Polymeric prodrug combination to exploit the therapeutic potential of antimicrobial peptides against cancer cells.

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Antimicrobial Peptides (AMPs) have unique anticancer properties, but their clinical application is currently limited by an inadequate margin of safety. A prodrug strategy associated with a combination therapy approach could address this limitation by increasing their therapeutic index and their efficacy. Accordingly, the first targeted anticancer polymeric prodrug candidates of AMPs, intended for combination therapy with another polymeric prodrug of an approved antineoplastic agent (doxorubicin), were synthesized as either a PEG-based dual-release prodrug or two individual pegylated prodrugs. The latter are based on a cathepsin B-labile peptide linker and an acid-sensitive acyl hydrazone bond for the AMP and doxorubicin prodrugs, respectively. Anticancer activities and toxicity differentials achieved with the free peptide and its polymer conjugates against ovarian, cancer and non-malignant, cells, indicate that protease-dependent reversible pegylation could be implemented to increase the therapeutic indices of AMPs in cancer therapy. The results obtained also show that this approach can be developed if the releasable PEG linker can be optimised to conciliate the attributes and restrictions of pegylation against proteases. In addition, combination of the polymeric prodrugs of the AMP and of doxorubicin provides additive antitumor effects which could be exploited to enhance the efficacy of the AMP candidate.

Introduction

Antimicrobial Peptides (AMPs), also called Host Defence Peptides (HDPs), are fundamental molecular mediators of innate immunity in multicellular organisms.[1] Their synthetic derivatives have also demonstrated promising and unique anticancer properties.[2] They can exert selective cytotoxic activities against cancer cells, essentially through their capacity to penetrate and destabilize the plasma membrane and, in some cases, to induce apoptosis through disruption of the mitochondrial membrane and/or interaction with intracellular targets. These properties are not significantly affected by molecular heterogeneities within a given tumour or between different tumours, nor are they counteracted by the adaptability of the signalling network and alternative survival pathways in cancer cells. AMP-based sequences have a broad spectrum of activity, rapidly killing sarcoma, leukaemia, carcinoma and neuroblastoma cells. In addition, on account of their unique mode of action and polypharmacology, innate immunity derived peptides should evade the common mechanisms of chemoresistance. Moreover, it is expected that they will have a low propensity to act as a selection pressure for the evolution of cancer cells to a resistant phenotype. Finally, as their membranolytic action is essentially independent of proliferation pathways, AMPs can exert cytotoxic activities against non-growing or slowly-growing neoplastic cells.[2] Lack of activity against these cell populations is part of the reason for tumour recurrence and the development of resistance to anticancer drugs.

However, AMPs are not without limitations.[3] Improvement of their clinical properties for systemic administration will need to address their rapid excretion and degradation and their potential toxicity. Apart from the immunogenic potential of these peptide-based drug candidates,[2] their characteristic polypharmacology could be associated with significant off-target toxicity.[3] Furthermore, because they can be rapidly metabolized and excreted, the doses required to maintain their therapeutic levels *in vivo* are likely to correlate with inadequate therapeutic windows.[1] Finally, AMPs' activities against cancer cells are generally achieved *in vitro* in the low micromolar range.

Nevertheless, the potency of AMPs can be additively or synergistically enhanced when used in combination with classical chemotherapeutic agents.[4] In addition, the safety barrier in the delivery of AMPs could be addressed by a prodrug approach.[5] Indeed, reversibly inactivating a

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therapeutic candidate with a promoiety can confine its activity to diseased tissues, if its activation is selectively triggered by a pathology-associated molecule or physicochemical condition.[6] For example, proteolytic enzymes such as cathepsin B Further benefits could be imparted to peptide therapeutics if a polymer is employed as a promoiety.[7,8] Indeed, polymers can improve the pharmacokinetic and pharmacodynamic properties of peptides and proteins, by prolonging their plasma circulation time, preventing their degradation by proteolytic enzymes and limiting their toxicity and immunogenicity through plasma and cell surface proteins binding inhibition. Polymer therapeutics can also achieve passive tumour targeting of anticancer agents by the Enhanced Permeability and Retention (EPR) effect and direct their intracellular delivery through the lysosomotropic route.[8] In the case of polyethylene glycol (PEG), these properties are essentially mediated by the capacity of each monomeric unit to associate with 2 to 3 water molecules, resulting in an increase of the conjugated peptide's or protein's hydrodynamic radius.[7]

Taken together, an approach based on chemotherapy combinations and polymeric (pegylated) prodrugs could capitalize on the unique properties of AMPs against cancer cells, while addressing their main shortcomings as single agents. Accordingly, the first targeted anticancer polymeric prodrug candidates of AMPs, amenable to combination therapy, are reported here. The synthesis of a dual-release prodrug and two individual prodrug candidates, combining a AMP and a classical anticancer agent, doxorubicin, as well as the preliminary evaluation of their activities against cancer cells are described.

