Gemcitabine versus Modified Gemcitabine: A Review of Several Promising Chemical Modifications.

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Abstract

Gemcitabine, an anticancer agent which acts against a wide range of solid tumors, is known to be rapidly deaminated in blood to the inactive metabolite 2',2'-difluorodeoxyuridine and to be rapidly excreted by the urine. Moreover, many cancers develop resistance against this drug, such as loss of transporters and kinases responsible for the first phosphorylation step. To increase its therapeutic levels, gemcitabine is administered at high dose (1000mg/m²) causing sides effects (neutropenia, nausea…).To improve its metabolic stability, its cytotoxic activity and to limit the phenomena of resistance many alternatives have emerged,such as the synthesis of prodrugs. Modifying an anticancer agent is not new, paclitaxel or ara-C have been subjected to such changes. This review summarizes the various chemical modifications that can be found in 4-(*N*)- and 5 ' position of gemcitabine. They can provide (i) a protection against deamination, (ii) a better storage and (iii) a prolonged release in the cell, (iv) a possible use in case of deoxycytidine kinase deficiency and (v) transporters deficiency. These new gemcitabine based sysems have the potential to improve the clinical outcome of a chemotherapy strategy.

KEYWORDS : *gemcitabine, prodrug, chemical modification, resistance, antitumor effect*

Content

1. INTRODUCTION

Cancer is the leading cause of death in developed countries. A major reason for this high mortality is the failure of current treatment that is in part attributed to the phenomenon of resistance but is also due to ineffective treatment against metastases. Current treatment of cancer using chemotherapy is largely based on the use of nucleoside analogues. These molecules are designed to mimic natural pyrimidine and purine nucleosides. Gemcitabine is one of these nucleoside analogues.

Gemcitabine acts against a wide range of solid tumours such as pancreatic, non-small lung, breast and ovarian cancers.[1–4] One of the major difficulties in cancer therapy is that tumours acquire resistance over time. This resistance is related to the functioning of gemcitabine. Gemcitabine is transported into cells by different transporters, such as hENT1, and a decreased expression of hENT1 is responsible for a lower level of activity of gemcitabine by blocking its uptake.[5] Once in the cell, gemcitabine undergoes a series of phosphorylations in order to be active; the first of these is carried out by deoxycytidine deaminase (dCK). A low level of dCK is correlated with low gemcitabine cytotoxicity.[6] Furthermore, gemcitabine is rapidly deaminated by cytidine deaminase (CDA) causing a short plasma half-life.[7] Therefore, strategies that provide both enhanced transport and high metabolic bioevasion by chemical modification could potentially lead to new therapeutic strategies.

In this review we look at the pharmacological parameters of gemcitabine, its mode of action (which remains the same even with modified forms), and we describe the state of the art of various chemical modifications of gemcitabine, exclusively on 2 sites of the molecule (4-(N) and 5’ sites, Figure 1), which have been carried out to improve

its efficiency. A prodrug is a biologically-inactive derivative of a parent drug molecule that usually requires an enzymatic or chemical transformation within the body in order to release the active drug, and has improved delivery properties in comparison to the parent molecule.[8] Drug modification has already been used with other anticancer drugs to overcome some disadvantages of the parent drug. For example, paclitaxel has been covalently attached to an acyl chain to obtain a lipophilic prodrug of paclitaxel to increase its encapsulation in lipid emulsions.[9] Another example is Ara-C, modified to facilitate Ara-C uptake and prolong its retention in the cell by grafting a fatty-acid chain at the 5' position of the nucleoside.[10]

Insert Figure 1.

In the 4-(*N*)- position of gemcitabine, we will describe modifications with PEG, with valproic acid, with 1,1',2-tris-nor-squalenoic acid (squalene), and with valeroyl, heptanoyl, lauroyl and stearoyl linear acyl derivatives. In the 5' position, grafting with elaidic acid, with cardiolipin, and a series of phosphoramidates will be reported. All these new gemcitabine-based molecules have the potential to improve the clinical outcome of traditional therapy.

Insert Figure 2.

1.1 Current state of affairs

Also called 2',2'-difluorodeoxycytidine (dFdC) (Figure 1), gemcitabine is a cell cycle-dependent (*S*-phase-specific) analogue of deoxycytidine (Figure 2.A). Even if it presents some similarities with other nucleoside analogues (cytosine arabinoside;

AraC) (Figure 2.B), it differs in many properties and in its own spectrum of activity. It was originally investigated for its antiviral effects but has since been developed as an active agent for cancer therapy.[11]

This molecule (Gemzar®: commercialised by Eli Lilly and Co.) was approved in 1996 by the Food and Drug Administration as first-line treatment for patients with locally-advanced (non-resectable Stage II or Stage III) or metastatic (Stage IV) pancreatic cancer.[1,12] Clinical trials comparing the use of Gemzar® and 5-FU for patients with locally-advanced or metastatic pancreatic cancer who had received no prior chemotherapy showed that patients treated with Gemzar® had significant increases in positive clinical response, survival, and disease progression time compared to 5-FU (Figure 3). In this clinical study, Gemzar® was administered intravenously with a 100mg/m²-dose for 30 minutes for 7 of the first 8 weeks as a first cycle. From the second cycle, gemcitabine was given weekly for 3 weeks in four-week cycles at the same dose. 5-FU was administered intravenously at a 600mg/m²-dose for 30 minutes.[13]

Insert.Figure 3.

The FDA approved the use of Gemzar® in 1996 in combination with cisplatin for the first-line treatment of patients with inoperable, locally-advanced (Stage IIIA or IIIB), or metastatic (Stage IV) non-small cell lung cancer.[2] Two schedules were investigated and the optimum schedule was sought. With the 4-week schedule, Gemzar® was administered intravenously at a 1,000mg/m^2-dose for 30 minutes on Days 1, 8, and 15 of each 28-day cycle. Cisplatin should be administered intravenously at a 100mg/m^2-dose on Day 1 after the infusion of Gemzar®. With the 3-week schedule,

Gemzar® should be administered intravenously at 1,250mg/m2 for 30 minutes on Days 1 and 8 of each 21-day cycle. Cisplatin should be administered intravenously at a 100mg/m^2-dose after the infusion of Gemzar on Day 1.

In 2004, the FDA approved the use of gemcitabine in combination with paclitaxel for the first-line treatment of patients with metastatic breast cancer,[3] and in 2006 in combination with carboplatin for the treatment of patients with advanced ovarian cancer.[4] For breast cancer, a 1,250mg/m^2-dose of Gemzar® (intravenous infusion for 30 minutes) was administered on Days 1 and 8 of a 21-day cycle with paclitaxel (175mg/m^2-dose) (intravenous infusion for 3 hours) administered prior to gemcitabine hydrochloride on Day 1 of each cycle. For ovarian cancer, Gemzar® was administered at a 1,000mg/m^2-dose on Days 1 and 8 of a 21-day cycle with carboplatin (AUC 4) administered on Day 1 of each cycle.

Until 2008, Eli Lilly had the exclusive rights of sale of gemcitabine in the US and Europe, and sales of Gemzar® increased constantly with a turnover of 1,720$ billion. Since 2008, sales have steadily declined with the introduction of generic alternatives. Indeed Gemzar® lost effective exclusivity in the U.S in November 2010 and in major European countries (France, Germany, Italy, Spain and the United Kingdom) in March 2009. In 2009 and 2010, sales outside the U.S. decreased by 37% and 31%, respectively; and in 2010 a decrease of 3% in the U.S was observed, driven by reduced demand and lower prices as a result of the entry of generic competition.[14]

1.2 Biodistribution

Gemcitabine has a very short plasma circulation time. The elimination half-life depends upon the infusion time, and the age and the gender of the patient (Table 1). For short infusions, the half-life is from 42 to 94 minutes. For infusions of seventy minutes, the half-life is 4 to 10 hours, and is associated with increased toxicity. At higher doses, major toxicity can be observed, such as: neutropaenia, reversible hepatic transaminase increases, proteinuria, nausea and vomiting, mild flulike syndrome, and mild skin rash.[15] Gemcitabine is rapidly cleared from the body upon its enzymatic conversion in the blood, liver, kidney and various tumour tissues.[16]

Insert Table 1.

1.3 Mechanisms of actions

Gemcitabine is transported across the plasma membrane by sodium-dependent (concentrative nucleoside transporter hCNTs) and by sodium-independent (equilibrative nucleoside transporter hENTs) mechanisms.[5,17] Gemcitabine is transported into cells by five nucleoside transporters, two equilibrative nucleoside transporters hENT1 and hENT2 and three concentrative nucleoside transporters hCNT1, hCNT2, and hCNT3. hENTs mediate bidirectional transport of nucleosides across biological membranes down a concentration gradient and are found in most tissues in the body. The hCNT family members are cation-dependent symporters that mediate unidirectional transport of nucleosides into cells.[18] Kinetic studies have shown that gemcitabine intracellular uptake is preferentially directed by hENT1 and, to a lesser extent, by hCNT1 and hCNT3.[19,20] Several studies have shown the importance of the presence of the hENT1 transporter for an optimal response to

gemcitabine.[21] One study has shown that in patients with pancreatic cancer, those with the highest level of hENT1 mRNA expression had a significant increase in survival time compared with patients expressing low hENT1 levels.[22]

In cells, gemcitabine undergoes a series of phosphorylations, essential to make it active (Figure 4). Firstly it is phosphorylated to a monophosphate compound (dFdCMP) by deoxycytidine kinase (dCK). It then undergoes a second modification to become gemcitabine diphosphate (dFdCDP), and finally to gemcitabine triphosphate (dFdCTP), all catalysed by nucleoside monophosphate kinase (UMP / CMP) and diphosphate kinase, respectively. Gemcitabine inactivation is catalysed by cytidine deaminase (CDA) as well as the deamination of gemcitabine monophosphate, which is catalysed by deoxycytidylate deaminase (DCTD). Phosphorylated metabolites of gemcitabine are reduced by cellular 5'-nucleotidase (5'-NT), and dFdCMP is also converted, and inactivated, by DCTD into 2'-deoxy-2',2'-difluorouridine monophosphate (dFdUMP).

