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Synthesis and biological activity of conjugates between paclitaxel and the cell delivery vector penetratin

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Abstract—Synthesis of paclitaxel–penetratin (pAntp) constructs, in which the 2′- or 7-position of paclitaxel was used as the attachment site for linker connecting the drug and peptide moieties, is described. Paclitaxel–2′-pAntp[43–58]-NH_2 **3b** and paclitaxel–2′-pAntp[52–58]-NH_2 **3c** showed excellent antitumour activity against human lung and breast cancer cell lines. These conjugates were highly soluble and stable with a half-life of >8 h under cell culture conditions. The drug–peptide conjugates may be therapeutically useful due to improved pharmaceutical properties.

The pAntp peptide, known as Penetratin®, is a novel cell-permeable peptide derived from the third helix of the *Drosophila* antennapedia homeoprotein.[1] It has been used as a nonviral, nontoxic and highly efficient vector for delivering bioactive substances, that by themselves are membrane-impermeable, to the cytoplasm or nucleus of cells.[2–6] It has been shown, for example, that doxorubicin, when coupled covalently to penetratin, was capable of crossing the blood–brain barrier in mice, thus significantly increasing drug uptake in comparison with free doxorubicin.[6,7] It has also been demonstrated that since doxorubicin–peptide conjugates bypassed P-glycoprotein, increased cellular drug uptake could be achieved, suggesting that vectorised drug conjugates may be capable of overcoming multi-drug resistance.[8]

The SARs of penetratin peptides in terms of minimum active peptide length and relative importance of amino acid residues required for membrane translocation have been studied thoroughly.[9] Cell penetration assays using human cell cultures with a series of truncated peptides revealed that the C-terminal segment ^{52}RRMKWKK^{58}-NH_2 (pAntp[52–58]-NH_2) of the parent 16mer sequence was necessary and sufficient for effective cell membrane translocation. In connection with a research programme directed at the assessment of the efficacy of penetratin derivatives as vectors for the delivery of poorly bioavailable therapeutic agents, particularly those which are to be used for cancer therapy, we designed and synthesised a series of penetratin-drug constructs,[10,11] in the hope that this system may improve the pharmaceutical properties of the drugs by, for example, improving solubility and bioavailability, or by minimising toxicity and overcoming drug resistance.

Paclitaxel **1**, a diterpene natural product,[12] has been shown to be highly cytotoxic to cancer cells and is a potent anticancer drug used in the clinic.[13,14] Paclitaxel acts by promoting the assembly of stable microtubules from tubulin and it inhibits the disassembly process, thus interfering with the G2- and M-phases of the cell cycle.[15,16] Therapeutic use of paclitaxel has, however, several drawbacks. These problems are mainly associated with extremely low water solubility, poor bioavailability and emergence of drug resistance in patients treated with paclitaxel. FTo date a great deal of research has been carried out to modify the chemical structure of paclitaxel in order to improve its physicochemical properties.[17–19] Among the most notable paclitaxel derivatives synthesised and characterised so far are prodrugs in which the 2′- and 7-hydroxyl groups of the molecule are engaged in a functional group that collapses, upon in vivo activation, to release paclitaxel.[20–22]

Keywords: Paclitaxel; Penetratin; Cell delivery vector; Drug–peptide conjugate; Antitumour activity.
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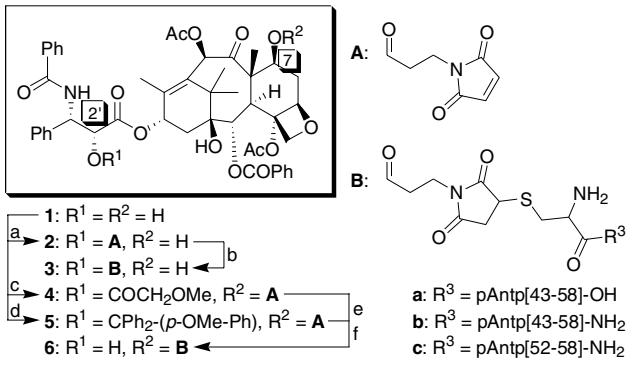
As a part of our vectorised drug conjugation programme[10,11] we synthesised a number of paclitaxel–peptide penetratin (paclitaxel–pAntp) constructs. Three forms of penetratin peptides, ^{43}RQIKIWFQNRRMKWKK^{58}-OH(pAntp[43–58]-OH), ^{43}RQIKIWFQNRRMKWKK^{58}-NH_2 (pAntp[43–58]-NH_2) and ^{52}RRMKWKK^{58}-NH_2 (pAntp[52–58]-NH_2), were used as delivery vectors.[9] Paclitaxel was attached through the 2′- or 7-hydroxyl positions to the end of the peptides via a succinimidopropionyl-sulfide linker, using chemo-selective maleimide-thiol chemistry. The synthesis and biological activities of these compounds are summarised herein.