Results and discussion

Preparation of a dual-release prodrug candidate

The first polymeric bioreversible derivative of an AMP targeting cancer cells was produced as a dual-release prodrug. This candidate contained an α-helical amphipathic peptide, P18, a hybrid sequence of 2 natural AMPs (cecropin A(1-8) and magainin 2(1-12)),[9] and an anthracycline antineoplastic agent, doxorubicin, conjugated at the two ends of a bi-functional linear PEG (Fig. S1). The peptide was synthesized by Solid Phase Peptide Synthesis (SPPS) from a Rink Amide PEGA resin. This poly(ethylene glycol-*co*-acrylamide) support allows the diffusion of macromolecules of molecular masses up to 35 kDa into the polymer matrix and was therefore selected to allow the pegylation of the peptide on the solid support. The P18 peptide **1** was assembled from D-amino acids (lys-trp-lys-phe-leu-lys-lys-leu-pro-phe-leu-lys-his-ala-leu-lys-lys-phe), with a homologous substitution at position 8 of isoleucine with leucine.[10] For its prodrug modification, the sequence was elongated at its N-terminus with a tetrapeptide motif (Gly-Phe-Leu-Gly), synthesized from natural amino acids. The latter sequence is stable to plasma proteases, but is a substrate for cathepsin B, a lysosomal cysteine protease highly up-regulated in a wide range of cancer cells.[11,12] The enantiomeric (D)-P18 sequence imparts proteolytic stability to the active peptide component and limits the cleavage of the peptide chain by the activating enzyme to the connection between the heterochiral residues.[8] To form a polymeric prodrug of P18, the N-terminal amino acid of the cathepsin B-sensitive linker was in turn amidated with a Fmoc-protected amino-PEG-acid, modified, after deprotection, by successive reactions with *S*-benzyl-thiosuccinic acid and hydrazine, to reverse the direction of the peptide chain and form a acyl hydrazone linker, respectively.[13,14] This pegylated peptide was then released from the resin as a fully deprotected sequence which was finally conjugated to doxorubicin in solution by hydrazone ligation. The latter bond is generally stable physiologically, only undergoing hydrolysis at pHs lower than 5. These acidic conditions are essentially limited to subcellular compartments such as the endosome or the lysosome.[15] Formation of the hydrazone was monitored by HPLC using Size-Exclusion Chromatography (SEC) with dual wavelength detection at 214 and 465 nm for the peptide and doxorubicin components, respectively. The final product was purified by SEC. SEC-HPLC and UV spectroscopy analyses confirmed that a dual-release prodrug candidate, based on a PEG of average molar mass of 2,000 g/mol, could be synthesized. However, scale-up of the linear multi-step synthetic route to produce materials for biological testing remained impractical. The typical synthetic scale of automated SPPS in a research laboratory provided only an analytical sample of the dual-release prodrug candidate. An alternative approach combining two individual polymeric prodrugs was therefore developed.

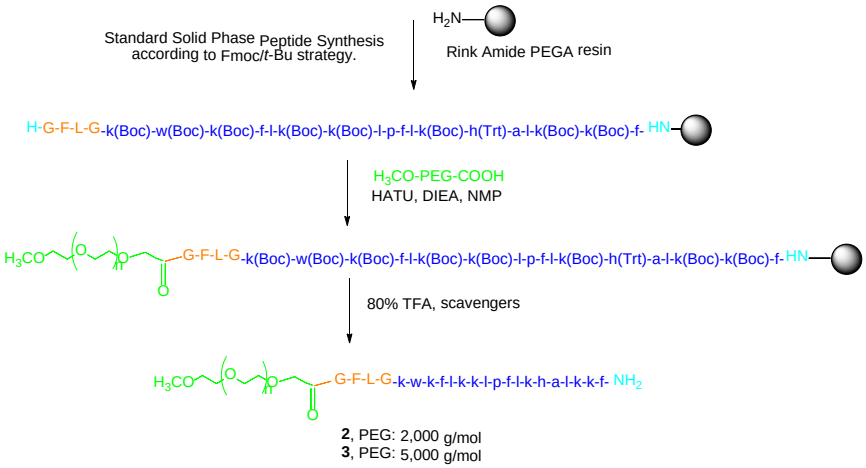

Fig. 1 Synthetic route to the polymeric prodrug candidates of the AMP P18. In blue is the P18 sequence, in turquoise the peptide termini, in orange the cathepsin B-sensitive linker and in green the PEG.

Preparation of the individual polymeric prodrug candidates

The polymeric bioreversible derivative of the peptide was produced as previously, by N-terminal modification of the (D)-P18 sequence with the cathepsin B-sensitive tetrapeptide linker, but with a mono-functional methoxy-PEG-acid in place of the bi-functional PEG, yielding MeO-PEG-G-F-L-G-(D)-P18-NH_2 (Fig. 1). Two polymeric prodrug candidates, **2** and **3**, were prepared by using PEGs of average molar mass of 2,000 and 5,000 g/mol, respectively. They were purified by SEC and analysed by RP- and/or SEC-HPLC and by MALDI-TOF Mass Spectrometry. The polymeric bioreversible derivative of doxorubicin was prepared by the same approach, but by omitting the P18 peptide, *ie*. by solid phase synthesis of a acyl carbohydrazide-PEG-amide, conjugated to doxorubicin by hydrazone chemistry in solution (Fig. 2). This polymer conjugate **4**, based on a PEG of average molar mass of 5,000 g/mol, was purified by SEC and analysed by SEC-HPLC and UV spectroscopy. Finally, a polymer conjugate of (D)-P18, which cannot be cleaved by cathepsin B, was synthesised by excluding the phenylalanyl and leucyl residues in the linker between the peptide and the polymer.[16] This yielded the pegylated peptide **5** (MeO-PEG-G-G-(D)-P18-NH_2), where the PEG has an average molar mass of 2,000 g/mol. Both the AMP and doxorubicin polymeric prodrug candidates were isolated in sufficient yields for preliminary biological testing.