Insert Figure 4

The triphosphate form of gemcitabine acts as a competitive substrate of deoxycytidine triphosphate. Its analogy allows it to be incorporated into DNA during replication, thus inhibiting chain elongation of DNA and causing cell death by apoptosis. Once gemcitabine triphosphate is incorporated at the end of the elongated DNA strand, one deoxynucleotide is added, and thereafter the DNA polymerases are unable to proceed. This action, called "masked chain termination", appears to lock the drug into DNA because proof-reading exonucleases are unable to remove

gemcitabine nucleotide from this penultimate position.[23,24] The inhibitory action of gemcitabine is enhanced by its non detection in the DNA chain.

Gemcitabine exhibits a unique property called self-potention which enhances its activation. The diphosphate form (dFdCDP) inhibits ribonucleoside diphosphate reductase (RNR), an enzyme of DNA synthesis, which permits the formation of nucleoside triphosphates. This results in a significant decrease in the concentration of cellular deoxycytidine triphosphate (dCTP) and a change in the ratio of dCTP / dFdCTP in favour of dFdCTP. The accumulation of gemcitabine triphosphate and the intra-cellular reduction of dCTP results in the inhibition of dFdCMP inactivation by DCTD, which requires sufficient concentrations of dCTP to be active.[25]

Thymidylate synthase (TS), which plays a key role in the synthesis of thymidine monophosphate, has been studied to investigate the possible inhibition of TS by gemcitabine exposure. The natural substrate of TS, 2′-deoxyuridine monophosphate (dUMP) is converted into 2'-deoxythymidine-monophosphate (dTMP). Thereby analogues of dUMP are potential TS inhibitors, and the deaminated product of gemcitabine dFdUMP resembles dUMP.[26] Studies with the human ovarian cancer cell line A2780 and the murine colon carcinoma cell line C26-10, have show inhibition of 90% of TS activity after 24h exposure to gemcitabine.[27] The expression of TS provides an alternative source of substrate for DNA synthesis and positively correlates with gemcitabine resistance and shortened patient survival time. Another study has proved that removing TS protein expression by siRNA induces a high degree of growth inhibition by gemcitabine, indicating the critical relation of TS to the enhancement of the therapeutic effect of gemcitabine.[28] A final, less well known and

less studied mode of action of gemcitabine is its incorporation into RNA which seems to be concentration-dependent.[29]

1.4 Resistance

Many forms of cancer show initial sensitivity to gemcitabine therapy followed by the rapid development of resistance, a feature that essentially characterises this disease. Thus, a better understanding of the origins of gemcitabine resistance is critical to the development of improved combination therapies to replace gemcitabine or to improve gemcitabine targeting. Resistance to antimetabolic drugs such as gemcitabine can be achieved by various genomic alterations.[29]

A major cause of resistance can be attributed to alterations in the transporter. The development of resistance to gemcitabine correlates strongly with a deficiency of hENT1 expression in human breast and pancreatic cancer cells.[5,30] Many studies have shown that the hENT1 but also hCNT3 expression determination can be used as a prognostic marker to provide prospective evaluations for patients receiving gemcitabine-based adjuvant therapy.[6,31,32]

In L1210 murine leukaemia cells made resistant to ara-C and cross-resistant to gemcitabine, altered action of dCK can be observed, due to genomic recombination.[27] These results suggest that a partial deletion of the dCK gene observed after selection in the presence of gemcitabine is involved with resistance to this agent both *in vitro* and *in vivo.* The expression of dCK has been postulated to be correlative to gemcitabine resistance.[33]

Another factor in gemcitabine resistance is the over-expression of ribonucleotide reductase (RR).[34] RR is a dimeric enzyme composed of regulatory subunit M1 (RRM1) and catalytic subunit M2 (RRM2). Ribonucleotide reductase is mainly responsible for the conversion of ribonucleosides to deoxyribonucleoside triphosphates (dNTPs), which are essential for DNA polymerisation and repair. RRM1 over-expression through transfection of a lung cancer cell line likewise resulted in gemcitabine resistance. Reduction of RRM1 expression through RNA interference abrogated the induced gemcitabine resistance.[35] Ribonucleotide reductase enzymatic activity is modulated by levels of its M2 subunit. An over-expression of RRM2 is associated with resistance to gemcitabine and down regulation of RRM2 by siRNA enhanced gemcitabine cytotoxicity, both *in vitro* and *in vivo* in pancreatic adenocarcinoma.[7] Gemcitabine monophosphate and triphosphate are reduced respectively by dCMP deaminase and by 5′-nucleotidase (5′-NT), and gemcitabine itself is inactivated by cytidine deaminase (CDA). High levels of these catabolic enzymes are associated with resistance to the drug.[36]

Apoptosis forms the principal cause of cell death in response to cytotoxic drug treatment. A variety of anticancer drugs have been shown to produce extensive apoptosis in sensitive malignant cells, but it has been suggested that the inability of some cells to undergo apoptosis is similar to the mechanism of gemcitabine resistance. The expression of p53, which plays an important role in apoptosis pathways, induces cell cycle arrest and, in the higher concentration ranges, p53 induces apoptosis. On the other hand, human lung cancer expressing the mutation of the p53 gene does not undergo apoptosis after gemcitabine treatment.[37] The

aberrant expression of genes associated with cellular survival and apoptosis are implicated in gemcitabine resistance.

New resistance mechanisms to gemcitabine implicate the stress-response protein Hu antigen R (HuR) which is an RNA-binding protein that post-transcriptionally regulates gene expression. A recent study has shown a relation between HuR and gemcitabine resistance. After treatment of a MiaPaCa-2 cell line with gemcitabine, increased HuR cytoplasmic levels and a reaction to the drug were observed. Since the dCK 3′UTR region contains 8 putative hits of an HuR recognition motif, HuR associated with dCK mRNA was tested and a bond of HuR with the dCK 3′UTR was obtained. A correlation between the level of HuR and the dCK protein levels, but not the dCKA mRNA, was observed. Regulation of dCK protein concentration by HuR and the prediction of gemcitabine response by cytoplasmic HuR levels was suspected.[38] Nevertheless a recent study has shown that HuR binds to VEGF mRNA, implying regulation of VEGF expression in pancreatic ductual andenocarcinoma after gemcitabine exposure.[39] Taken together, these data suggest that the genes encoding proteins involved in the transport and metabolism of gemcitabine and in the metabolism of targets can be potential candidates to predict sensitivity to gemcitabine. Quantitative analyses of these genes can be a potent tool to perform individualised chemotherapy.

Gemcitabine is a polar drug with low membrane permeability and which is extensively degraded by cytidine deaminase into an inactive metabolite in the liver. Moreover, the increasing amount of resistance also reduces its cytotoxicity. Thus, a frequent administration schedule at high drug doses is required, and this leads to serious side

effects such as: myelosuppression, high levels of hepatoxicity, renal toxicity, thrombocytopaenia and anaemia.[40] To date, various innovative approaches have been developed to overcome these disadvantages. We will focus on chemical modifications at the 4-(*N*)- and 5 ' position of gemcitabine.

2. 4 - (*N*) – MODIFICATION OF GEMCITABINE

2.1 PEG-gemcitabine

For 40 years, several antitumour agents, either proteins, peptides or low molecular-weight drugs, have been considered for PEG conjugation. PEGylation can give a number of relevant advantages such as considerable *in vivo* half-life prolongation, a reduction or removal of immunogenicity, and a reduction of aggregation.[41]

The technique has become the leading approach for overcoming most of the aforementioned biological limits, and the number of PEGylated products on the market and in clinical trials is increasing constantly.[42,43] The PEGylation process presents many advantages: (i) PEG is a polymer with high solubility in water and excellent biocompatibility (it is FDA-approved for human administration by mouth, injection or dermal application [44]), (ii) plasma half-life of pegylated product is increased, (iii) it provides enzymes protection, (iv) it accumulates in tumour zones according to the “enhanced permeability and retention” effect (EPR).[45,46] However a number of drugs encapsulated or solubilizing with pegylated agents can activate the complement system, the nonspecific, humoral arm of antimicrobial immune defense. Complement activation has been recently proposed as a major underlying or contributing cause of infusion reactions, referred to as complement-activation related pseudoallergy (CARPA). CARPA may be a major underlying cause, or contributing

factor to the hypersensitivity reactions (HSRs) caused by many successful drugs, such as Taxol® and Doxil®.[47]

