovative approach attempted in order to improve the biopharmaceutical properties of gemcitabine was its conjugation to squalenic acid molecules. Squalene is a polyunsaturated triterpene containing six isoprene units and is a biochemical precursor of cholesterol and other steroids. Squalene is abundantly diffused in nature (olives, shark liver oil, etc.) and it is well tolerated after both intravenous and oral administration [76]. Squalenoyl gemcitabine (Sq-gemcitabine) was derived from attempts to protect the nucleoside from the deamination process and to improve its loading capacity inside the liposomal device by covalently coupling acyl chains to its 4-amino group [77]. These new compounds were more cytotoxic and less water soluble, and, therefore, difficult to administer by the i.v. route (i.e., stearoyl, valeroyl and lauroyl derivates). Surprisingly, though, Sq-gemcitabine allowed the achievement of novel colloidal nanoassemblies having a mean size of 150 nm and a narrow size distribution (polydispersity index of about 0.1), following dispersion in an aqueous environment (**Figure 5**), while the other gemcitabine lipophilic derivates precipitated as large aggregates [78]. In particular, molecular modeling studies of Sq-gemcitabine in an aqueous medium provided the theoretical evidence of the formation of an inverse hexagonal phase characterized by nanoaggregates with a central core made up of water and gemcitabine molecules surrounded by a layer of squalenic molecules (**Figure 6**) [79]. The research team of Prof. Couvreur thoroughly investigated the pharmacological activity of Sq-gemcitabine nanoparticles with respect to the free drug and analyzed its pharmacokinetic profile.

Initially, *in vitro* tests demonstrated that Sq-gemcitabine nanosystems were six- to eightfold more cytotoxic with respect to the free compound on MCF-7 and KB3-1 cells. Moreover, they increased the antitumoral effect of gemcitabine on L1210K cells [80], a cell line less sensitive to the drug than the parent wild-type cell line because of its deficiency in the dCK [78]. An important mechanism of gemcitabine resistance in leukemic cells is strictly related to the absence of the nucleoside transporters, that is, hENT1, hENT2, hCNT1. These transporters made part of molecular mechanism involved in gemcitabine sensitivity/resistance in cells and tumor tissues. In particular, hENT1 transporter is required to translocate drug across the cell surface and is generally involved in gemcitabine transport in tumors as its expression correlates with cellular proliferation [81]. Moreover, this nucleotide transporter, associated with hCNT1, plays a crucial role in the internalization and anticancer activity of gemcitabine [82]. The increase in cytotoxicity exhibited by Sq-gemcitabine on L1210 10K leukemia cells compared with gemcitabine can be generally correlated to lower resistance (a factor of 32) of gemcitabine nanoaggregates than native anticancer drug and this effect probably depends on the slow intracellular release of gemcitabine from squalenoyl derivate [80].

In vivo experiments in human PC showed that adenoviral-mediated overexpression of hENT1 enhanced gemcitabine sensitivity [83], while carcinoma cells with mutated or deleted nucleotide transporter exhibited a great resistance to gemcitabine and other nucleotide analog anticancer drug [84], thus suggesting as the balance between anticancer activity and resistance in human carcinoma cell lines depends on the expression of genes encoding proteins involved in gemcitabine transport. Moreover, a study published by Gusella and coworkers showed that hENT1 genotype is correlated with pharmacokinetic features of gemcitabine in Caucasian patients with solid tumors [85]. In particular, the genotype role is very important in patients with low cytidine deaminase activity and clearance, and plasma concentration of gemcitabine in these subjects is reduced probably due to drug reabsorption at the renal tubule level. Additionally, clinical study in patients with PC treated with gemcitabine showed that low expression