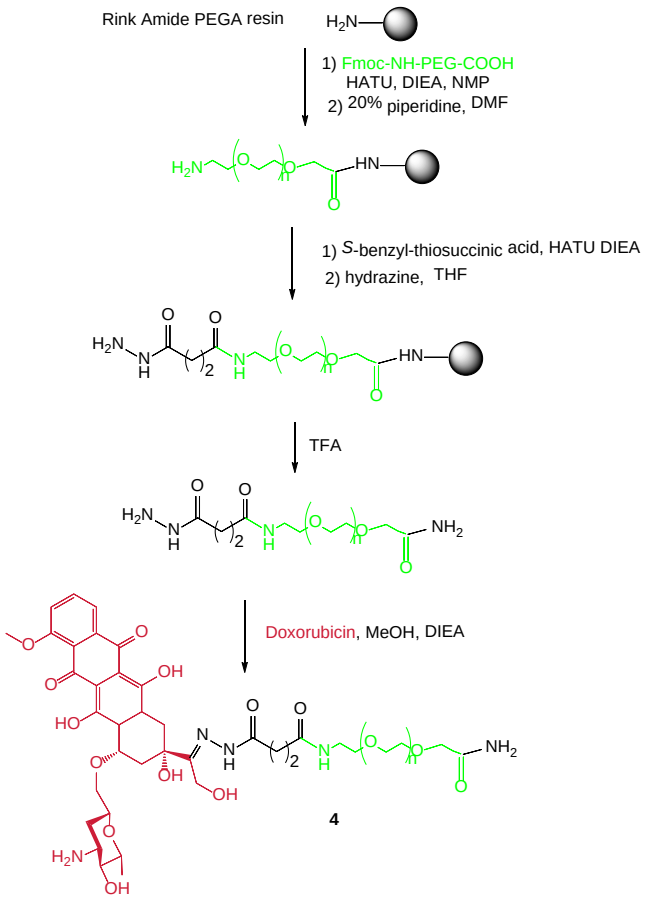

Fig. 2 Synthetic route to the polymeric prodrug candidate of doxorubicin, based on a PEG of average molar mass of 5,000 g/mol.

***In vitro* cytotoxicity of the polymeric prodrug candidates**

Cell viability studies based on a tetrazolium dye reduction assay, using different ovarian cell lines, were performed to determine if the therapeutic index of (D)-P18 could be increased by reversible pegylation (Table 1). Peptide **1** was used as a positive control and the pegylated peptide **5** as a negative control. Doxorubicin was also tested in its free form. One non-malignant Hs832 and two cancer, the doxorubicin-sensitive A2780P and doxorubicin-resistant SK-OV3, cell lines were included in these studies.

Results obtained with (D)-P18 reveal that this AMP is active in the low micromolar range against the doxorubicin-sensitive A2780P cancer cell line but also against the non-malignant Hs832 cells. This latter activity is unexpected and inconsistent with the selectivity reported for (L)-P18 and for this general class of peptides.[2] On the other hand, the SK-OV3 cell line showed reduced sensitivity to the AMP. Results in Table 1 also show that the 2 agents, peptide and doxorubicin, have lower cytotoxic activities as polymer conjugates. This is particularly significant for the anthracycline candidate, with nearly a 300-fold reduction in activity between doxorubicin and its pegylated version. This difference is higher than those (5- to 200-fold) reported for other pegylated doxorubicin derivatives based on acyl hydrazone linkers.[17] Whereas the PEG size impacts on the activity of the doxorubicin conjugate, it is unlikely to be the reason for the higher activity differential between free and conjugated doxorubicin, as compound **4** is based on a PEG 5 to 14 times shorter than those of the reported conjugates.[17] The difference could be due to the lower electron withdrawing strength of the succinamidyl group in conjugate **4**, compared to those of the benzoyl and phenylacetyl groups in reported hydrazones.[17,18]

Since (D)-P18 displays a reduced level of anticancer activity against the SK-OV3 cells, the effect of the reversible pegylation on its therapeutic index are primarily examined here with the A2780P cell line. The activity differentials reported in Table 1 for (D)-P18 and its polymer conjugates indicate that an increase in its therapeutic index, defined as the ratio of the IC_{50s} for the Hs832 and A2780P cells, may be achieved when a PEG of relatively low molar mass is linked to the peptide through a glycyl-phenylalanyl-leucyl-glycyl sequence (compound **2**). An increase in the polymer size is detrimental to the anticancer activity and therefore therapeutic index of the corresponding prodrug candidate. This indicates that access of cathepsin B to its substrate in the polymeric prodrugs might be hindered by the PEG. The lowest activities are observed when a cathepsin B-insensitive linker is introduced between the peptide and the PEG, despite the lower length of the polymer in the negative control **5**. As PEG allows the reduction of the AMP toxicity towards non-malignant Hs832 cells to a greater degree than towards malignant A2780P cells, it could indeed be a promoiety for the generation of polymeric prodrugs of AMPs.

On the other hand, the PEG might hinder the access of cathepsin B to the tetrapeptide motif inserted between the polymer and (D)-P18. This might place constraints on the PEG

Table 1 Ovarian cell viability studies. IC_{50} values in [μM], determined using the MTT assay at 24 h for Hs832 and SK-OV3 and MTS assay at 72 hours for A2780P; experiments were carried out in triplicate, in three independent experiments. Therapeutic Indices for A2780P (IC_{50} Hs832 / IC_{50} A2780P) and SK-OV3 (IC_{50} Hs832 / IC_{50} SK-OV3) cells are indicated below the IC_{50} values. ND: not determined.

Candidate	Hs832 cells	A2780P cells	SK-OV3 cells
(D)-P18 (**1**)	5.8 ±1.0	2.7 ±3.2	17.4 ±10
Therapeutic Index		2.1	0.3
MeO-$PEG_{2,000}$-G-F-L-G-(D)-P18-NH_2 (**2**)	19.0 ±5.3	6.8 ±0.7	39.4 ±4.2
Therapeutic Index		2.8	0.5
MeO-$PEG_{5,000}$-G-F-L-G-(D)-P18-NH_2 (**3**)	42.3 ±2.1	54.5 ±0.4	65.3 ±15.9
Therapeutic Index		0.8	0.6
Doxorubicin-$PEG_{5,000}$-NH_2 (**4**)	> 100	29 ±4.6	>100
MeO-$PEG_{2,000}$-G-G-(D)-P18-NH_2 (**5**)	51.8 ±9.8	87 ±2.3	77.9
Therapeutic Index		0.6	0.7
Doxorubicin	>100	0.098 ±0.015	68.3 ±25
(**2**) + (**4**)	ND	2.9 ±0.7	ND
G-(D)-P18 (**6**)	ND	4.6 ±0.6	ND