Clinical trials of several derivatives of PEG coupled to anticancer drugs are already under way or have been completed.[43] Pegamotecan (Enzon Pharmaceuticals, Inc.) is a prodrug obtained by coupling two molecules of camptothecin to a PEG of 40kDa. An amphiphilic polymer-docetaxel conjugate was prepared by attaching PEG to docetaxel through an ester linkage. The PEG-docetaxel was used to form nano-sized micelles for the solubilisation of free docetaxel. A 1.8-fold higher area under the curve (AUC) for docetaxel equivalent plasma concentration *vs.* time was obtained, in comparison with free docetaxel. The maximum tolerated dose of PEG-docetaxel was also 2.5-fold higher than that for free docetaxel in healthy mice.[48] Studies with paclitaxel were also conducted: PEG (5000 Da) was bound to the 2' position of paclitaxel through a spacer succinyl group and this prodrug increased the half-life of PEG-paclitaxel.[49] Other studies have shown the importance of conjugation with PEGs with a molecular weight ≥ 30kDa, in order to prevent rapid elimination by the kidneys.[43] Because of encouraging results from studies with other anticancer agents and because the anti-cancer effects of gemcitabine is limited by its short half-life, its rapid metabolisation and its low tumour uptake, the addition of PEG on gemcitabine has been tested by several research teams. In a recent paper, a synthesis of a PEG-gemcitabine, by conjugating the amino groups at 4-(*N*)-position of gemcitabine to N-hydroxysuccinimide derivative of PEG, has been carried out (Figure 2.C).[50]

Confocal analysis showed PEG-gemcitabine colocalisation in lysosome and endosome after 24h incubation and an enhanced retention in cancer cells after 3 days of incubation in comparison to native gemcitabine. It is known that the cellular

uptake of PEG-drugs occurs through endocytosis and they are retained in transport vesicles which traffic along the endo-lysosomal scaffold which are acidic in nature.[51,52] The endo-lysosomal transport vesicles allow cleavage of the amides bounds between PEG and gemcitabine, thanks the acidic nature of these vesicles, and thereby allow its prolonged release. Pharmacokinetic studies have shown consistently higher bioavailability (21 times) of PEG-gemcitabine over native gemcitabine, after 1h of intravenous administration in mice (Figure 5). In MiaPaCA 2 and PANC-1 PEG-gemcitabine was more effective in all cases in comparison with native gemcitabine.

To improve the effect of PEG-gemcitabine, folic acid has recently been conjugated to PEG-gemcitabine (Figure 2.E) to evaluate their active targeting and cytotoxic superiority compared the non-targeted PEG-gemcitabine.[53] The drug linkage involved the 4-(*N*)-amino group of gemcitabine and the COOH of PEG, while folic acid was linked through its carboxylic function to PEG amino group. Folic acid was chosen as the targeting agent because its receptor is over-expressed in several types of cancers (lung, breast, kidney and ovarian),[54–56] while in normal human tissues its receptors maybe have limited distribution. Effective targeting with folate has been proven by using folate on the surface of many nanocarriers such as gold nanoparticles, liposomes or magnetic nanoparticles for the detection of cancer cells and the release of anticancer drugs.[57–59]

All conjugates were able to release the drug in a pH-dependent manner with no role played by enzymes. The presence of a plasma enzyme does not accelerate the conversion of different compounds in dFdU which confirms previous studies showing

that the protection of the amino group of gemcitabine prevents this.[60] Polymer conjugation of gemcitabine increases drug plasma half-life, which is dependent on the polymer's molecular weight, by reducing its kidney clearance. An increase of $t_{1/2}$ α, $t_{1/2}$ β and the area under the curve was obtained. The results of cytotoxicity tests showed no improvement of PEG-gemcitabine (with or without folic acid), compared to native gemcitabine, on tumour cell lines which did not over-express the folate receptor. Folic acid derivatives are less toxic than PEG derivatives. This is explained by a slower entry into the cell by endocytosis and a cytotoxic activity only after the release of gemcitabine. Moreover, the polymer derivatives targeting folate receptors need a receptor-mediated endocytosis mechanism for cell penetration in cells that do not over-express the folate receptor. Folate-targeted poly(ethylene glycol) (PEG)-coated nanoparticles are found to bind to folate receptors triggering for caveolae-assisted endocytosis, followed by the formation of intracellular vesicles which can be visualised by confocal microscopy.[61] In contrast, in KB-3-1 cells which over-express folate receptors, derivatives with folate are certainly less cytotoxic than native gemcitabine but are more cytotoxic than gemcitabine only coupled to PEG.[53]

PEGylation is currently considered to be one of the most successful techniques to prolong the residence time of drugs in the bloodstream. In a few cases, polymer conjugation has also shown that it can confer targeting properties to the disease site, such as tumour masses, by passive diffusion (EPR effect).[62] Different studies have proven the interesting use of PEG-like drug carriers. They permit an increase of drug plasma half-life, prevent the degradation action of cytidine-deaminase enzyme, increase the availability after intravenous injection, and in certain cases increase the

cytotoxicity against cancer cells. The easy addition of an agent that specifically targets cancer cells in the case of folic acid is also a benefit of the use of PEGylation.

Insert Figure 5.

2.2 LY2334737

Gemcitabine has poor oral bioavailability due to an extensive first pass metabolism by cytidine deaminase. To circumvent this rapid deamination into 2',2'-difluorodeoxyuridine (dFdU), a gemcitabine prodrug has been developed, LY2334737 (Figure 2.D). LY2334737 is an oral gemcitabine prodrug in which gemcitabine is linked to valproic acid *via* an amide bond at 4-(N)-position, enabling it to bypass hydrolysis in enterocytes and portal circulation, thereby avoiding the extensive first pass metabolism that occurs with unmodified gemcitabine. Circulating levels of LY2334737 are detectable several hours after oral administration. In addition, a gradual release of gemcitabine following cleavage of the amide bond should enhance efficacy, since more cancer cells should be exposed to a continuous effective cytotoxic level of gemcitabine. The stability of the prodrug was tested on a pH range from 1 to 8 to check the possibility of delivering an intact prodrug into systemic circulation after passing through the gastrointestinal tract after oral administration. LY2334737 is pH-dependent with about 21% degradation at pH 1 and no degradation between pH 6 to 8, after 4h of incubation at 40°C.[63] *In vitro* hydrolysis profiles showed a slow hydrolysis in the liver (subcellular fractions S9 that contain drug-metabolising enzymes) and in crude homogenates of small intestinal epithelial cells with 27 and 11pmol/mg.min, respectively. *In vivo* antitumour activity with mice bearing HCT-116 human colon tumour xenografts, indicated the same efficacy of

LY2334737 after an oral administration at a 7.55mg/kg-dose for 14 days *vs* 4 doses of an intraperitoneal administration of gemcitabine at a 160mg/kg-dose. Phase I trials of LY2334737 either as monotherapy or in combination with other agents are currently under way to determine the maximum tolerated dose and dose limiting toxicities of daily administration.[64]

2.3 Squalenoylation

Squalene is a triterpene that is an intermediate in the cholesterol biosynthesis pathway. Squalene is a structurally-unique triterpene compound that is one of the main components (about 13%) of skin surface lipids. It was so called due to its first historical isolation from shark liver oil, where it is contained in large quantities, and is considered its richest source.[65] In humans, about 60 percent of dietary squalene is absorbed and is distributed ubiquitously in human tissues in small amount.[66] Recent *in vitro* and *in vivo* model experiments suggest a tumour-inhibiting role for squalene.[67]

Pr. Couvreur's team developed the concept of squalenoylation involving the chemical linkage of squalene with various nucleoside analogues which allowed the formation of novel colloidal nano-assemblies of 100-300nm with a narrow size distribution, after dispersion in an aqueous environment.[68] They were interested in the pharmacological activity of gemcitabine covalently coupled at 4-(N)-position with 1,1', 2-tris-nor-squalenoic acid to obtain Sq-gemcitabine (4-(N)-Tris-nor-squalenoyl-gemcitabine, SQdFdC) (Figure 2.F). A study by X-ray diffraction (SAXS) combined with molecular modelling identified the supramolecular organisation of these nano-assemblies, which form an inverse, hexagonal phase, in which the central aqueous core consisting of water and gemcitabine molecules was surrounded by the squalene

moieties.[69] They investigated the anticancer activity of Sq-gemcitabine *in vitro* on resistant murine and human leukaemia cells (L1210 10K and CEM/ARAC8C, respectively). The L1210 10K cells were characterised by a lower expression of cytoplasmic dCK and CEM/ARAC8C by a deficiency in hENT1 transporters,[70,71] these two ways representing two major resistance factors to gemcitabine. After 72h of incubation with different concentrations, Sq-gemcitabine demonstrated 3.26 and 3.22-fold higher cytotoxicity compared to gemcitabine with L1210 10K and CEM/ARAC8C, respectively.[72] After intravenous injection of Sq-gemcitabine in aggressive leukaemia-bearing mice, an increase in survival time compared to gemcitabine was obtained. This significant increase was attributed to the high degree of localisation of Sq-gemcitabine in the liver and spleen which are the major metastatic organs. Sq-gemcitabine was found to be more efficient than gemcitabine, suggesting the considerable potential of this treatment for leukaemia.