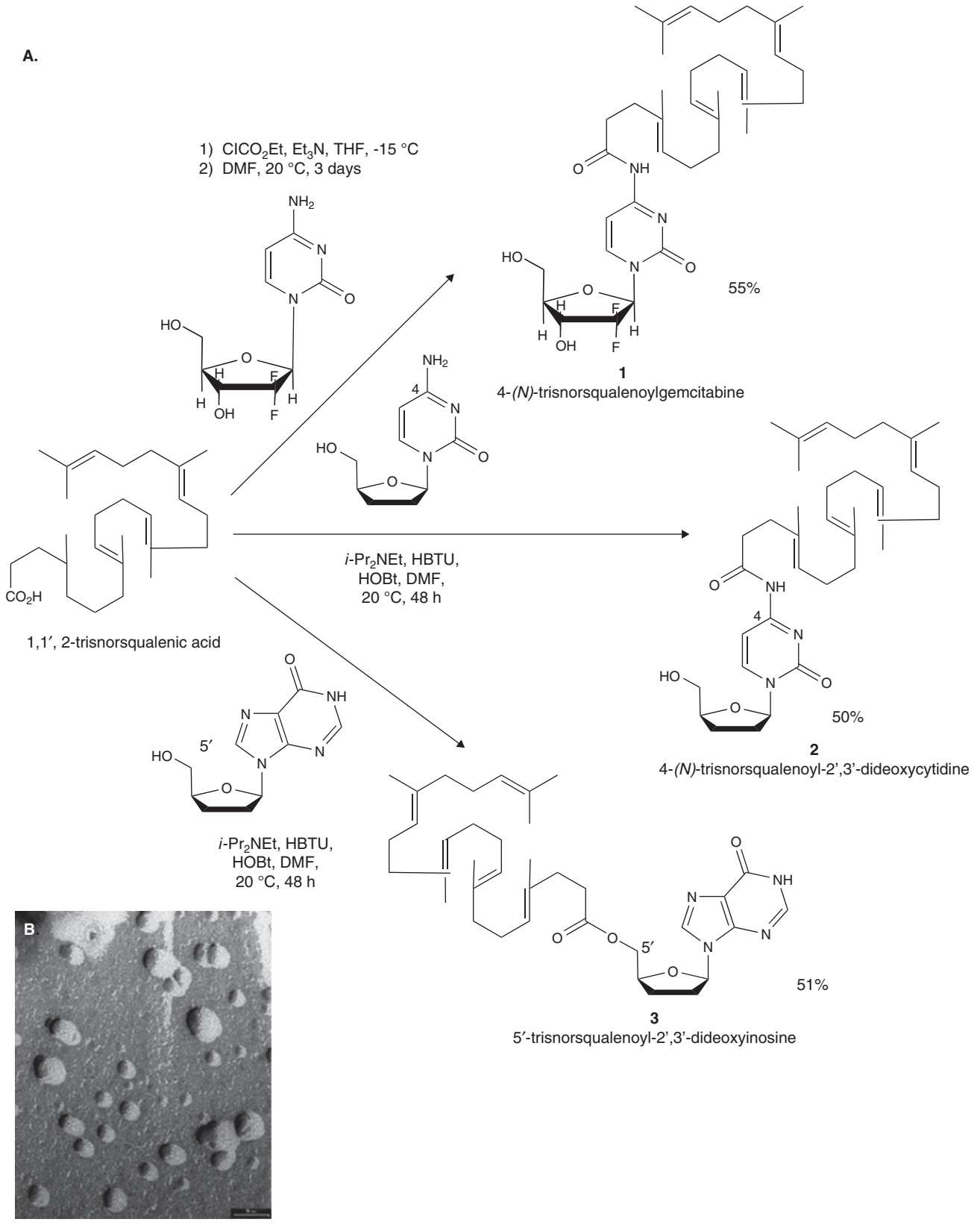

Figure 5. (A) Synthesis of 4-(*N*)-trisnorsqualenoylgemcitabine (SQdFdC) (**1**), of 4-(*N*)-trisnorsqualenoyl-ddC (**2**) and of 5′-trisnorsqualenoylddI (**3**), (**B**) Transmission electron micrograph of 4-(*N*)-trisnorsqualenoylgemcitabine nanoassemblies after freeze-fracture. Bar, 50 nm.