size which can be used and limit it below the values required to maximise the peptide's therapeutic index, enhance its blood circulation time and sustain the EPR effect. Although this linker was successfully used in polymeric prodrugs based on a *N*-(2-hydroxypropyl)methacrylamide copolymer of molecular weight of approximately 30,000,[8] the rapid and/or quantitative release of the active AMP from its prodrug doesn't seem to occur here even with a $PEG_{2,000}$. This could be due to a lack of expression of cathepsin B in the cancer cell lines selected and/or to inadequate kinetics of activation. Western blot analysis demonstrated the presence of the cathepsin B proenzyme in both the A2780P and SK-OV3 cells (Fig. S14), undermining therefore the first assumption. Higher expression levels found in the former cell line were consistent with the higher activity of compound **2** against A2780P cells. To assess the second hypothesis, an activation study of the prodrug candidate **2** and the negative control **5** was performed with purified enzyme. It showed that proteolysis only occurred with the former pegylated peptide, confirming that compound **2**, but not **5**, is a cathepsin B-dependent bioreversible derivative of P18. However, it also revealed that activation kinetics were slow (approximately 43% conversion after 24 hours incubation at 37°C and pH of 5). This study also showed that the peptide released from **2** retained a residual glycine from the tetrapeptide linker at its N-terminus (Fig. S17). This peptide, G-(D)-P18, (**6**) was synthesized and tested against the A2780P cell line, displaying an IC_{50} of 4.6 μM, comparable to the parent peptide **1**. In addition, no statistically significant difference was observed in the IC_{50s} of **1**, **6** and also **2**, while the difference in the activities of the prodrug candidates and the negative control against A2780P cells reached statistical significance (Fig. S18).

Finally, the pegylated prodrug candidates of doxorubicin and the AMP, **4** and **2**, respectively, were combined against the doxorubicin-sensitive ovarian cancer cell line (A2780P). The results, expressed as a fractional inhibitory concentration (FIC) index of 0.65, indicated that the combination of these polymeric prodrugs is additive.

AMPs are new biologics which have the potential to address some of the issues of drug resistance in the anti-cancer and anti-infective therapeutic fields. However, they have limitations, such as unknown systemic toxicity and low anticancer activity as single agents. Combinations of polymeric prodrugs of AMPs and of classical anticancer agents could provide some solutions to these clinical shortcomings. As described here, both agents could be preferentially delivered to solid tumour cells by the EPR mechanism and a lysosomotropic route. The synthetic feasibility of this polymeric prodrug approach was initially established by completing the assembly of a dual-release prodrug based on a bi-functional PEG and subsequently of 2 individual prodrugs based on mono-functional PEGs. The second option is economically more viable and does not limit the combination of the two agents to a 1:1 ratio. The co-delivery of these 2 individual polymeric prodrugs would rely essentially on a passive targeting mechanism (EPR effect), but the possible entanglement of the 2 polymer chains could also contribute to their combination. Activation of the peptidic and anthracycline prodrugs should ultimately occur in the same organelle, but rely on independent mechanisms, enzymatic and chemical, respectively.[8,15] Acid-sensitive prodrugs of the AMP could also be generated by using a glyoxylyl-peptide.[19] The non-enzymatic activation of these prodrugs could circumvent some limitations of proteolytically activated prodrugs associated with variable levels of expression of cathepsin B in cancer cells, but could have a lower site-specificity.[20] On the other hand, cathepsin B-dependent prodrugs of the peptide and of doxorubicin[21] could be combined, but their activation would compete for the same enzyme.

It is generally accepted that AMPs have selective cytotoxicities against cancer cells, although a therapeutic index of only 2.1 is observed here for (D)-P18 against Hs832 and A2780P cells. An

increase in the toxicity of P18 against non-malignant cells (fibroblasts) has however already been reported, when the peptide is assembled from D-amino acids and modified by a leucine at position 8, as in **1**. The dominant mechanism of action of this peptide was shown to be, in pancreatic carcinoma and cervical cancer cell lines, by induction of the mitochondria-associated pathway of apoptosis.[10] It is expected that disruption of the mitochondrial membrane of cancer cells is conserved with the polymeric prodrugs of (D)-P18. Indeed, it has been established with another α-helical amphipathic peptide, a magainin 2-derived sequence, that pegylation reduced its antimicrobial activity and abolished its cytotoxicity against CHO-K1 cells, but did not modify its basic mechanism of action, the plasma membrane permeabilization in this case.[22] Moreover, the active entity obtained by cathepsin B-activation of the prodrug candidate **2**, was shown to be the parent peptide (D)-P18 (**1**), only modified by a N-terminal glycine residue (peptide **6**). For the SK-OV3 cells, the lower sensitivity of (D)-P18 against them offsets the effects of the pegylation on its therapeutic index, but it is interesting to note that the resistance mechanism of these cells seems to affect AMPs as well.

Further improvement of the AMP's therapeutic index could potentially be achieved by optimisation of the prodrug activation. The latter depends on the kinetics of hydrolysis of the leucyl-glycyl amide bond and the expression levels of cathepsin B, and possibly on the presence of other proteases, including the lysosomal neprilysin which can hydrolyse a glycyl-phenylalanyl-leucyl-glycyl linker.[12] The low IC_{50} of the prodrug **2** against the Hs832 cell line and the statistically significant difference in the activities of compounds **2** and **5** (Fig. S18) seems to indicate that cleavage of the linker occurs to some extend with these cells. Unfortunately, the slow growth rate of this benign cell line precluded the analysis of cathepsin B expression. While this cysteine protease is generally highly up-regulated in cancer cells, it is noteworthy that it is expressed constitutively and can be active both intra- and extra-cellularly.[12] With the A2780P and SK-OV3 cells, the hydrolysis kinetics of the prodrugs can be affected by the presence of the synthetic polymer, as well as the heterochirality of the residues in the linker and the AMP sequence, separated by one achiral residue only. One of the main benefits resulting from the pegylation of biopharmaceuticals is their protection against proteolytic degradation.[7] Here, protection of the active peptide component against protease-mediated hydrolysis is not a concern as its sequence is assembled from D-amino acids. Therefore, for protease-dependent pegylated prodrugs of AMPs, an optimization of the hydrodynamic radius increase is essentially required, to reduce the peptide's toxicity, enhance its blood circulation time and sustain the EPR effect, while still allowing the proteolytic activation. Whereas this optimization appears to rely on antagonistic requirements, the development of other pegylated antimicrobial peptides released from their polymeric carrier by proteolysis substantiates its feasibility.[23] Furthermore, tolerance of cathepsin B, or other activating enzyme candidates such as matrix metalloproteinase 2, to large PEGs could be enhanced by the inclusion of additional residues between the tetrapeptide linker and the P18 sequence.[24] Finally, the kinetics of AMP prodrug activation, and the generality of this approach, could be enhanced by combination with a pegylated-cathepsin B, according to the PDEPT strategy, although this approach would require the co-administration of 3 polymer conjugates, for the AMP prodrug, its activating enzyme and the anthracycline prodrug.[25]