Paclitaxel–pAntp constructs were prepared as outlined in Scheme 1. Selective acylation of the sterically less hindered secondary 2′-hydroxyl of paclitaxel with 3-maleimidopropionic anhydride in pyridine at room temperature gave paclitaxel-2′-maleimidopropionate **2** in good yield. Cysteine was introduced as an N-terminal residue of the pAntp peptides in order to facilitate unambiguous conjugation. Reaction of paclitaxel-2′-maleimidopropionate with Cys-pAntp peptides proceeded smoothly in the presence of base to afford 3.

In order to prepare paclitaxel-7-maleimidopropionate, the 2′-hydroxyl was masked as a methoxyacetate ester.[23] A solution of paclitaxel and DIEA in $CH_2Cl_2$was treated with methoxyacetic acid *N*-hydroxysuccinimidyl ester to afford 2′-methoxyacetyl paclitaxel, which was acylated at the 7-hydroxyl with 3-maleimidopropionic anhydride in the presence of a catalytic amount of 4-*N*,*N*-dimethylaminopyridine (DMAP) to provide **4** (Scheme 1). This was followed by coupling to Cys-pAntp, then treatment with ethanolamine, to give the final conjugates **6**. *p*-Methoxytrityl as an alternative acid labile protecting group was also introduced at the 2′-position of paclitaxel in quantitative yield. Elimination of this group, following acylation of the 7-OH to give **5**, was effected with dilute solution of chloroacetic acid/CH_2Cl_2, using anisole to scavenge the trityl cation liberated during the deprotection.



Scheme 1. Reagents: (a) 3-maleimidopropionic anhydride, pyridine, CH_2Cl_2; (b) H-Cys-R^3, Et_3N, DMF; (c) i—*N*-hydroxysuccinimidomethoxyacetate, DIEA, CH_2Cl_2; ii—3-maleimidopropionic anhydride, DMAP, CH_2Cl_2; (d) i—*p*-methoxytrityl chloride, pyridine, CH_2CH_2; ii—3-maleimidopropionic anhydride, DMAP, CH_2Cl_2; (e) i—H-Cys-R^3, Et_3N, DMF; ii—$H_2N(CH_2)_2OH$, MeOH; (f) i—chloroacetic acid, anisole, CH_2Cl_2; ii—H-Cys-R^3, Et_3N, DMF.

The pAntp peptides, paclitaxel–2′-pAntp **3a**–**3c** and paclitaxel–7-pAntp **6a**, were tested for their antitumour effects against human lung and breast carcinoma cell lines using a standard cellular proliferation MTT assay (Table 1).[24] The free peptides were inactive up to 20 μM concentration in this assay. The constructs **3b** and **3c** were shown to be as potent as the unconjugated parent compound paclitaxel. The peptide–acid conjugate **3a** was modestly cytotoxic in A549 cells with an IC_{50} value of 0.27 μM. Paclitaxel–7-Antp[43–58]-OH **6a** showed significantly reduced potency compared to the 2′-Antp[43–58]-OH **3a** counterpart. These results are in line with what has been found with other paclitaxel 2′- and 7-OH ester prodrugs, for example, the acetates[25] and succinates,[19] where the 7-OH-conjugated isomers were generally found to be less cytotoxic than the 2′-OH conjugates.[17,26,27] The inactivity of the 7-OH esters presumably emanates from the much higher hydrolytic stability of the sterically hindered 7-OH esters. Esterification of both the 2′- and 7-OH groups appears to reduce or abolish innate paclitaxel activity and only the 2′-linked conjugates release paclitaxel intracellularly at a productive rate. It should be noted, however, that some non-dissociated 7-OH paclitaxel ester conjugates have been reported to retain the ability to induce microtubule assembly in vitro.[25,26]