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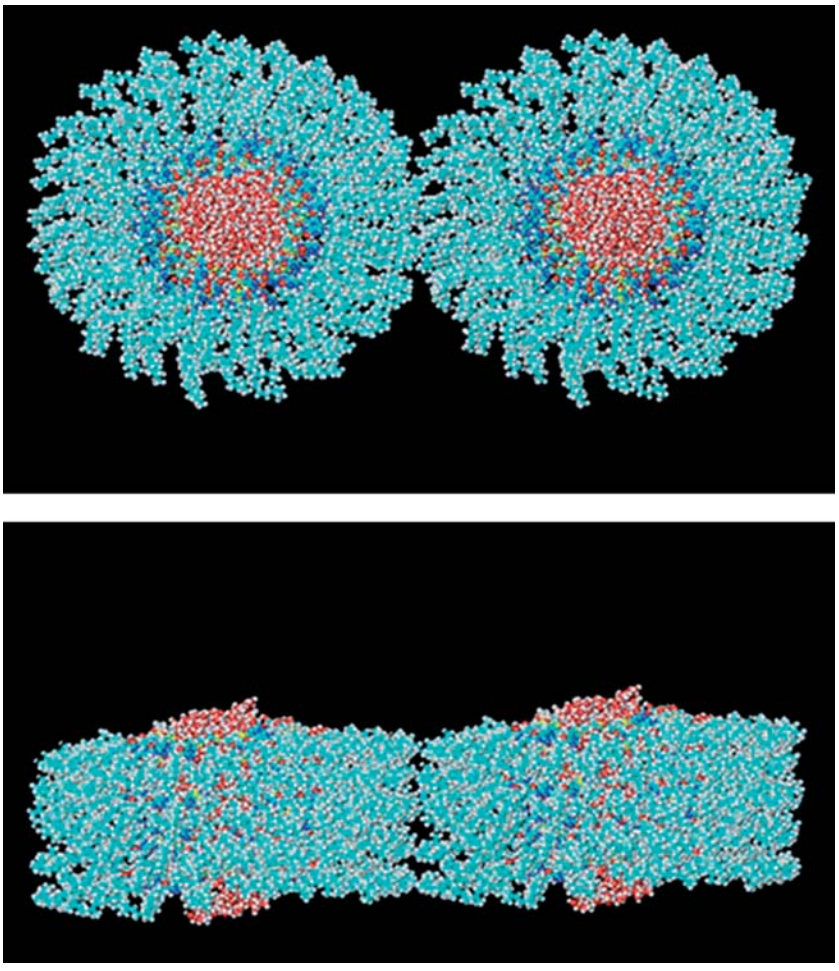

Figure 6. Face (upper part) and top (lower part) views of a construction of two sections of column, each made of six layers of a disk-like assembly composed of 20 conjugates. The whole system encompasses 31,178 atoms with 240 conjugates and 2556 water molecules. On the face view, the aqueous core is clearly visible in the center (oxygen/red), and the extent of the polar heterocyclic part can be appreciated by the presence of a circle of pyrimidinone nitrogen atoms (blue) and fluorine atoms (yellow). The top view shows the central inclusion of the water spindle surrounded by lipophilic squalene side chains. Reproduced with permission from John Wiley and Sons [79].

of hENT1 in tumor tissue is generally associated with reduced survival [86]. At the same time, the success of anticancer therapy with gemcitabine alone or in combination with cisplatin in patients affected by bladder cancer depends on magnitude expression of hENT1 in the cytoplasma of tumor cells. The median overall survival of these patients, who received gemcitabine in combination therapy with cisplatin, is increased for high expression of hENT1, thus suggesting as this nucleotide transporter receptor is significantly associated with antitumor response of gemcitabine in combined chemotherapy [86].

For this reason, the anticancer efficacy of Sq-gemcitabine on a human cancer cell line (CEM/ARAC8C) (a nucleoside transporter-deficient variant of the CCRF-CEM leukemia cell line) was evaluated and an antitumoral effect of approximately threefold higher than that of the free compound was observed [80].

The cellular uptake mechanism of squalenoyl nanoaggregates was recently investigated and a prevalent localization of the free ^{3}H-gemcitabine in the cytoplasm was observed while ^{3}H-Sq-gemcitabine nanoparticles passively accumulated in the cellular membranes as a consequence of their lipophilic features. Microautoradiography, differential contrast and confocal laser microscopy showed that the endoplasmic reticulum is the main intracellular reservoir of Sq-gemcitabine [87]. Therefore, cell membranes act as accumulating compartments in which Sq-gemcitabine is metabolized into gemcitabine, which is, successively, phosphorylated, distributed in the cell nucleus and/or excreted through the hENT nucleoside transporter (**Figure** 7). Biodistribution study in leukemia-bearing mice showed that ^{3}H-Sq-gemcitabine nanoparticles are extensively accumulated in liver and spleen, the major sites of metastasis in the case of leukemia, after *in vivo* injection