Studies on human pancreatic adenocarcinoma models were also performed. *In vitro*, Sq-gemcitabine showed higher antiproliferative and cytotoxic effects compared to native gemcitabine, on chemoresistant tumour cells (Panc-1) and sensitive cell lines (Capan1 and BxPc3), which were associated with significant DNA synthesis inhibition, S-phase arrest, and higher induction of apoptosis (caspase 3 activation). *In vivo* experiments were performed with subcutaneous tumour models (Panc1 and Capan1) and orthotopic tumours (Panc1). Sq-gemcitabine treatment exerted significant inhibition of tumour growth in all types of tumour models compared to gemcitabine, and avoided the formation of metastatis spreading in the peritoneum, and prolonged the survival time of mice with orthotopic pancreatic tumours (Figure 6).[73]

The impact of the encapsulation of Sq-gemcitabine in liposomes on a model of subcutaneous leukaemia (L1210wt) was also studied. PEGylated liposomeS were employed to modify the drug pharmacokinetics and biodistribution to enhance the anticancer activity (accumulation in tumours due to their ability to extravasate into these tissues by the EPR effect) and to decrease the capture by macrophages in organs of the reticuloendothelial system.[74] PEGylated liposomal formulations exhibited a hydrodynamic diameter of 133 ± 24nm with a polydispersity index of 0.035 and were stable for 2 weeks. A similar *in vivo* anticancer activity of Sq-gemcitabine in pegylated-liposomes compared to native gemcitabine was demonstrated, with a drug dosage 5-fold lower than free gemcitabine on a subcutaneous grafted L1210wt leukaemia model. They explained this by the protection of gemcitabine from deamination in the blood.

A novel innovative nanoparticle system is currently being developed using Sq-gemcitabine: nanocarriers are able to target the tumour due to the presence of magnetite nanocrystals formed in the self-assembly of the squalenoyl gemcitabine bioconjugate.[75] These particles were thought to be compatible with parenteral administration. After intravenous injection, in L1210 subcutaneous tumour model mice, the new assembly of magnetite-Sq-gemcitabine was guided to the tumour by a magnet placed at the tumour location. Considerable inhibition of tumour growth and an accumulation of the product in the tumour periphery was observed using T_2-weighted imaging in magnetic resonance imaging.

While the mechanism of entry and metabolisation of gemcitabine into cells is known, the cellular uptake mechanism of Sq-gemcitabine, its subcellular localisation, and its metabolisation pathway have only been studied recently. An *in vitro* passive entry in cancer cells (MCF-7: human breast adenocarcinoma) and a preferential accumulation in endoplasmic reticulum, thanks the high lipophilic level of squalene, were observed.[76] This passive input may explain the efficacy of Sq-gemctiabine on cell lines deficient in active transporters.[72]

In addition to storage, a gradual cleavage of Sq-gemcitabine in native and active form also explains his efficiency. Cathepsin B, a lysosomal enzyme often overexpressed in cancer cells,[72] was shown to be responsible for the amidic-linkage degradation of this nanosystem inside the cells. The role of cathepsin B and D in the cleavage of the Sq-gemcitabine has been shown, increasing half-life in blood from 1.5 h to 8h, for native gemcitabine and Sq-gemcitabine, respectively,[79] thus increasing its anticancer activity through its longer presence in the blood. Two important elements have contributed to the efficiency of this new drug: (i) the storage of gemcitabine in the endoplasmic reticulum have allowed it to be protected from deamination by the presence of squalene and (ii) the progressive cleavage in its native form allowed the metabolisation of the cancer compound.The concept of squalenoylation has also been applied to other nucleoside analogues.[69] Whatever the squalene binding position (heterocycle or sugar), these molecules also self-organise in water as nanoparticles of 100-200nm. A variety of novel squalene-based prodrugs of the anticancer compound paclitaxel have been synthesised and have produced nanoparticles in water.[80] Preliminary results show a notable cytotoxicity on a murine lung carcinoma cell line (M109).

Insert Figure 6.

2.4 Lipophilic prodrugs

An approach to improve the stability and cytotoxic activity of gemcitabine is to protect the amine group by forming a prodrug and by incorporating it into particles like the Sq-gemcitabine loading in liposomes. Many native gemcitabine-loaded particles have already been studied. Liposomes, PLGA-, polycyanoacrylate-, chitosan- or albulmin-nanoparticles, and carbon nanotubes can be cited.[81–85] In addition, the co-encapsulation of gemcitabine with other anticancer drugs inside a particle to obtain a synergistic effect has been achieved.[86] Nanoparticles have the advantages of: high stability, high carrier capacity, incorporation of hydrophobic and hydrophilic compounds, and being injectable through various routes of administration. Encapsulation improves the cytotoxic activity of the drug with protection against metabolic inactivation.[87] In addition, extended circulation in the blood by the addition of PEG on the surface of nanocarriers and an active targeting by grafting peptides or antibodies to the shell of nanocarriers result in sustained exposure to tumour cells and enhanced efficacy.[88,89]

In 1998, Eli Lilly patented the synthesis of lipophilic gemcitabine. To protect against the deamination of gemcitabine, it was proposed to covalently link the amino group in position 4 with saturated and mono-unsaturated, long-chain C18 and C20. The results showed better cytotoxicity of lipophilic derivatives in comparison to native gemcitabine. A few years later, the synthesis of a series of 4-(*N*)-acyl derivative prodrugs of gemcitabine was carried out, firstly to prevent diffusion through liposome bilayers, and later to be encapsulated into other particles.[90] Tokunaga Y *et al*.

improved metabolic stability with the synthesis of a series of prodrugs of gemcitabine, increasing lipophilicity, by linking the 4-(N)-position with valeroyl, heptanoyl, lauroyl and stearoyl derivatives (Figure 2.G) and encapsulated them into liposomes.[91] Liposomal formulations containing these lipophilic prodrugs of gemcitabine increased the drug entrapment efficacy with respect to conventional liposomes but their encapsulation efficiency (EE) closely depended on the length of the 4-(N)-acyl chain, the phospholipids and the presence of cholesterol. Better results were obtained by incorporating 4-(*N*)-lauroyl-gemcitabine (GemC12) and 4-(*N*)-stearoyl-gemcitabine (GemC18) in liposomes composed by DSPC/DSPG 9:1 (EE of 94.4±7.9% and 97.7±2.3%, respectively). Native gemcitabine is well known to rapidly convert to inactive metabolite by cytidine deaminase which is widely distributed in plasma but C12 and C18 derivatives are both stable in plasma. After 24h, more than 60% of unmodified prodrugs were still present, with GemC12 and GemC18 derivatives, so that after 8h of incubation in plasma, only 40% of unmodified drug was present with native gemcitabine. pH stability was obtained in the pH range 4-9, which confirms the stable amide linkage. *In vitro* studies have shown that cytotoxicity of free or encapsulated GemC12 and GemC18 derivatives were 2- and 7-fold (in KB and HT-29 cells line, respectively) greater than that of native gemcitabine. Encapsulation of the C18 derivatives into liposomes produced an increase of plasma availability: the AUC was 50 times higher than for native gemcitabine, resulting in the increased accumulation in tumour cells and a high level of antitumoural efficacy in mice grafted with HT-29 and KB 396p cells.[92]

The feasibility of adding GemC18 into other particles to overcome gemcitabine resistance in cancer cells has been evaluated recently in different studies.[93–95] GemC18 was incorporated into lipid nanoparticles (NPs) engineered from

lecithin/glycerol monostearate-in-water emulsions.[93] *In vitro* studies were performed with a deficient hENT1 cell line (CCRF-CEM-AraC-8C).[94] Native gemcitabine was not able to enter the cells efficiently, but the GemC18-NPs were able to efficiently deliver the stearoyl gemcitabine into cells by endocytosis and caused apoptosis *via* caspase-3 activation. Thus in the hENT1 deficient cell line, the GemC18-NPs were 15-fold more cytotoxic than gemcitabine. A second resistant cancer cell line, TC-1-GR, that over-expressed ribonucleotide reductase subunit M1 (RRM1), was tested *in vitro* and *in vivo*. In both cases treatment with GemC18-NPs efficiently inhibited the growth of cancer cells.[96]

PEGylated nanoparticles were also formulated and evaluated *in vitro* and *in vivo* with a pancreatic cancer cell line, BxPc-3.[93] *In vitro*, GemC18-NPs and PEG- GemC18-NPs were less cytotoxic than native gemcitabine and the addition of PEG did not show any difference in toxicity. However *in vivo,* GemC18-NPs and PEG- GemC18-NPs were more efficient than gemcitabine in controlling the growth of tumours. The *in vitro* decrease in cytotoxicity was explained by a longer uptake into the cell of the particles (by endocytosis), then a gradual release and finally hydrolysis of the GemC18. This explains a lower cytotoxicity level compared to free gemcitabine with an equivalent incubation time. *In vivo*, PEG-GemC18-NPs significantly increased the accumulation of nanoparticles in the tumours (6.3-fold) and blood circulation (5.3-fold after 24h) (Figure 7.A and B). The addition of PEG also caused a decrease in the accumulation of nanoparticles in the reticulo endothelial system such as the liver and spleen (Figure 7.C). Despite an increase in circulation time and tumour accumulation, the PEG-GemC18-NPs did not significantly show different antitumour activities with GemC18-NPs.