Conclusions

Overall, the relative activities of the polymeric bioreversible derivatives of P18 and of the peptide itself are consistent with a prodrug activity and indicate that an additive combination with an anthracycline antineoplastic agent can be achieved. Although these results are preliminary, the trend observed in the cell viability data is coherent with results expected for prodrugs and options for their optimisation exist. Combination with a classical antineoplastic agent can also enhance the inherent anticancer activity of the AMP and compensate the increase in the quantity of polymeric prodrug required to achieve equimolar doses with the free peptide. The additive effect in this study may have been limited by the low activity of the pegylated doxorubicin component. Results could be further improved through the use of acyl hydrazones derivatives based on aromatic carboxyls[17] or by combination with a pegylated liposomal formulation of doxorubicin.[26] Combination therapies are more complex to develop than single drug regimens, but have been the mainstays in the treatment of most types of cancer at advanced stages in the past half century. Tumour cells are also less likely to develop resistance against two agents with different mechanisms of action than against single agents.[27] Combining an agent with activity against non- or slowly-growing tumour cells (AMP) with an agent targeting rapidly dividing cancer cells (anthracycline), could circumvent mechanisms of tumour resistance to single agents and prevent tumour recurrence developing from quiescent/dormant cancer cells.

The initial results obtained with bioreversible pegylated derivatives of an AMP and an anthracycline indicate that a polymeric prodrug combination approach could realise the therapeutic potential of AMPs. The development of the polymeric prodrug candidates generated by this approach will primarily require the selection of the optimal activating enzyme and the optimisation of the linkers in these nanomedicines.

Experimental

Materials and methods

Materials. Fmoc-protected amino acids, Rink Amide MBHA and Rink Amide PEGA resins were sourced from Novabiochem (Merck Millipore). HATU was obtained from ChemPep Inc. NMP was purchased from BioSciences. All other reagents and solvents were supplied by Sigma-Aldrich. Human ovarian adenocarcinoma (SK-OV-3) cells and benign ovarian cyst (Hs832) cells were purchased from

LGC Standards. Ovarian carcinoma (A2780p) cells were a gift of Professor Celine Marmion (Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland). Cell culture media were obtained from BioSera. 0.2 μm Filters were purchased from Millipore.

Analytical and purification techniques. NMR spectra were recorded on a BRUCKER Avance 400 spectrometer. Samples were prepared in $CDCl_3$ (referenced to 7.26 ppm for ^{1}H and 77.0 ppm for ^{13}C). Chromatographic analysis and purification by RP-HPLC were performed on a Varian Galaxy HPLC and PerSeptive Biosystems Biocad Sprint Perfusion Chromatography HPLC, respectively, using Phenomenex Jupiter 5μm C5 300Å or Gemini 5μm C18 110Å columns (4.6 mmDx250mmL analytical, 10 mmDx250mmL semi-preparative). The mobile phase consisted of buffer A: 0.1% TFA in water and buffer B: 0.1% TFA in acetonitrile, with a linear gradient of 5 to 65% B in 30 min at a flow rate of 1 ml/min (analysis) or 4 ml/min (semi-preparative). SEC-HPLC was performed on a Varian Galaxy HPLC, using a Phenomenex BioSEP S2000 column (6.5mmDx300mmL), with an isocratic gradient at a flow rate of 1ml/min for 45 minutes, using a PBS buffer 0.05 M, pH 2-8 with 50% acetonitrile. UV single or dual wave wavelength detection were performed at 214 nm or 214 and 280 nm for the Biocad Sprint, while the Varian Galaxy was equipped with a Diode Array Detector (PDA) operating from 190 nm to 950 nm. Purities were ascertained from the percent area of the synthetic product relative to the total area of all UV absorbing components. Mass spectrometry (MS) was performed by Matrix Assisted Laser Desorption Ionization-Time of Flight MS (MALDI-TOF MS) on a Reflex Bruker Spectrometer. Two matrices were used, 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxy-cinnamic acid, dissolved in 50% acetonitrile and 50% water at a concentration of 10 mg/ml or 15 mg/ml, respectively. One μl of each 1:1 solution of matrix peptide were applied to the MALDI plate. Values of n reported in the MS results correspond to the number of PEG units for polydisperse polymers. Electrospray ionization mass spectra were recorded on an Advion Expression Compact Mass Spectrometer or a Waters Micro mass LCT, at 80 eV.