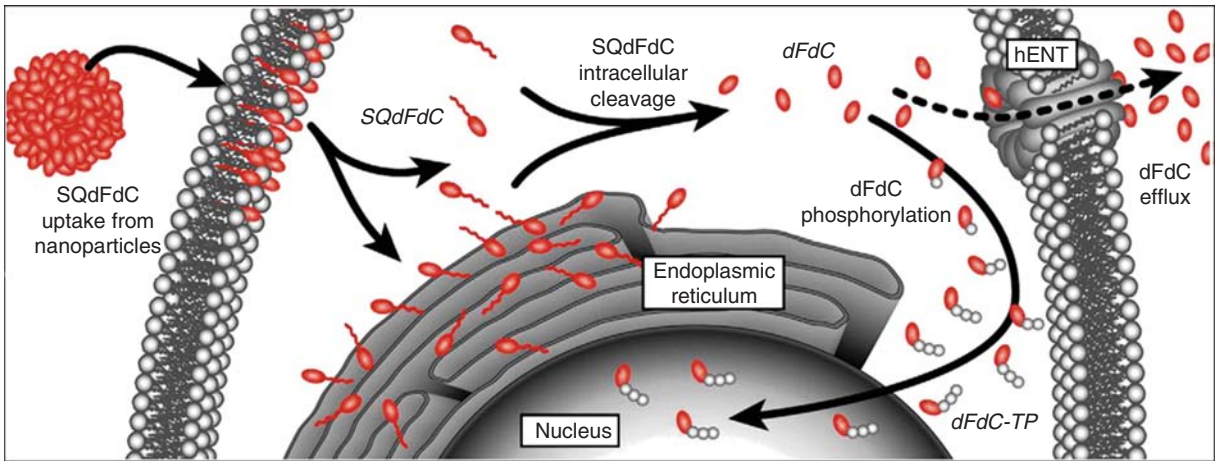

Figure 7. Intracellular metabolization pathway of Sq-GEM.
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if compared with ^{3}H-gemcitabine, thus warranting the effectiveness of Sq-gemcitabine in anti-leukemia therapy [80].

The *in vivo* potentialities of Sq-gemcitabine were evaluated using a mouse xenograft model, obtained after injection of cancer cells. The anticancer activity of Sq-gemcitabine at a drug dosage threefold lower than that of the free drug (5 vs 15 mg/kg, respectively) was tested on DBA/2 mice, previously i.v. inoculated with P388 leukemia cells and L1210K cells. In both cases, the colloidal nanoaggregates improved the mouse survival rate and reduced weight loss with respect to the free form of gemcitabine [78]. It was also interesting to note that the squalenoyl derivates allowed an increase in the percentage of apoptotic cells, as observed in an ascitic mouse model obtained by carrying out an i.p. cell injection and taking a sample of ascitic fluid a few days later.

A possible explanation of these results could be the better plasmatic stability of the nanosystems with respect to the free drug and the intracellular presence of particular proteases. In particular, 80% of the bioactive compound of Sq-gemcitabine remained unmodified while the free drug was completely metabolized in human plasma at 37°C after 24h incubation [78]. It was also demonstrated that the lysosomal enzymes cathepsin B and D (cysteine and aspartic proteases, respectively), which are overexpressed in cancer cells, are the principal factors responsible for the amidic-linkage degradation of the nanosystems inside the cells [78,88].

