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Gemcitabine anti-proliferative activity significantly enhanced upon conjugation with cell-penetrating peptides

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ABSTRACT

Gemcitabine proven efficiency against a wide range of solid tumors and undergoes deamination to its inactive uridine metabolite, which underlies its low bioavailability, and tumour resistance was also associated with nucleoside transporter alterations. Hence, we have conjugated gemcitabine to cell-penetrating peptides (CPP), in an effort to both mask its aniline moiety and facilitate its delivery into cancer cells. Two CPP-drug conjugates have been synthesized and studied regarding both the time-dependent kinetics of gemcitabine release and their anti-proliferative activity on three different human cancer cell lines. Results obtained reveal a dramatic increase in the anti-proliferative activity of gemcitabine in vitro, upon conjugation with the CPPs. As such, CPP-gemcitabine conjugates emerge as promising leads for cancer therapy.

In developed countries, cancer is one of the major causes of death, and one of the main reasons to which this high mortality can be attributed is failure of current treatment options. The hard distinction between normal and cancer cells, as well as the phenomenon of resistance and the inefficacy to treat metastases, are the main obstacles when treating cancer patients. Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC, **1**, Scheme 1) is a chemotherapeutic nucleoside analogue used in the treatment of solid tumours in various cancers, as pancreatic, nonsmall lung, breast and ovarian.[1] Gemcitabine is a hydrophilic drug and its cellular uptake is primarily facilitated by the human equilibrative nucleoside transporter 1 (hENT1).[2] The drug is activated via phosphorylation to its 5′-monophosphate (dFdCMP, **2**, Scheme 1) by deoxycytidine kinase (dCK), and then undergoes further phosphorylation by intracellular kinases to the diphosphate (dFdCDP, **3**) and triphosphate (dDdCTP, **4**) forms.[3] However, gemcitabine may also undergo deamination to its inactive uridine metabolite, 2′,2′-difluorodeoxyuridine (dFdU, **5**), by cytidine deaminase (CDA), which is present at high levels in both human plasma and liver.[4] Hence, the major impediment to gemcitabine efficacy is its rapid inactivation, since upon administration more than 90% of the drug (**1**) is converted to **5** and **2** (Scheme 1).[5] Another important drawback in gemcitabine therapy is that, after initial tumour regression, tumour cells develop different forms of drug resistance, as related to nucleoside transporter deficiency.[6]

Many efforts have been aimed at enhancing the therapeutic index of gemcitabine, namely through chemical modifications either on the cytosine's aniline or on the 5′-hydroxyl group of the 2,2′-difluoro-2′-deoxyribose moiety.[7] Several of the reported modifications at position 5′ of gemcitabine include amino ester prodrugs,[8] liposaccharide esters (CP-4126),[9] cardiolipine conjugates (NEO6002)[10] and, more recently, l-alanine-based phosphoramidates (NUC-1031),[11] and conjugates with receptor-binding peptides.[12] Modifications at the aniline group of 1 have been mainly carried out by amidation, to take advantage of the slow drug release provided by the hydrolysable amide bond; such would, in principle, enhance bioavailability and uptake, while also providing resistance to enzymatic deamination.[13–18]

In connection with the above, and as part of our effort to identify orally active prodrugs, we have now addressed the conjugation of cell-penetrating peptides (CPP) to the aniline moiety of gemcitabine, through suitable bio-/chemo-reversible bonds. The use of CPP is intended to facilitate intracellular delivery of the conjugates, as a common virtue of all CPP is that they are able to efficiently pass through cell membranes while being non-cytotoxic and able

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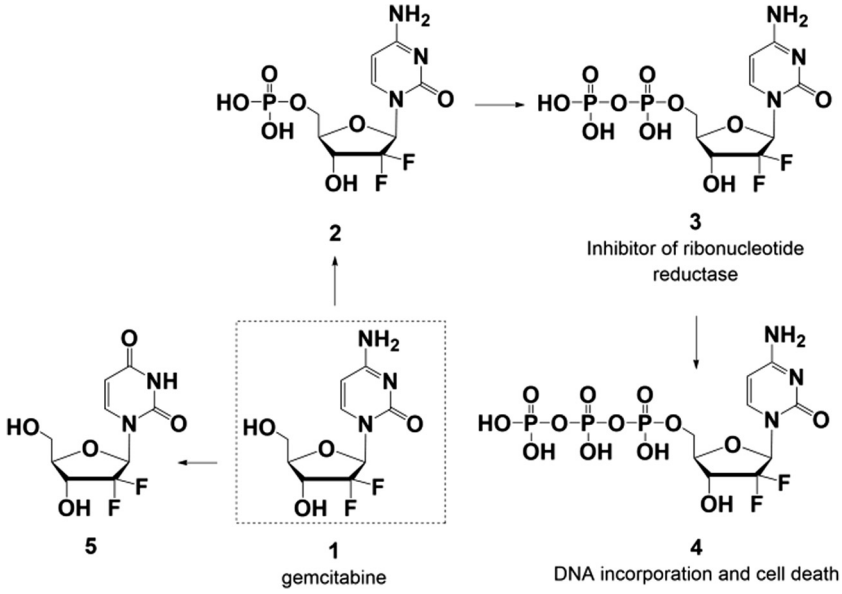
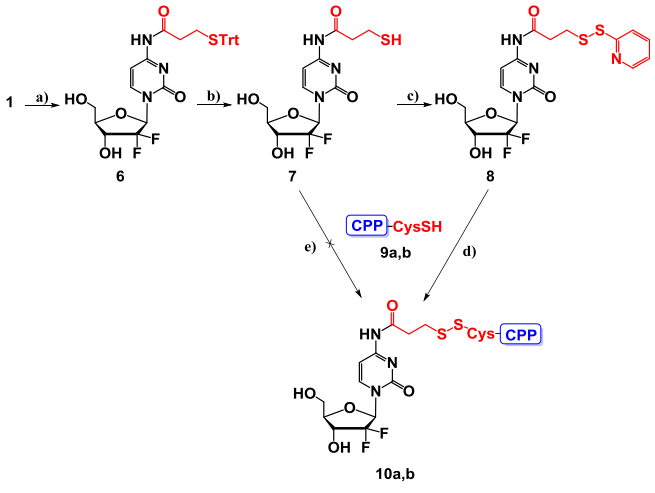