Peptide synthesis. Peptide sequences were assembled from their C- to N-termini by standard Solid Phase Peptide Synthesis according to the Fmoc-*t*Bu strategy with HATU/DIEA coupling chemistry, in NMP solvent. Single coupling cycles, using a total 10-fold excess of Fmoc-amino acid derivatives to resin-bound peptide were used, except for amino acids following a proline residue (leu^{8} in the P18 sequence) for which a double coupling cycle was used. The side-chain protecting groups were Boc for lysine and tryptophan and Trt for histidine. Assembly of the amino acid sequences starting from a Rink Amide MBHA resin or a Rink Amide PEGA resin, for the P18 sequence or its pegylated derivatives, respectively, were carried out on a 100 μmol scale, on an automated peptide synthesizer (433, Applied Biosystems). Peptides were deprotected and cleaved from the resin, precipitated with diethyl ether from this cleavage cocktail and subsequently washed three times with diethyl ether, air dried, dissolved in distilled water and lyophilized. Manual peptide synthesis was performed in a fritted reaction vessel placed on an orbital shaker. The synthetic scale, depending on the resin type and its swelling properties was 0.1-0.25 mmol. The resin was swelled beforehand, in DCM for 20 min and DMF for 10 min. Deprotection of Fmoc-protected amino acids was performed with a solution of 20% piperidine in DMF. Approximately 10 ml of the deprotection solution and a reaction time of 10 min were used. The deprotection reaction was repeated twice for 5 min each. The resin was then washed 3 x 5 min with DMF and 1 x 5 min with DCM. Coupling steps with the Fmoc-protected reagents (amino acids/PEGs) were performed by dissolving the coupling reagents and amino acids/PEGs in 10 ml of DMF/NMP and using reaction times of 1-3 hours. The numbers of equivalents of reagents used are based on the initial substitution of the resin. Deprotection and coupling cycles using amino acids were monitored by the qualitative Kaiser test.

Synthetic procedures

Peptide 1. (D)P18 was synthesized from a resin with a substitution of 0.7mmol/g. It was cleaved from the resin using a mixture of 85% trifluoroacetic acid (TFA), 5% triisopropylsilane (TIS), 2.5% thioanisole, 2.5% 1,2-ethanedithiol (EDT) and 5% water. The cleavage reaction was performed at RT for 2 h. It was purified and characterized as described above. Analytical HPLC (C18) showed a homogenous peak (98.12 %) with retention time of 22.85 min. MALDI-TOF MS (*m/z*) (α-cyano-4-hydroxy-cinnamic acid): 2300.9330 (M^{+}).

Pegylated peptide 2. Pegylation of the P18 peptide, elongated with the GFLG linker, was performed by manual solid phase synthesis using a Rink amide PEGA resin. Polydisperse MeO-$PEG_{(2000)}$-COOH (0.2 mmol, 400 mg) was added to HATU (72 mg, 0.19 mmol) and DIEA (69.6 μL, 0.4 mmol) in DMF. The solution was transferred to the resin-bound peptide in a solid phase reaction vessel, which was agitated on a shaker for 3 hours. The reaction was monitored by the Kaiser test to ensure complete coupling. The pegylated peptide was cleaved from the resin using a cleavage cocktail containing 5100 μl of TFA, 150 μl of EDT, 150 μl of thioanisole, 300 μl of water and 300 μl of TIS, and a reaction time of 2.5 hours. It was then precipitated with diethyl ether as a white solid, which was collected by centrifugation, dissolved in water, lyophilized and purified by SEC using Sephadex G-25 with de-ionized water as the mobile phase. TLC staining by iodine crystals was used to identify fractions containing the pegylated peptide. It was analysed by RP-HPLC on a C5 column, showing a homogenous peak (97.02 %) with retention time of 28.43 min, and by MALDI-TOF MS (*m/z*) (α-cyano-4-hydroxycinnamic acid): (n=40) 4076.5, (n=42) 4164.5, (n=44) 4252.7, (n=45) 4297.7, (n=46) 4341.7, (n=47) 4385.7, (n=48) 4429.7, (n=50) 4571.8, (n=52) 4605.9, (n=53) 4649.9, (n=54) 4694.0, (n=56) 4782.0, (n=59) 4914.13 (n=60) 4959.2, (n=61) 5003.2, (n=62) 5047.2, (n=65) 5178.3, (n=67) 5267.4, (n=68) 5311.4 (n=70) 5399.5 (n=71) 5443.5.

Pegylated peptide 3. This candidate was synthesised as described above for **2** using polydisperse MeO-PEG$_{(5000)}$-COOH (0.2 mmol, 1 g). Analytical RP-HPLC (C5) showed a homogenous peak (97.50) with retention time of 29.27 min. MALDI-TOF MS (*m/z*) (α-cyano-4-hydroxycinnamic acid): (n=105) 6929.2, (n= 106), (n=107) 7018.0, (n=110) 7149.1, (n=111) 7199.9, (n=113) 7282.3, (n=116) 7414.1, (n=118) 7501.7, (n=120) 7590.1, (n=125) 7811.1, (n=128) 7898.3, (n=133) 8121.8, (n=135) 8207.0, (n=138) 8386.0, (n=139) 8428.2, (n=141) 8518.2, (n=142) 8561.2.

Pegylated doxorubicin 4. Pegylation of doxorubicin was carried by solid phase synthesis, using a Rink amide PEGA resin, on a 0.1 mmol synthetic scale, loaded with Fmoc-NH-PEG$_{(5000)}$-COOH, as described above for **3** (omitting the peptide and linker sequences). *S*-benzyl thiosuccinic acid (112.5 mg, 0.5 mmol) was coupled to the resin-bound PEG, using a solution of HOBt (74.9 mg, 0.49 mmol), HBTU (185.7 mg, 0.49 mmol) and DIEA (174 µl, 1 mmol) in DMF and a reaction time of 3 hours. After a wash step, hydrazine hydrate (2 ml, 7.4 mmol) and 1,4-dioxane (2 ml) were added to the solid phase reaction vessel containing the resin pre-swelled in DCM. The reaction vessel was agitated on a shaker for 2 hours. The resin was then washed (3 x 5 min each) with DCM and the reaction was repeated. The hydrazido-succinamidyl-PEG-amide was released from the resin by treatment with a cleavage solution consisting of 5700 µl of TFA and 300 µl of DCM for 2.5 hours. After evaporation of the cleavage solution under a stream of nitrogen, the product was dissolved in water and lyophilized. Conjugation of doxorubicin was carried out by reacting the latter product (221 mg, 0.049 mmol) with doxorubicin hydrochloride (53 mg, 0.092 mmol) in presence of a few drops of acetic acid in anhydrous methanol, for 24 hours, under argon and in the dark. The methanol was then evaporated and the residue dried on a high vacuum line. The product was then purified by SEC using Sephadex LH-20 and methanol as the mobile phase. The separation was monitored by SEC-HPLC using a PBS buffer pH 6.95 at 0.5 ml/min, at wavelengths of 214 and 480 nm for the peptide's amide bonds and doxorubicin, respectively. Successful conjugation of doxorubicin was confirmed by UV-VIS spectrophotometry by the shift in the wavelength of its maximum absorbance. SEC- HPLC showed a peak (96.46%) with retention time of 13.85 min.