Recently, the pharmacokinetic and biodistribution profiles of Sq-gemcitabine as compared with the free drug provided further evidence on the improvement of the biopharmaceutical properties of the nucleoside after squalenoylation. The half-life of free gemcitabine passed from 1.5 to ~8 h in the case of the squalenoyl formulation, thus evidencing a great increase in the bioactive presence of the drug in the bloodstream [89]. Although the B and D enzymes of cathepsin are overexpressed in tumoral cells, other plasmatic amidases and deaminases could be involved in the cleavage of the amidic linkage of Sq-gemcitabine, thus allowing the metabolization of the anticancer compound. However, in the case of the deaminases, the affinity of squalene for low-density lipoproteins (LDLs) favored a great reduction of their shares in Sq-gemcitabine, thus allowing a prolonged concentration of gemcitabine in the blood [76]. It was interesting to note that ^{3}H-Sq-gemcitabine localized approximately fivefold and approximately twofold more than ^{3}H-gemcitabine in the spleen and liver, respectively, thus furnishing another advantage for the rational therapeutic use of the nanoaggregates considering that these organs are the major sites of metastasis in the case of leukemia [80,89]. Furthermore, it was very interesting to note that Sq-gemcitabine induced no histological modification of the liver and no alteration of the hematological parameters with respect to the free compound [80] and also reduced the drug's gastrointestinal toxicity [90].

Another great advantage of using Sq-gemcitabine had to do with its activity after oral administration; in fact, squalene is absorbed through the lymphatic vessels in a similar manner to that of cholesterol after its passage thorough the intestinal barrier [76]. So the oral administration of the nanoaggregates (15 mg/kg) in F344 Fischer rats, previously inoculated intravenously with RNK-16 large granular lymphocyte leukemia cells, heightened the antitumoral activity of this bioactive compound as compared with its free form, thus opening new strategies for the oral administration of the nucleoside analog [78].

Considering the aforesaid investigations and the ease of starting a scale-up procedure, which is a consequence of the freeze-drying process that preserves the pharmacological activity of Sq-gemcitabine, further preclinical studies have been initiated in order to exploit the real potentialities of this new nanomedicine [91,92].

5. Polymeric nanoparticles

Another strategy abundantly used to modify the biopharmaceutical properties of gemcitabine was the encapsulation of polymeric nanoparticles. They are colloidal suspensions in which the mean particle size is less than 1 µm and can be morphologically classified as spheres and capsules. In the first case, the particles are made up of a porous/dense matrix in which the drug can be adsorbed and/or entrapped, while capsule systems are formed by a core (aqueous or lipophilic) containing drugs surrounded by a polymeric shell [93,94]. Generally, nanoparticles are made up of biocompatible, biodegradable, nontoxic polymers, such as albumin, cyanoacrylates, dextran, chitosan, polylactic acid (PLA) and poly(lactic-co-glycolic acid). Different approaches were used to deliver gemcitabine by means of these types of colloids.

In 2007, Stella and coworkers encapsulated different gemcitabine lipophilic derivates both in capsule and in matrix nanodevices. A series of lipophilic derivatives of gemcitabine, in which an acyl chain was covalently coupled to the 4-amino group of the drug (i.e., valeroyl-, lauroyl-, stearoyl- species) were incorporated into poly[aminopoly (ethylene glycol)cyanoacrylate-co-hexadecyl cyanoacrylate] (poly(H_2NPEGCA-*co*-HDCA)) nanosystems [95]. The matrix systems were obtained by the nanoprecipitation method, solubilizing the copolymer and the gemcitabine derivates in an organic solvent that was added to water, while the nanocapsules were realized by the addition of Miglyol 812N to the organic phase in order to obtain an oily core. The results showed that the flexibility of the gemcitabine derivates increased with the length of the lateral chain linked in position 4 of the drug; moreover, quasi-elastic light scattering analysis showed that their encapsulation in nanospheres determined the formation of colloids having a mean diameter of 150 – 190 nm with a unimodal size distribution, while in the case of nanocapsules, this result was obtained only with the stearoyl derivate. This was in agreement with the drug-loading efficiency that increased with the lipophilicity of the gemcitabine derivates, thus evidencing that the stearoyl nucleoside was completely entrapped inside the nanodevices. As far as cytotoxicity goes, investigated on KB3-1 and MCF-7 cells in comparison with the free compound and the two nanoparticle formulations containing the nucleoside pro-drug, stearoyl gemcitabine furnished the lowest IC_{50} value (comparable with that obtained using the colloidal samples). In the same investigation, differential scanning calorimetry analysis confirmed the effective presence of stearoyl gemcitabine in the nanoparticles as a consequence of the decrease in the melting temperature and enthalpy [95].