In a recent study, the active targeting of nanoparticles has been tested to increase the antitumour effect of the particles. For this, the epithelial growth factor (EGF) was conjugated to the particle surface to target the epidermal growth factor receptor (EGFR).[95] Elevated levels of the epidermal growth factor receptor (EGFR), a growth-factor-receptor tyrosine kinase, was identified as a common component of multiple cancer types and appeared to promote solid tumour growth. The EGFR was found to act as a strong prognostic indicator in head and neck, ovarian, cervical, bladder and oesophageal cancer.[97] The epidermal growth factor receptor (EGFR) is over-expressed in 80% of non-small cell lung cancer (NSCLC),[98] 80-100% of human head and neck cancer cells, 14-91% of human breast cancer cells, and 30-50% of human pancreatic cancer cells.[95] Epidermal growth factor receptor (EGFR) is an important anti-cancer therapy target that is applicable to many cancer types.[99] Anti-EGFR antibodies and EGF have been conjugated onto liposomes, lipid nanoparticles, chitosan particles and magnetic particles to target cancer cells over-expressing EGFR and the delivery of anticancer drugs or siRNA. *In vitro* uptake and cytotoxicity of EGF-GemC18-NPs realised with different human breast adenocarcinoma cell lines showed a correlation between EGFR density on the cell surface and cell uptake and toxicity.[86,98,100,101] In cell culture, EGF-GemC18-NPs uptake by tumour cells was correlated to the EGFR density, whereas the uptake of untargeted GemC18-NPs exhibited no difference among those same cell lines. The relative cytotoxicity of the EGF-conjugated GemC18-NPs to tumour cells in cell culture was correlated to EGFR expression as well, with more internalised EGF-GemC18-NPs and higher expected cytotoxicity. *In vivo* efficacy, with mice bearing MDA-MB-468 tumours (human breast adenocarcinoma cell lines over-express EGFR with $1x10^6$ receptors per cell), confirmed the effectiveness of EGF-GemC18-NPs. Mice treated with EGF-GemC18-

NPs had a longer life and significantly slower tumour growth than mice treated with untargeted GemC18-NPs, due to the EGF's ability to increase the accumulation of EGF-GemC18-NPs in the tumours.

The increase of anticancer activity observed with these lipophilic derivatives compared with native gemcitabine was obtained at the expense of their solubility in aqueous media. Indeed, with their highly lipophilic properties, these compounds proved difficult to reconcile with intravenous administration and hence encapsulation is necessary. The modification of lipophilic behaviour and encapsulation could be considered a good and versatile antitumoral approach against several tumours which become less sensitive to the native drug.

Insert Figure 7.

3. 5' MODIFICATIONS OF GEMCITABINE

3.1 CP-4126

In order to enhance cellular uptake, to prolong cell retention, and increase the half-life of gemcitabine with a less hydrophilic drug, derivatives containing a fatty acid side chain have been developed.[102,103] Gemcitabine containing a fatty acid chain at the 5' position of the nucleoside (CP-4126) has been developed (Figure 2.H). The fatty acid was esterified to the 5' position on a sugar moiety. CP-4126 contains a fatty acid with a chain length of 18 carbon atoms and one trans-double bond (elaidic acid) in position 9. Due to its different molecular design, CP-4126 is absorbed by cancer cells independent of hENT1 levels, which improves its efficacy in tumours with low or no hENT1 expression. Altered membrane transport is also a mechanism of 1-β-D-

arabinofuranosylcytosine (Ara-C) resistance. Similarly, in order to facilitate ara-C uptake and prolong retention in the cell, lipophilic prodrugs have been synthesised and in particular CP-4055 with the same modification as CP-4126.[10] CP-4055 showed a higher activity compared to Ara-C in several human solid tumourS and leukaemia xenografts. *In vitro* studies with inhibitors of nucleoside carrier-dependent transport, nitrobenzylmercaptopurine riboside and dipyridamol, strongly reduce the cellular sensitivity to Ara-C, but not to CP-4055, indicating that CP-4055 uses an alternative/additional mechanism for internalisation into the cell compared with Ara-C.[104] CP-4055 or ELACYT® is currently being tested in several clinical studies: phase II in Acute Myeloid Leukemia (AML), phase I in haematology in combination with idarubicin (Idamycin®), phase II in colorectal cancer, phase II in malignant melanoma in combination with sorafenib (Nexavar®) and phase II in ovarian cancer.[105–107]

In vitro tests have shown that IC50 of gemcitabine increased up to 200-fold in deficient nucleoside transport cell lines, but there was no difference with CP-4126, suggesting a nucleoside transporter-independent transport in the cell of the fatty acid derivative.[108] Inside the cell, CP-4126 was localised in the membrane and the cytosolic fraction, leading to long retention after removal of the cell culture medium. This accumulation caused a slower and prolonged release of the gemcitabine from the lipophilic analogue. CP-4126 needs to be converted into gemcitabine by non-identified esterases, releasing the fatty acid, in order to be phosphorylated.[109] CP-4126 is active in cells with deficient nucleoside membrane transport.[110] On the other hand, activity of native gemcitabine and CP-4126 was comparable in the cell lines without resistance, while in dCK-deficient cells both compounds were inactive. CP-4126 is, like native gemcitabine, dependent upon activation by dCK. *In vivo* studies

have also shown highly effective action of gemcitabine and CP-4126 in sarcoma, lung, prostate, pancreatic and breast cancer. In contrast to native gemcitabine which was highly toxic *via* the oral route, CP-4126 was administered orally with various schedules and an efficient antitumour activity (Figure 8).[111] CP-4126 was also evaluated for a potential synergy with several clinically-active cytotoxic drugs such as docetaxel, oxaliplatin and pemetrexed.[112] *In vitro* preliminary results have shown a synergistic effect in the lung cancer cell line (A549) and the colon cancer cell line (WiDR) with the combination of CP-4126 and oxaliplatin. Furthermore, the combination of docetaxel with CP-4126 induced an accumulation in the G2/M phase in the A549 cell line, but a G0/G1 phase accumulation in the WiDR cell line. Pemetrexed with CP-4126 induced in the A549 cell line an increase of cells in the G0/G1 phase and the S phase.

CP-4126 is currently in a phase II clinical trials in solid tumour patients.[109] This trial will investigate the use of CP-4126 as a second-line treatment for advanced, metastatic pancreatic cancer in patients refractory to first line gemcitabine treatment, where the resistance mechanism is likely to be due to impaired drug entry into tumour cells. The trial is progressing in Europe, the US, South America and Australia, and results are anticipated for the end of 2012.

Insert Figure 8.

3.2 NEO6002

To enhance the uptake and efficacy by a prolonged release of gemcitabine in cancer cells, NeoPharm has synthesised a novel gemcitabine-cardiolipin conjugate.[113] This

approach was to conjugate the ether analogue of synthetic cardiolipin with gemcitabine *via* a succinate linker. Cardiolipin (CL) is a major membrane phospholipid specifically localised in mitochondria. At the cellular level, CL has been shown to have a role in mitochondrial energy production, mitochondrial membrane dynamics, and the triggering of apoptosis.[114] The conjugate is called NEO6002 (Figure 2.I).

In vitro studies showed that NEO6002 can improve the efficacy and potentially overcome NT-deficient (nucleoside transporters) gemcitabine-resistant tumours, indicating a different internalisation route of NEO6002.[115] NeoPharm's studies on NEO6002 showed evidence of cytotoxicity against various cancer cell lines, including: A549 (human lung); BxPC-3 (human pancreas); MX-1 (human breast); HT-29 (human colon) and P388 (murine leukaemia).[116] Mice bearing or not BxPC-3 tumour xenografts were treated with NEO6002 or Gemzar® and the toxicities for each were evaluated by the mortality, body weight loss, peripheral blood cell counts, and plasma levels of the liver enzymes at the end of the study. Mice treated with Gemzar® for six daily 27µmol/kg-doses were all moribund whereas no mouse treated with NEO6002 died. This suggested that NEO6002 was less toxic at equimolar dosage when compared with Gemzar®. In mice bearing BxPC-3 tumours, at a dose of 18µmol/kg, NEO6002 inhibited the growth of BxPC-3 xenografts by 52%, while only 32% of tumour inhibition was achieved with Gemzar®.

3.3 Phosphoramidate Gemcitabine

The obligatory phosphorylation is often the rate-limiting step in the activation process of many anti-cancer drugs and is therefore still one of the limiting factors for the

therapeutic use of nucleoside analogues. Hence, different strategies to improve the antitumour efficacy of nucleoside analogues are being investigated.

The use of modified nucleotide prodrugs incorporating a phosphate function protecting group, has led to the selective release of the mono-phosphorylated nucleoside analogue.[117] This is the case of AraC grafted with an S-pivaloyl-2-thioethyl (tBuSATA).[118] This prodrug has been found to be more efficient than native Ara-C against L1210 10K cells, being totally dCK-deficient. This modification was tested on gemcitabine to get (i) resistance to chemical degradation, (ii) passive diffusion across a cell membrane and (iii) release of the mono-phosphorylated metabolite, independent of kinase expression. These prodrugs are designed to undergo intracellular activation to generate an unstable phosphoramidate intermediate anion, followed by a spontaneous cyclisation and P–N bond cleavage by water to liberate the nucleoside monophosphate.[119] In a patent from 2009, Perigaud *et al.* explained the synthesis of four derivatives of gemcitabine , *N*-(n-butylamino)-O-(S-pivaloyl-2-thioethyl)-O-5' gemcitabine phosphoramidate diester (Gem-1), *N*-(isopropylamino)-O-(S-pivaloyl-2-thioethyl)-O-5'-gemcitabine phosphoramidate diester (Gem-2), *N*-(benzylamino)-O-(S-pivaloyl-2-thioethyl)-O-5'-gemcitabine phosphoramidate diester (Gem-3), and *N*-(benzylamino)-O-(S-(2,2-dimethyl-3-hydroxypropionyl)-2-thioethyl)-O-5'-gemcitabine phosphoramidate diester (Gem-4) (Figure 2.J-M). A better cytotoxicity level was obtained in preliminary *in vitro* tests with Gem-1, Gem-2 and Gem-3 compared to the native gemcitabine in L1210 10K cell line (23.7 ± 1.2µM, 18.3 ± 1.5µM, 9.7 ± 9.0µM, 36.7 ± 11.6µM respectively).[120]