Control pegylated peptide 5. Pegylation of the P18 peptide extended with a diglycine motif was performed by manual solid phase synthesis using MeO-PEG$_{(2000)}$-COOH as described for **2**. Analytical RP-HPLC (C5) showed a homogenous peak (97.92%) with retention time of 28.12 min. MALDI-TOF MS (*m/z*) (α-cyano-4-hydroxycinnamic acid): (n=36) 4024.5, (n=37) 4.68.6, (n=38) 4112.5, (n=39) 4156.6, (n=41) 4289.6, (n=43) 4333.7, (n=44) 4377.7 (n=45) 4421.7 (n=46) 4465.8, (n=48) 4553.8, (n=49) 4597.9 (n=50) 4641.9 (n=51) 4685.9 (n=54) 4818.0 (n=55) 4862.2 (n=56) 4906.1.

G-(D)-P18 peptide 6. This peptide was assembled from a Rink Amide MBHA resin with D-amino acids by automated peptide synthesis and isolated as described for **1**. Analytical HPLC (C18) showed a homogenous peak (97.10) with retention time of 22.8 min. ESI MS (*m/z*): 786.2 ($[M +3 H]^{3+}$).

Biological procedures

Activation study with purified cathepsin-B. The enzyme was dissolved at a concentration of 10 µg/ml in 400 µl of PBS buffer with 0.05 M EDTA at pH 5. Pegylated peptides **2** and **5** were dissolved in water at 1mg/ml. 100 µl of each peptide solution was added to 100 µl of the enzyme solution in a 96-well plate and incubated for 24 hours at 37° C in a 5% CO_2 humidified atmosphere. Results were determined by analytical HPLC using an analytical C18 column for **2** or a Biosep-S2000 column for **5**. Mass spectrometry analysis was performed on an ESI low resolution mass spectrometer.

Quantification of Cathepsin B expression in A2780P and SK-OV-3 cell lines by western blotting. RIPA lysis and extraction buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, at a quantity of 1 ml per T175cm2 flask) was used to extract protein from cultured cells with the addition of a Protease Inhibitor Cocktail (Sigma-Aldrich) before use in order to prevent proteolysis. The lysate was centrifuged for 15 minutes at 14000 rpm to pellet the cell debris. BCA assay (Sigma-Aldrich) was carried out to determine the concentration of the isolated protein. 40 micrograms of cell lysate was separated using 12% SDS PAGE gel. The blot was then transferred to a PVDF membrane and non-specific binding sites were blocked by incubation for two hours at room temperature with blocking buffer (5% non-fat dry milk in TBS containing 0.05% Tween-20). The membrane was probed at 4°C overnight with primary cathepsin B antibody (Santa Cruz) diluted to 1:100 in blocking buffer. The membrane was then washed with TBS+0.1% tween 20. The secondary antibody (goat anti-mouse, Santa Cruz) was then diluted to 1:1000 in blocking buffer and added to the membrane. The membrane was incubated for 2 hours at room temperature and the washing steps were repeated. The protein was detected using ECL reagent, then visualised in a dark room. Beta-Actin was used to confirm equal protein loading.

Cell culture and viability assays. The A2780p cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) FBS. The SK-OV-3 and the Hs832 cells lines were both grown in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM glutamine, supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin and 10% (v/v) heat inactivated Foetal Bovine Serum and (FBS). All cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cells were routinely passaged by trypsinization in 0.5% (w/v) trypsin / 0.2% (w/v) EDTA mixture in phosphate-buffered saline (PBS). To inactivate trypsin, an equal volume of complete medium was added. Detached cells were collected by centrifugation (3 min) at 1000 rpm. Harvested cells were re-suspended in an appropriate volume depending on the size of the cell pellet. An aliquot of the single cell suspension was mixed with an equal volume of 0.4 % (w/v) trypan blue and counted using a haemocytometer (Improved Neubauer model) under a phase contrast microscope. Viable cells were counted in four quadrants; the mean value was multiplied by two (dilution factor) and then by factor of 104, which accounts for the volume of the haemocytometer. Using this method, a value