Scheme 1. Activation and inactivation pathways for gemcitabine (**1**).

to carry a wide variety of cargos inside cells,[19–21] and also to enhance the water solubility of drugs.[22] Moreover, it has been suggested that cancer cell metabolism and additional pathological conditions provide a unique cellular environment that can be used as triggers for CPP exposure to then affect intracellular drug delivery.[23] As starting point to this approach, we have synthesized two novel peptide-gemcitabine conjugates (**10a,b**), using two well-known CPP sequences, Penetratin (Pen, RQIKIWFQNRRMKWKK) and pVEC (LLIILRRRIRKQAHAHSK), which are both amphipathic but differ in their net charge at physiological pH (7.4) and in their secondary structure;[24,25] moreover, both peptides have been reported in numerous cancer studies over the last two decades.[24,26] An additional cysteine residue (Cys) was coupled to the *N*-terminus of both CPP, producing Cys-Pen (**9a**) and Cys-pVEC (**9b**), in order to provide the thiol group required for subsequent binding to gemcitabine through a 3-sulfanylpropanoyl linker (Scheme 2).



Scheme 2. Synthesis route to CPP-gemcitabine conjugates (**10**). a) 3-(*S*-tritylsulfanyl)propanoic acid, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyl-aminium tetrafluoroborate (TBTU), *N*-ethyl-*N*,*N*-diisopropylamine (DIEA), *N*,*N*-dimethylformamide (DMF), 0 °C to rt, 24 h (45%); b) dichloromethane (DCM)/trifluoroacetic acid (TFA) 1:1 (v/v), triethylsilane (TIS), 0 °C, 1 h (95%); c) 2,2-dithiodipyridine, methanol, acetic acid, rt, 24 h (60%); d) DMF, rt, 24 h (95% for **10a**, Cys-Pen conjugate; 60% (1 h) or 89% (72 h) for **10b**, Cys-pVEC conjugate); e) 5% dimethylsulfoxide (DMSO) in H_2O/acetonitrile 3:1 (v/v), pH 8 (diluted NH_4OH), 24 h (target product not formed).

The synthesis of the target conjugates[27] was accomplished through a convergent route in which the gemcitabine derivative **8** and the Cys-modified CPPs **9a,b** were synthesized in parallel and then coupled to obtain conjugates **10a,b**. Compound **8** is a gemcitabine derivative modified with a thiopyridyl group, and was prepared in three steps from the commercially available drug. The first step was the TBTU-mediated acylation of gemcitabine (**1**) with 3-(*S*-tritylsulfanyl)propanoic acid to obtain intermediate **6**, whose trityl *S*-protecting group was removed by mild acidolysis to afford the free thiol **7**; this thiol was further reacted with 2,2′-dithiodipyridine to provide **8** in 60% yield, through a recently reported method.[17] Cys-modified CPP **9a,b** (Table 1) were synthesized by solid-phase peptide synthesis (SPPS) employing 9-fluorenylmethoxycarbonyl (Fmoc)-based chemistry, according to our previously reported procedures,[28] and showed a high water solubility.

A disulfide exchange reaction between **8** and **9** produced the target conjugates **10a**,**b**, respectively, whose structure and purity were confirmed by analytical high-performance liquid chromatography (HPLC), both as stand-alone technique as well as coupled to mass spectrometry (LC-MS). Noteworthy, oxidative formation of a disulfide bond between intermediate **7** and Cys-CPPs **9a**,**b** was attempted by standard methods [e) on Scheme 2],[29] but failed to provide conjugates **10a**,**b**.

The 3-(S-sulfanylpropanoyl) linker between drug and CPP was chosen to provide two cleavable disulfide and amide linkages, whose hydrolytic stability at physiological pH and temperature were assessed, as follows. A semi-quantitative study of the degradation kinetics of both conjugates **10a,b** in phosphate buffer saline (PBS), pH 7.4, at 37 °C was carried out by HPLC (Fig. 1).[30]

Fig. 1 depicts time-dependent release of gemcitabine from its CPP conjugates, demonstrating that the parent drug is released upon hydrolytic cleavage of the aromatic amide in conjugates **10a** and **10b**, whose half-lives were of approximately 9.6 days and 42 h, respectively.

Both conjugates were also evaluated against three different human cancer cell lines: MKN-28 (gastric cancer), Caco-2 (colorectal adenocarcinoma) and HT-29 (colon cancer).[31] The anti-proliferative activity of the conjugates was assessed according to the