In another investigation, gemcitabine was loaded in bovine serum albumin (BSA) nanoparticles in order to increase its efficacy against PC [96]. In particular, gemcitabine nanospheres were obtained by different desolvation-crosslinking methods (direct drug loading and indirect drug absorption), obtaining two particle populations of ~100 and ~400 nm, respectively, characterized by an encapsulation rate of 80 – 90% and a zeta potential of -15/-24 mV. It was interesting to note that a pH environment of 8.0 – 9.0 allowed the formation of particles with the best morphological and dimensional parameters because at pH ≥ 8, albumin demonstrates augmented viscosity and decreased dissolution. In these systems, gemcitabine release was characterized by an initial burst effect (after 30 min), which induced a drug leakage of about 20%, and was very similar to that observed in the case of PEGylated liposomes [53]. Both populations of BSA nanoparticles elicited a great reduction of BxPC-3 PC cell viability. Although the real benefit of using this type of colloid in *in vivo* xenograft models is under investigation, it should be considered that a nanosystem with a mean diameter greater than 200 nm is rapidly phagocytosed by the macrophage phagocytic system after systemic administration [54].

Recently, gemcitabine was also encapsulated in nanoparticles made up of poly(ethylene glycol)-block-poly(D,L-lactide), realized by the double emulsion (w/o/w) technique, and tested on the SW1990 human PC cell line [97]. The nanoparticle morphology was not perfectly spherical and a mean diameter of about 200 nm was observed. In this case, the encapsulated gemcitabine showed a degree of cytotoxicity similar to that of the free compound.

Gemcitabine was also entrapped in polymeric nanodevices so that it could be taken up by the brain. The presence of the blood brain barrier (BBB) precludes or reduces the access of many bioactives and colloids to the brain [98,99]. In particular, polybutylcyanoacrylate (PBCA) nanoparticles were prepared by the emulsion polymerization method and coated with 1% (w/v) Tween 80 in order to bypass the BBB and to make possible the treatment of glioma with the nucleoside analog [100]. The use of Tween 80, in fact, was necessary in order to favor the penetration of the nanosystems through the BBB, as a result of the endocytic process promoted by the plasmatic apolipoproteins (adsorbed on the colloidal surfaces) and the LDL receptors [101]. The cytotoxic activity of gemcitabine was investigated in C6 glioma cells and compared with that occurring in PBCA nanoparticles and Tween 80-coated PBCA nanoparticles containing the drug. *In vitro* tests evidenced that the colloidal formulations increased the antitumoral efficacy of gemcitabine and that the empty nanoparticle formulations did not cause variations in cell morphology. On the other hand, the Tween 80 mixture and empty nanoparticles induced a certain amount of cell suffering, probably as a consequence of the action of the free surfactant agent.

The comparison of the antitumoral effect of gemcitabine-loaded Tween 80-coated nanoparticles with respect to the free drug in an *in vivo* glioma model (obtained by the stereotaxic inoculation of C6 cells into the right caudate nucleus of Sprague Dawley rat brains) evidenced a greater anticancer

efficacy of the nanoparticle formulation, as demonstrated by the increased survival rate and the improvement of the physiological conditions of the rats [100].

A new gemcitabine-nanoparticle formulation was obtained through chitosan coacervation. The resulting nanoparticles were characterized by a well-stabilized spherical shape and a mean diameter of ~140 nm, an ideal polydispersity index (< 0.1) and a positive zeta potential, which was influenced by the pH of the environment. As concerns the drug-loading capacity, the incidence of gemcitabine entrapment was very low if the drug was added after the coacervation procedure (adsorption), probably because the amino groups of the nucleoside contrasted those of the polymer. But if the gemcitabine was added during the formation of the nanoparticles, the loading capacity increased due to the mechanical entrapment of the drug. The nanoparticles prepared using the best method are characterized by a pH of 7.4 and a biphasic release of gemcitabine with an initial burst effect (40% of drug leakage in the first 2 h) followed by a prolonged drug release for up to 96 h, as a consequence of the drug desorption from the colloidal surface. The release profiles varied only slightly at lower pHs. The gemcitabine-loaded chitosan nanoparticles were tested on DBA/2 mice, subcutaneously inoculated with L1210 wild-type cells in Matrigel and compared with the free compound (both of them at a drug dosage of 20 mg/kg) and with the empty nanosystems. The nanoparticles favored a significant decrease in tumoral mass growth in comparison with the free drug while the empty carriers showed no antitumoral action. The authors attributed these results to the protection of the gemcitabine-loaded chitosan nanoparticles against their plasmatic metabolization and to the ability of the nanocarriers to induce the drug's accumulation in the tumor through bypass of the hENT1 active transporter [102]. Recently, chitosan nanoparticles, obtained by gelation method, were used to increase the selective targeting of gemcitabine on PC cells after the conjugation of Herceptin (antihuman EGFR-2 or anti-HER2) on their surface [103]. The antibody allowed to augment the colloidal localization inside Mia Paca 2 and PANC 1 cells, thus favoring a better pharmacological action of the drug. Moreover, it was very interesting to note that HER2 nonexpressing cell line HEK293 did not show any increased cytotoxicity by using gemcitabine-loaded herceptin nanoparticles with respect to the formulation without the antibody, thus demonstrating the efficacy of anti-HER2 for the pancreas selective targeting.