Another approach to deliver nucleoside 5'-monophosphate intracellularly was developed by using other phosphoramidate conjugations. Many phosphoramidate prodrugs have been synthesised with an increase of biological activity in various therapeutic domains like fluoro-2'-deoxyuridine. The cytostatic activity of NUC-3073, a phosphoramidate prodrug of 5-fluoro-2'-deoxyuridine, has been found to be independent from activation by thymidine kinase and non-sensitive to degradation by phosphorolytic enzymes.[121] Recently INX-08189 has entered human clinical trials. INX-08189 is a phosphoramidate motif and a 6-O-methoxy based-prodrug moiety which are combined to generate lipophilic prodrugs of the guanine monophosphate nucleoside.[122] The *in vitro* and *in vivo* data indicated that INX-08189 was a highly potent inhibitor of the hepatitis C virus with a high barrier for resistance and good oral pharmacokinetic properties.[123] This approach was extended to Ara-C and *in vitro* studies indicated that the phosphoramidate Ara-C was significantly more potent than native Ara-C against transport- and kinase-deficient CEM leukaemia cell lines.[124] Finally, this approach was used with 2′-β-D-Arabinouridine (AraU), the uridine analogue of the anticancer agent AraC. Unfortunately, neither the parent compound (AraU) nor any phosphoramidate drugs showed antiviral activity, or potent inhibitory activity against any of the cancer cell lines.[125] The phophoramidate prodrug approach was extented to gemcitabine to form 5'-(2'-deoxy-2',2'-difluorocytidyl) 5-nitrofurfuryl *N*-methyl-*N*-(4-chlorobutyl) phosphoramidate (Figure 2.N). The purpose of this modification was to overcome resistance in tumours deficient in dCK by delivering intracellulary a gemcitabine 5'-monophosphate, entering the cell by passive diffusion.[126] *In vitro* tests on many cancer cell lines have shown that the prodrug is less active than native gemcitabine in wild-type cell lines but more active than native gemcitabine in dCK-deficient cell lines (AG6000 and CEM-dCK-). However, after

blocking the equilibrative nucleoside transport, inhibition of tumour growth was no longer observed with the prodrug, indicating that the prodrug antitumour activity was mediated by cell entry implying equilibrative nucleoside transport.

4. CONCLUSION

Since the 90s, gemcitabine has become the standard treatment for pancreatic cancer. Studies show a therapeutic benefit from the use of gemcitabine compared to fluorouracil and have led to the prescription of gemcitabine as a standard treatment in advanced or metastatic pancreatic cancer.[127] It is also widely used in combination for other solid cancers such as lung, bladder, ovary or breast.

It undergoes a series of phosphorylations to become active and thus brings the cell into apoptosis. However the rapid deamination after intravenous injection of gemcitabine induces the formation of its inactive metabolite (dFdU) which is then excreted, mainly in urine. Thus repeated injections and high concentrations to maintain a sufficient concentration for antitumour activity cause a number of side effects.[40] Repeated exposition does not directly lead to clinical improvement. The current clinical are relies on a balance of anticancer activity vs. Toxicity to normal tissue, to achieve an efficient therapeutic scheme. The short half-life is not the only drawback: numerous tumours develop resistance mechanisms: resistance by a lack of transporters and resistance by a lack of kinase required for phosphorylation and thus activation. The chemical modification of a drug is a smart solution to try to override this resistance and improve the resulting pharmacokinetic parameters.

All changes at 4-(*N*)-position with PEG, squalene, valproic acid and linear acyl derivatives (valeroyl, heptanoyl, lauroyl and stearoyl) have been characterised to protect the amine function and thus block CDA action. The enhanced bioavailability

of prodrugs, thanks to the storage in various cytosolic fractions followed by a prolonged release, has been obtained by the addition of PEG, squalene in 4-(*N*)-position and elaidic acid in 5’ position. Increased plasma availability is also observed due to the encapsulation of lipophilic GemC18 in liposomes. An independent nucleoside transporter route can be observed with PEG, squalene, elaidic acid and derivatives, limiting the phenomenon of resistance. And finally, only the phosphoramidate function on 5’ position provides a mono-phosphate gemcitabine, initiating pathway activation.

In conclusion, gemcitabine prodrugs have beneficial antitumoural effects by using independent nucleoside transport, by reducing the catabolic effect of CDA, by prolonging the release of the native or mono-phosphate gemcitabine, and finally by enhancing the cytotoxicity effect. Gemcitabine modification seems to be an innovative and interesting approach to treat less-sensitive cancers.

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Figure captions

Figure 1. Modification sites in gemcitabine molecule.

Figure 2. Chemicals representation of gemcitabine analogues and modification presented in this review. **A**: Deoxycytidine. **B**: Cytosine arabinoside (AraC): **C**: PEG-gemcitabine. **D**: LY2334737. **E**: Folate-PEG-gemcitabine. **F**: Squalenoyl-gemcitabine. **G**: 4-(N)-acyl-gemcitabine. **H**: CP-4126. **I**: NEO6002. **J**: N-(n-butylamino)-O-(S-pivaloyl-2-thioethyl)-O-5' gemcitabine phosphoramidate diester (Gem-1). **K**: N-(isopropylamino)-O-(S-pivaloyl-2-thioethyl)-O-5'-gemcitabine phosphoramidate diester (Gem-2). **L**: N-(benzylamino)-O-(S-pivaloyl-2-thioethyl)-O-5'-gemcitabine phosphoramidate diester (Gem-3). **M**: N-(benzylamino)-O-(S-(2,2-dimethyl-3-hydroxypropionyl)-2-thioethyl)-O-5'-gemcitabine phosphoramidate diester (Gem-4). **N**: 5'-(2'-Deoxy-2',2'-difluorocytidyl) 5-nitrofurfuryl *N*-methyl-*N*-(4-chlorobutyl) phosphoramidate.

Figure 3. Kaplan-Meier survival curve.[13]

Table 1. Gemcitabine Clearance and Half-life for "Typical" Patients.[13]

Figure 4. Phosphorylation and dephosphorylation of gemcitabine in the cell. ENTs: equilibrative nucleoside transporter, CNTs: concentrative nucleoside transporter, dFdCMP: gemcitabine monophosphate, dFdCDP: gemcitabine diphosphate, dFdCTP: gemcitabine triphosphate, dFdUMP: 2'-deoxy-2',2'-difluorouridine monophosphate, dFdU: 2', 2'- difluorodeoxyuridine, CDA: cytidine deaminase, DCTD:

deoxycytidylate deaminase, 5'NT: 5'-nucleotidase, UMP/CMP kinase: nucleoside monophosphate kinase.

Figure 5. *In vivo* bioavailability of gemcitabine and PEGylated gemcitabine. The mice were divided in two groups. Equivalent concentration of gemcitabine and PEGylated gemcitabine (6.74 mg/kg) was given to group 1 and group 2, respectively. Gemcitabine and PEGylated gemcitabine were administered intravenously and blood was collected at different time intervals. Serum was separated and the concentration of gemcitabine was determined by RP-HPLC analysis, as described in materials and methods. (**) $p < 0.005$ and (*) $p < 0.05$ gemcitabine versus PEGylated gemcitabine.[50]

Figure 6. SQ-Gem improves inhibition of tumour growth and increased survival. **(A)** Mice ($n = 8$) bearing subcutaneous tumours were treated twice with Gem or SQ-Gem (20 mg/kg). After 1 month, statistical analysis of tumour volumes showed superior antitumour efficacy of SQ-Gem compared to untreated or SQ-treated mice (***$P < 0.001$) and to Gem ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$). **(B)** The same experiment was performed on Panc1 orthotopic tumour model ($n = 14$). Tumours were significantly reduced by SQ-Gem treatment (**$P < 0.01$ vs. untreated mice and $^{\dagger}P < 0.05$ vs. Gem-treated mice). **(C)** Kaplan-Meier survival curves of orthotopic Panc1 tumour-bearing mice showed significant enhanced median survival after SQ-Gem treatment (vs. Gem-treated mice, $p < 0.5$ and vs. control mice, ***$P < 0.001$).[73]

Figure 7. *In vivo* and *ex vivo* imaging of GemC18-NPs and PEG-GemC18-NPs. (A) IVIS images of athymic mice 24 h after injection of fluorescein-labeled GemC18-NPs

or PEG-GemC18-NPs. (B) Relative fluorescence intensity values in BxPC-3 tumours (circular ROI in A). [a]$p = 0.0006$, GemC18-NPs vs. PEG-GemC18-NPs. (C) Tissue distribution of fluorescein-labeled GemC18-NPs and PEG-GemC18-NPs 24 h after injection.[b]GemC18-NPs vs. PEG-GemC18-NPs, $p = 0.003$, 0.021, and 0.002 for blood, liver, and spleen, respectively.[93]

Figure 8. Antitumour efficacy of CP-4126 as an oral drug in the human colon cancer xenograft Co6044. Mice treated with saline, qd 1–5 (▲), 100 mg/kg CP-4126(●), 20 mg/kg CP-4126 (∇) and 10 mg/kg CP-4126 (□). The curve for the optimal schedule of gemcitabine (not shown) was in between that of the control and q3d schedule. (from [102])

Figures

Figure 1.