representative of the number of cells per ml in the original suspension was obtained. Cell viability was assessed by the MTT/MTS colorimetric assay. Relative numbers of viable cells were determined spectrophotometrically following solubilisation of the formazan crystals in DMSO. For the MTT cell viability assays with the Hs832 and SK-OV-3 cells, one day prior to treatment with test compounds, cells were seeded at a density of 40,000 cells per well in a volume of 150 µl of media per well. The following day, media was aspirated from cells and replaced with media containing test compounds. Serial dilutions were from 100 µM to 0.78 µM for **1** – **5**, against both cell lines and for doxorubicin against the SK-OV3 cells; from 1 µM to 0.19 nM for doxorubicin against Hs832 cells. Plates were then placed in an incubator for 24 hours. On the day of analysis, 50 µl of a 20 µM fresh sterile filtered solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml) was added to each well of treated plates using a multi-channel pipette. The MTT solution was prepared using sterile 0.1 M PBS pH 7.4 and filter sterilised using a 0.2 µm filter. Plates were then returned to the incubator for 4 hours. The media/MTT solution was aspirated from the well, with care taken not to dislodge the formazan crystals from the bottom of each well. The crystals were dissolved following addition of 200 µl DMSO to each well. Finally, absorbance of the resulting solution was measured at 550 nm for 1 s in a Varioscan microplate reader (Perkin-Elmer). The MTS assay is a similar, one-step, process. A2780P cells were seeded at a density of 5,000 cells per well. Serial dilutions of test compounds were from 100 µM to 0.78 µM for **1**, **2**, **5** and **6**; from 1 µM to 7.8 nM for doxorubicin. For the combination study, 75 µl of **2** at concentrations serially diluted from 200 µM to 1.56 µM and 75 µl of **4** added at double the IC_{50} concentration of 59.6 µM, were used. After incubation for 72 hours, 30 µl of MTS was added to the wells, the plates were returned for incubation for 3 hours and the absorbance was read at 490 nm for 1 s. Absorbance values in treated plates were expressed as a percentage of untreated controls in order to obtain percentage viability values. Assays were repeated in triplicate. Statistical analyses of the data were carried out using GraphPad Prism software and the two-tailed unpaired *t* test.

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References

1 M. Zasloff, *Nature,* 2002, **415**, 389.
2 J. S. Mader and D. W. Hoskin, *Expert Opin. Investig. Drugs*, 2006, **15**, 933.
3 R. E. W. Hancock and H.-G. Sahl, *Nat. Biotechnol.*, 2006, **24**, 1551.
4 N. Papo and Y. Shai, *Cell. Mol. Life Sci*., 2005, **62**, 784.
5 (*a*) R. E. W. Hancock, *Lancet Infect. Dis.*, 2001, **1**, 156; (*b*) V. J. Stella, *Expert Opin. Ther. Patents*, 2004, **14**, 277; (*c*) A. P. Jallouk, R. U. Palekar, J. N. Marsh, H. Pan, C. T. Pham, P. H. Schlesinger and S. A. Wickline, *Bioconjug. Chem*., 2015, **26**, 1640; (*d*) J. H. Jang, M Y. Kim, J. W. Lee, S. C. Kim and J. H. Cho, *Peptides*, 2011, **32**, 895.
6 J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Järvinen and J. Savolainen, *Nat. Rev. Drug Discov.*, 2008, **7**, 255.
7 J. M. Harris and R. B. Chess, *Nat. Rev. Drug Discov*., 2003, **2**, 214.
8 R. Duncan, *Nat. Rev. Cancer*, 2006, **6**, 688.
9 S. Y. Shin, J. H. Kang and K.-S. Hahm, *J. Pept. Res.*, 1999, **53**, 82.
10 S. O'Connor, E. Szwej, J. Nikodinovic-Runic, A. O'Connor, A. T. Byrne, M. Devocelle, N. O'Donovan, W. M. Gallagher, R. Babu, S. T. Kenny, M. Zinn, Q. R. Zulian and K. E. O'Connor, *Biomaterials,* 2013, **34**, 2710.
11 G. M. Dubowchik, R. A. Firestone, L. Padilla, D. Willner, S. J. Hofstead, K. Mosure, J. O. Knipe, S. J. Lasch and P. A. Trail, *Bioconjug. Chem.*, 2002, **13,** 855.
12 M. M. Mohamed and B. F. Sloane, *Nat. Rev. Cancer*, 2006, **6,** 764.
13 D. A. Stetsenko and M. J. Gait, *J. Org. Chem.*, 2000, **65**, 4900.
14 T. S. Zatsepin, D. A. Stetsenko, A. A. Arzumanov, E. A. Romanova, M. J. Gait and T. S. Oretskaya, *Bioconjug. Chem*. 2002, **13,** 822.
15 A. M. Wu and P. D. Senter, *Nat. Biotechnol*., 2005, **23**, 1137.
16 A. Nori and J. Kopeček, *Adv. Drug Deliv. Rev.* 2005, **57,** 609.
17 P. C. A. Rodrigues, U. Beyer, P. Schumacher, T. Roth, H. H. Fiebig, C. Unger, L. Messori, P. Orioli, D. H. Paper, R. Mülhaupt and F. Kratz, *Bioorg. Med. Chem.*, 1999, **7,** 2517.
18 J. Kalia and R. T. Raines, *Angew. Chem. Int. Ed. Engl.* 2008, **120**, 7633.
19 A. Dirksen, T. M. Hackeng and P. E. Dawson. *Angew. Chem. Int. Ed.* 2006, **45**, 7581.
20 P. Ettmayer, G. L. Amidon, B. Clement and B. Testa *J. Med. Chem.*, 2004, **47**, 2393.
21 M. J. Vicent, F. Greco, R. I. Nicholson, A. Paul, P. C. Griffiths and Ruth Duncan. *Angew. Chem. Int. Ed.* 2005, **44**, 4061.
22 Y. Imura, M. Nishida and K. Matsuzaki, *Biochim. Biophys. Acta*, 2007, **1768** , 2578.
23 F. I. Nollman, T. Goldbach, N. Berthold and R. Hoffmann, *Angew. Chem. Int. Ed. Engl.*, 2013, **52**, 7597.
24 T. Jiang, E. S. Olson, Q. T. Nguyen, M. Roy, P. A. Jennings and R. Y. Tsien, *PNAS*, 2004, **101**, 17867.
25 R. Satchi, T. A. Connors and R. Duncan, *Br. J. Cancer*, 2001, **85**, 1070.
26 T. A. Lawrie, R. Rabbie, C. Thoma and J. Morrison, *Cochrane Database Syst Rev.* 2013, CD010482. DOI: 10.1002/14651858.CD010482.pub2.
27 J. E. Dancey and H. X. Chen, *Nat. Rev. Drug Discov.* 2006, **5**, 649.

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