The same aim prompted our research team to design and prepare PEGylated polymeric nanocapsules made up of PLA, which was used to entrap a great amount of gemcitabine. These nanocapsules were tested *in vivo* on HEK293 xenograft tumors and were found to provide an antitumoral effect similar to that of the free compound, but at a drug dosage 10-fold lower (data under publication). Further experiments are in progress in order to appreciate the real advantages of this formulation.

6. Conclusion

The entrapment of gemcitabine in colloidal devices seems to be a natural way of improving the biopharmaceutical properties of the nucleoside analog (**Table 2**). Equally promising, the innovative concept of squalenoylation opens up new perspectives for the design of original gemcitabine formulations. Both strategies allow the increase in the antitumoral action of the drug and its plasmatic half-life, thus retracing the steps that allowed the realization of a commercial product available for the treatment of different cancer pathologies: liposomal doxorubicin (Doxil, Caelyx).

7. Expert opinion

The introduction of nucleoside analog in the anticancer therapy represents an important innovation in the therapeutic treatment of tumors. Since its introduction in clinical practice, gemcitabine has demonstrated significant activity against different kind of solid tumors and its pharmacodynamic features made this anticancer drug a useful and important tool for anticancer chemotherapy with respect to the other nucleoside anticancer drug. The narrow pharmacokinetic parameters and the loss of therapeutic activity after systemic administration, due to the rapid metabolization of gemcitabine into an inactive metabolite, represent a drawback of this drug, thus hampering its effective and fully successful employment in clinical practice. The limitation of gemcitabine kinetic blood stream distribution and its rapid metabolism has been overcome in the clinical practice though a therapeutic schedule with repeated administration regimens in such a way as to maintain suitable drug level in the plasma, in order to achieve an antitumor response. As a consequence of this therapeutic regimen, serious drawbacks can occur and hence prejudice the success of the therapy.

As undeniably demonstrated, the encapsulation of gemcitabine in suitable colloidal carriers can be a smart solution for the pharmacokinetic limitations of the native drug. In fact, nanocarriers, that is, liposomes and nanoparticles, can not only improve the biological fates of anticancer drug, thus not limiting their advantages to the pharmacokinetic issues of the drug, but also extend the possibility of improving biodistribution profile and accumulation of gemcitabine at the level of the tumor mass, as is the case of PEGylated systems. In particular, PEGylation, formulate features, that is, biomaterial compositions and targeting properties, and preparation procedures, that is, entrapment methods, can modulate the effective success of gemcitabine chemotherapeutic approach and they have been taken into consideration in the attempt to describe an overview of different peculiarities of nanocarriers and to discover objective evidence as to which of these is the most promising.

Biocompatible and self-assembled properties of squalenic acid derivates in physiological conditions suggested that the squalenoylation of gemcitabine and other analog nucleoside anticancer drugs could represent an innovative approach suitable for potential clinical application. Nevertheless, this

Table 2. Most important delivery strategies of gemcitabine.