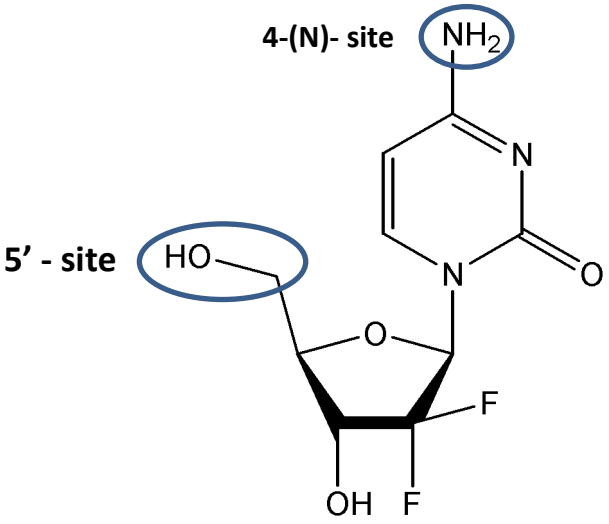

Figure 2.

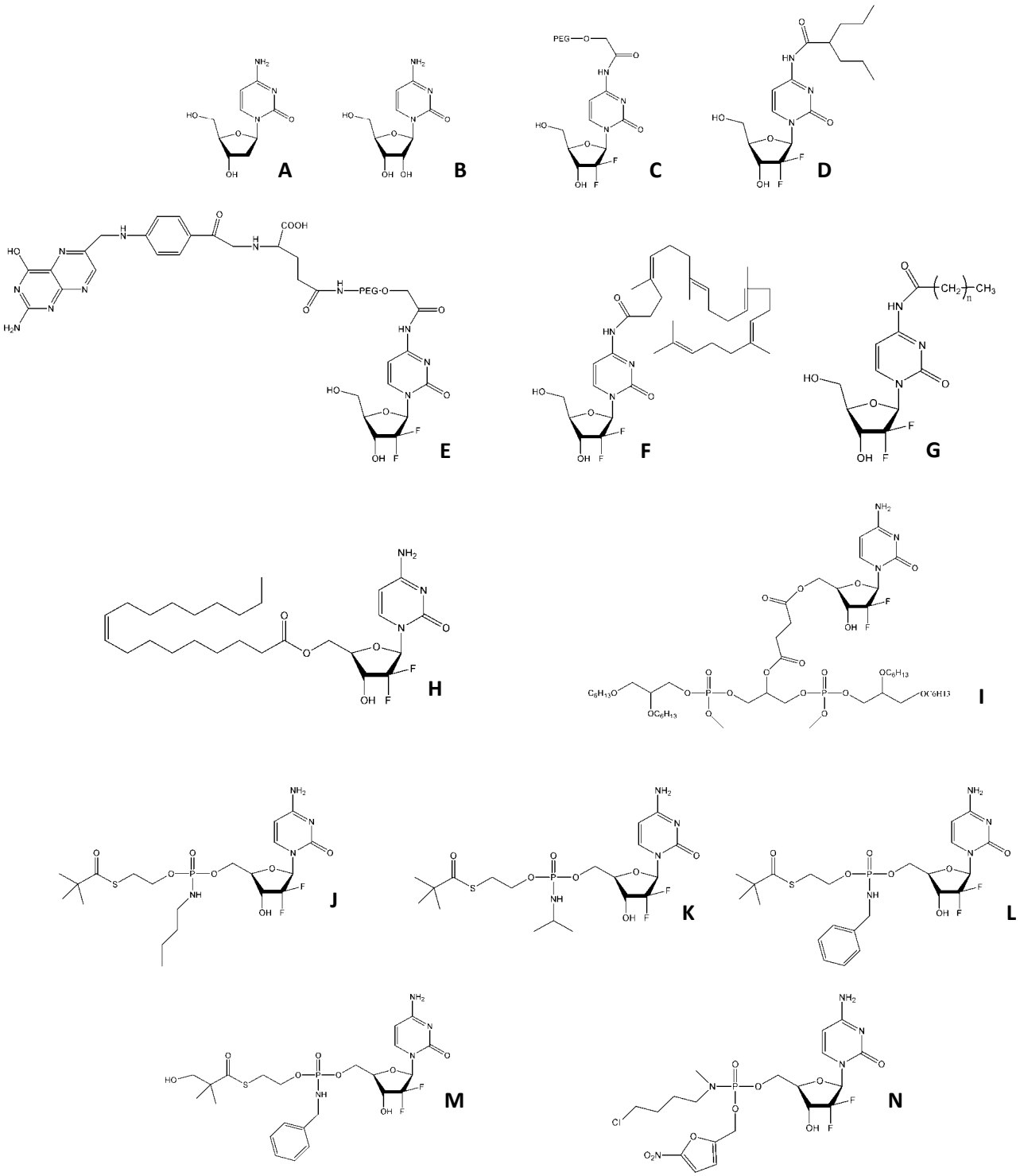

Figure 3.

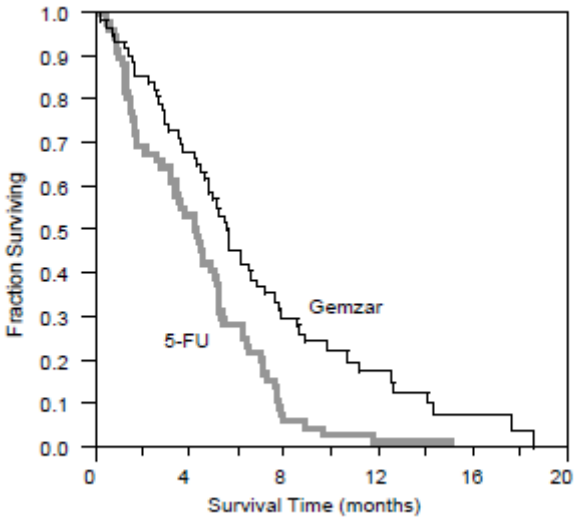
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Fraction Surviving
Gemzar
5-FU
0
4
8
12
16
20
Survival Time (months)

Table 1.

Age	Clearance Men ($L/hr/m^2$)	Clearance Women ($L/hr/m^2$)	Half-Life[a] Men (min)	Half-Life[a] Women (min)
29	92.2	69.4	42	49
45	75.7	57.0	48	57
65	55.1	41.5	61	73
79	40.7	30.7	79	94

[a] Half-life for patients receiving a short infusion (< 70min)

Figure 4.

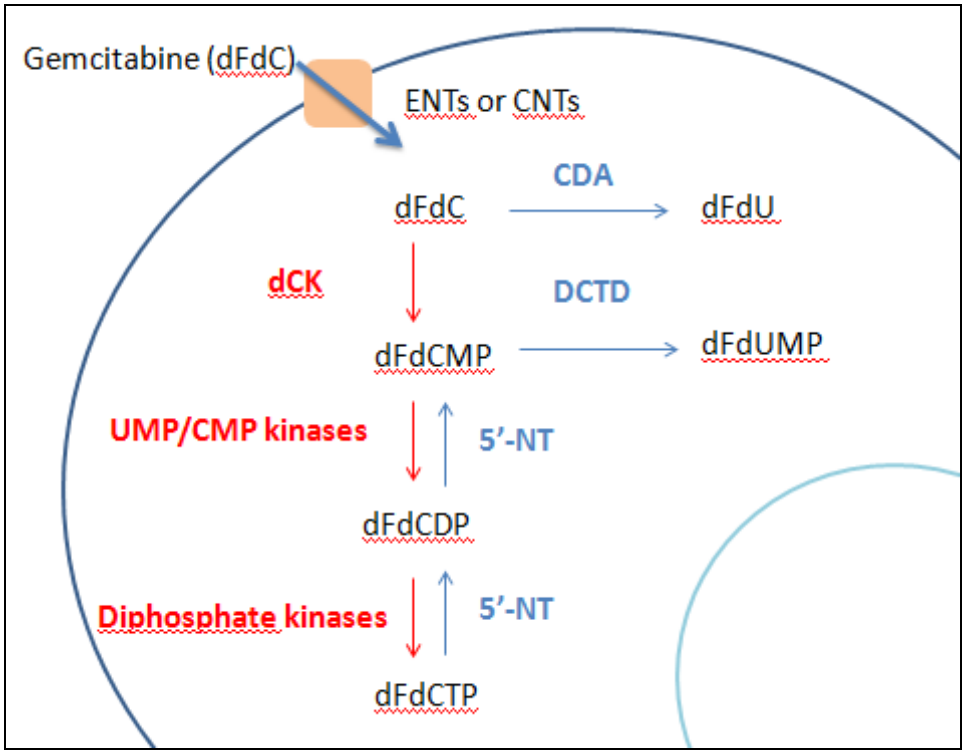
Gemcitabine (dFdC)
ENTs or CNTs
CDA
dFdC
dFdU
dCK
DCTD
dFdCMP
dFdUMP
UMP/CMP kinases
5'-NT
dFdCDP
Diphosphate kinases
5'-NT
dFdCTP

Figure 5.