Carrier	Most relevant obtained results	Ref.
Liposomes	PEGylated unilamellar liposomes increase the apoptotic effects of gemcitabine on thyroid carcinoma cells	[27]
	PEG-coated unilamellar liposomes reduce the EC_{50} dose of gemcitabine in multiple myeloma cells	[28]
	Pretreatment with ammonium sulfate allows high gemcitabine entrapment in vesicular devices	[29]
	PEGylated unilamellar liposomes increased the *in vivo* antitumoral efficacy of gemcitabine in pancreatic xenograft models	[30]
	PEG-coated liposomes improved the biopharmaceutical properties of gemcitabine with respect to GEMZAR®	[31]
	The co-encapsulation of gemcitabine and paclitaxel in a liposomal device favored the synergic action of the two drugs	[44]
	The release profile of gemcitabine-loaded liposomes is biphasic with an initial burst effect	[53]
	Ideation, liposomal encapsulation and pharmacokinetic investigations of lipophilic gemcitabine pro-drugs	[58,62]
	Liposomal gemcitabine localized in tumor sites by EPR effect	[61]
	pH-sensitive EGFR-targeted immunoliposomes increased the drug accumulation and its efficacy in lung cancer tissue	[69]
Supramolecular vesicular aggregates (SVAs)	Technological characterization of gemcitabine-loaded novel SVAs based on PAHy copolymers.	[66]
	Gemcitabine selectively targeted to breast cancer cells after encapsulation in folate SVAs	[72]
Squalenoyl nanoaggregates	The conjugation of gemcitabine to squalenic acid allowed a higher half-life, an improved anticancer efficacy and reduced side effects of the drug	[60,76-80,87,89-92]
Polymeric nanoparticles	Encapsulation of lipophilic gemcitabine derivates in nanomatrix and nanocapsules of cyanoacrylate favored a better anticancer effect on different cell lines	[95]
	Gemcitabine entrapped in albumin nanoparticles elicit a great antitumoral effect against pancreatic cancer cell lines	[96]
	Entrapment of gemcitabine in PEG–PLA nanoparticles caused the obtainment of colloids with mean diameter of 200 nm	[97]
	Tween 80 coated-polybutylcyanoacrylate nanoparticles containing gemcitabine were used to efficaciously treat *in vivo* glioma	[100]
	Gemcitabine-loaded chitosan nanoparticles were efficient against leukemic diseases	[102]
	Herceptin conjugated-chitosan nanoparticles increased pharmacological action of gemcitabine on pancreatic tumor cells	[103]

EGFR: Epidermal growth factor receptor; EPR: enhanced permeability and retention; PEG: Poly(ethylene glycol); PLA: Polylactic acid; PAHy: α,β-polyaspartyl [(hydrazide)-co(butyrylhydrazide)]copolymer.

scenario seems to be an upcoming approach for the cancer therapy; it may not really be so near due to the fact that a completely new compound was synthesized and hence the long road of regulatory affairs has to be undertaken before approval can be granted for its use in clinical practice. In the field of supramolecular system for gemcitabine anticancer therapeutic delivery, other very interesting carriers are SVAs, which are very promising but suffer the same problem of squalenoyl derivates.

The therapeutic use of gemcitabine-loaded SVAs represents an extremely interesting field because of the huge number of possibilities that exist to modulate the carrier's features as a function of the therapeutic requirements and the goals to be reached.

Therefore, thinking in terms of a near-future prospective for potential clinical application among the various colloidal carriers analyzed in this review for the delivery of gemcitabine, it is the opinion of the authors that the less innovative but most clinically tested PEGylated liposomes (a PEGylated liposomal formulation of doxorubicin – Doxil – is already in clinical use) have a greater possibility of being employed as a nanomedicine in the effective therapeutic treatment of various cancer diseases. In fact, the different components of liposomal devices, suitable for clinical practice, that is, phospholipids, cholesterol and polyethylene glycol copolymers, are largely approved by international commits for safety drugs and administration, that is, EMEA and FDA, and are extensively used in other drug formulations already presented in pharmaceutical marketing or enrolled in the Phase II, III and IV clinical trials.

The success in *in vivo* tumor model of gemcitabine-loaded nanocarriers, and particularly that of liposomal formulations, seemed to suggest an upcoming substitution of GEMZAR® with gemcitabine liposomal therapy in the

anticancer therapeutic schedule and to open future perspective for nanocarriers in the cancer therapy. Moreover, combination anticancer drug in liposomal devices could also represent the new frontier of anticancer therapy in nanomedicine.

Declaration of interest

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