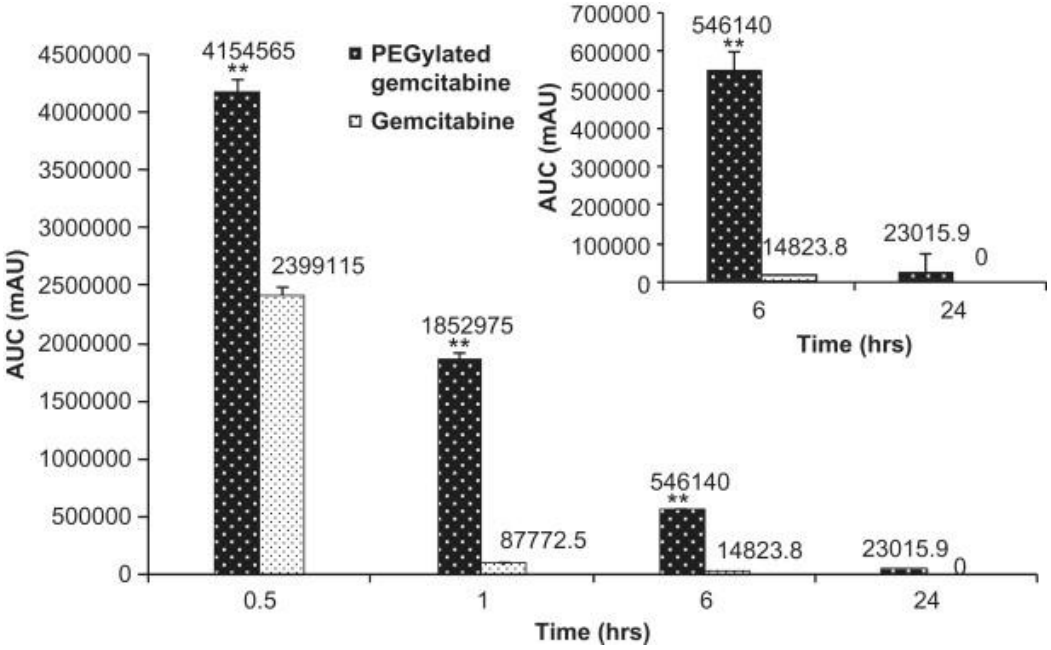
AUC (mAU)
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
4154565
**
2399115
1852975
**
87772.5
546140
**
14823.8
23015.9
0
0.5
1
6
24
Time (hrs)
PEGylated gemcitabine
Gemcitabine
700000
600000
500000
400000
300000
200000
100000
0
546140
**
14823.8
23015.9
0
6
24
Time (hrs)

Figure 6.

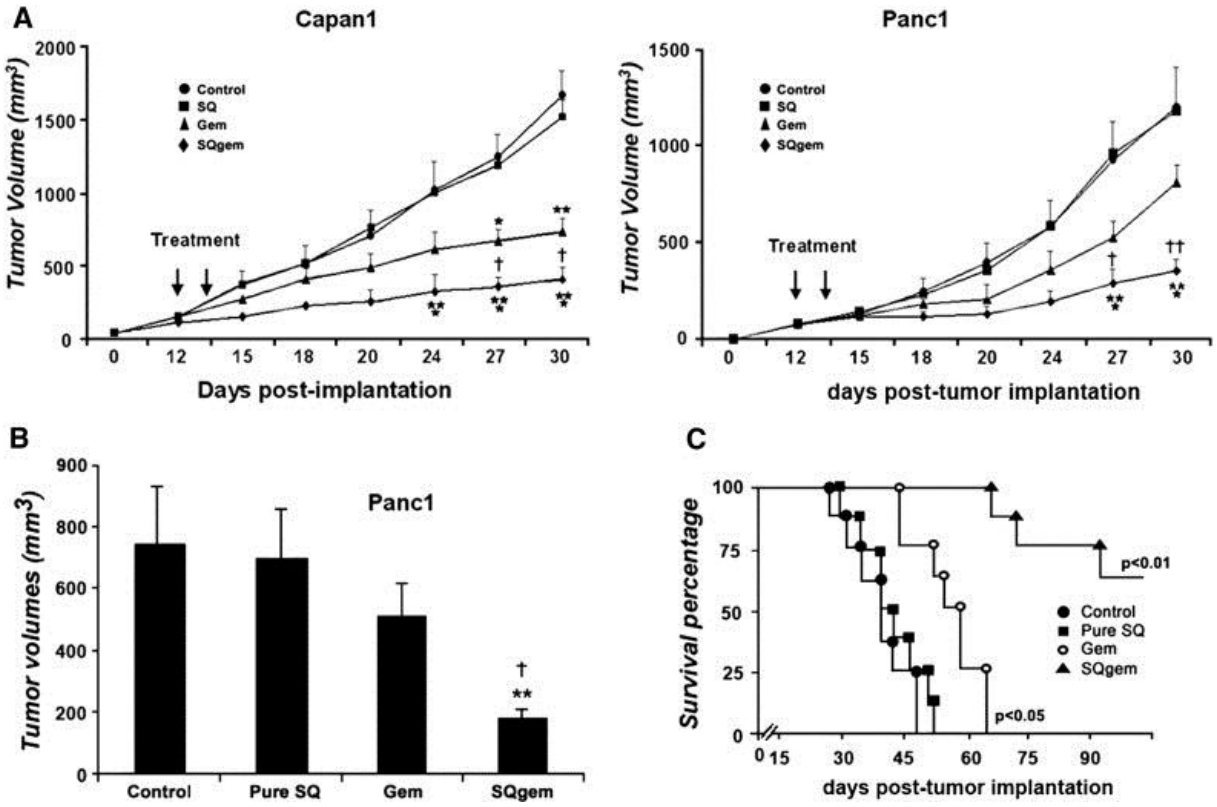
A
Capan1
Tumor Volume (mm3)
Control
SQ
Gem
SQgem
Treatment
Days post-implantation
Panc1
days post-tumor implantation
B
Panc1
Tumor volumes (mm3)
Control
Pure SQ
Gem
SQgem
C
Survival percentage
p<0.01
p<0.05
days post-tumor implantation

Figure 7.

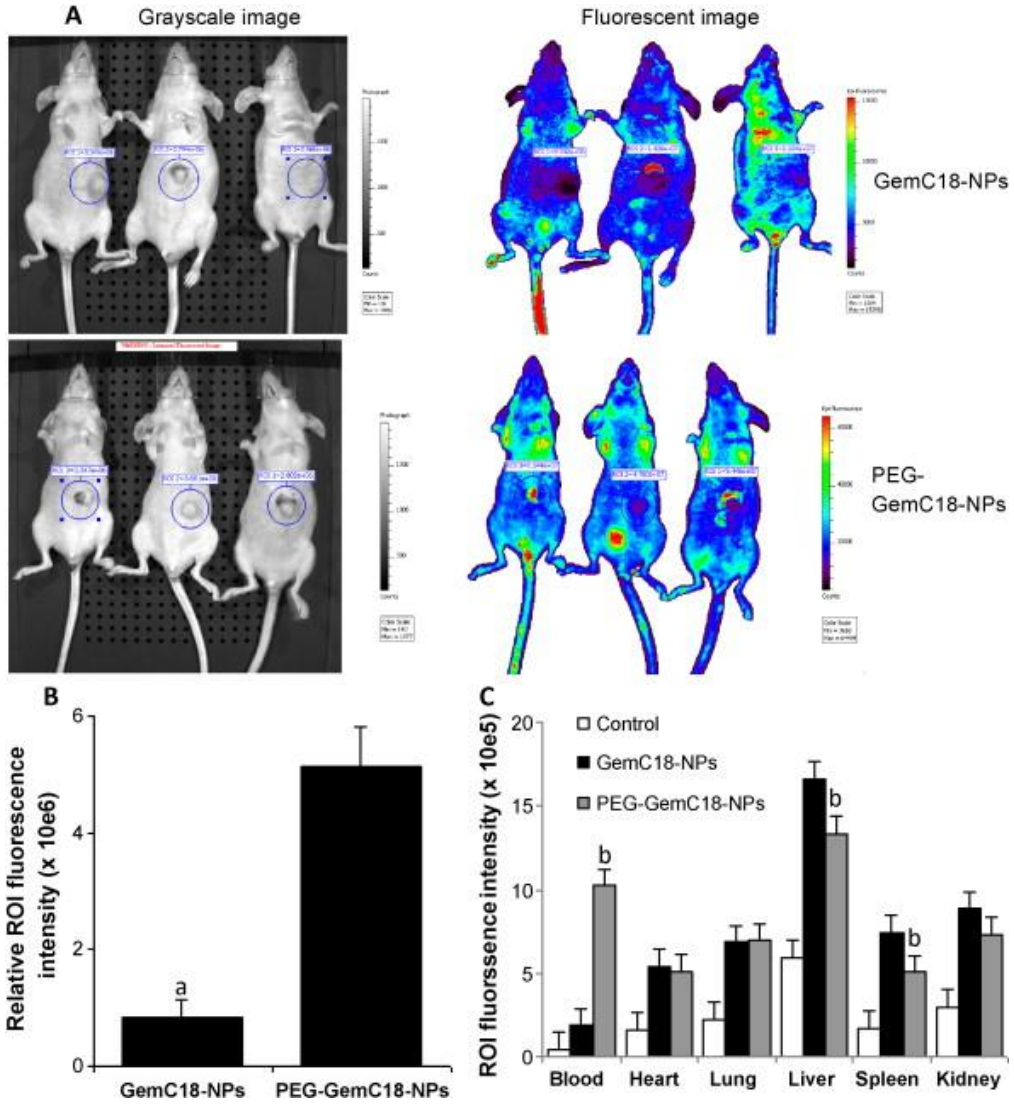
A
Grayscale image
Fluorescent image
GemC18-NPs
PEG-
GemC18-NPs
B
Relative ROI fluorescence intensity (x 10e6)
6
4
2
0
a
GemC18-NPs
PEG-GemC18-NPs
C
ROI fluorssence intensity (x 10e5)
20
15
10
5
0
Control
GemC18-NPs
PEG-GemC18-NPs
b
b
b
Blood
Heart
Lung
Liver
Spleen
Kidney

Figure 8.