As expected, the aqueous solubility of the conjugates **3** and **6** was enhanced considerably (e.g., >10 mg/mL, >4.5 mM for **3c**) in comparison to unconjugated paclitaxel (<0.01 mg/mL). The stability of the peptides and constructs in tissue culture media was examined next. Solutions of the compounds in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum at concentrations varying from 1 to 40 μM were incubated at 37 °C. Samples were taken at various time points and were analysed. Degradation of pAntp[43–58]-OH was observed after incubating for periods as short as 10 min (Fig. 1). Mass spectral analysis of the degradation fraction, isolated by RP-HPLC, indicated that cleavage of the C-terminal lysines had occurred. Apparently removal of Lys^{58} is rapid, followed by further C-terminal proteolytic truncation. It was, however,

Table 1. Antiproliferative activity of pAnp peptides and conjugates **3** and **6** against human tumour cell lines

Compound	IC_{50}[a] (μM)	
	MCF7[b]	A549[c]
Antp(43-58)-OH	na	na
Antp(43-58)-NH_2	na	na
Antp(52-58)-NH_2	na	na
3a	nt	0.27
3b	<0.017	<0.015
3c	<0.015	<0.015
6a	nt	~10
Paclitaxel **1**	<0.015	<0.015

[a] Concentration of test compound producing 50% growth inhibition using an MTT assay. Cells were treated with test compounds for 72 h. The stated values are means of three independent experiments (na, not active; nt, not tested).

[b] Human breast cancer cell line.

[c] Human lung cancer cell line.

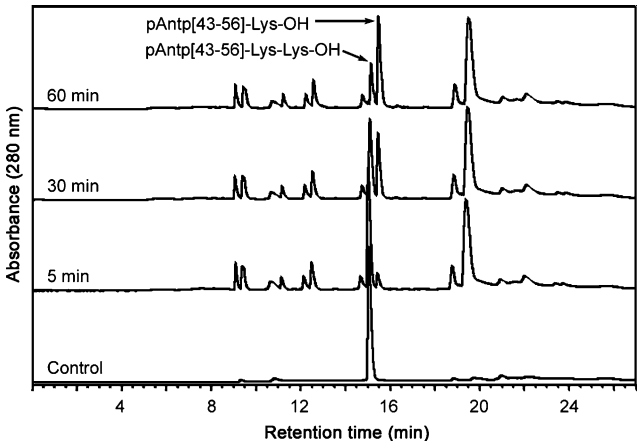


Figure 1. Degradation of pAntp[43-58]-OH in tissue culture medium. See text for conditions. Aliquots were withdrawn and analysed by RP-HPLC. The elution positions of the intact peptide and the C-terminal Lys-truncation product are indicated, as is the incubation time.

discovered that proteolysis of these lysine residues was blocked when the terminal lysine was present as the carboxamide rather than the carboxylate. Thus pAntp-NH_2 peptides were stable with half-lives of >12 h under the same conditions. These findings are significant, since presence of the terminal Lys[58] residue in penetratin variants is required absolutely for membrane translocation activity, as we have shown.[9] Penetratin is very commonly employed as a cellular delivery vector for a variety of cell biology applications and it is frequently unclear if the peptide acid or amide has been used.[28] Clearly use of the peptide acid severely limits the cell delivery potential of penetratin peptides.

We next looked at the stability of the bioactive paclitaxel–peptide conjugates in tissue culture medium under similar conditions. The stability of each conjugate correlated to that of its peptide counterpart. Thus, paclitaxel–2′-pAntp[43–58]-NH_2 **3b** and paclitaxel-2′-pAntp[52–58]-NH_2 **3c** were found to be stable with half-lives of >8 h by RP-HPLC and mass spectrometric assessment. Again this is in agreement with the reported hydrolytic stability at pH 7.4 of other paclitaxel prodrugs with similar ester functions.[19,27,29]

Paclitaxel–pAntp conjugates **3b** and **3c** were further subjected to time-exposure cellular experiments. A549 and MCF7 cells were treated with the constructs for 1 h and 3 days, respectively, and quantified by MTT assay (Table 2). When the tumour cells were exposed to **3b** and **3c** for 1 h, a period during which the conjugates were shown to be stable under the culture conditions, both compounds exhibited excellent cytotoxicity with IC_{50} values ranging from 0.043 to 0.618 μM. It was noted that **3c** showed activity comparable to that of paclitaxel in A549 cells, although less potent in MCF7 cells. These results show that cytotoxicity is not due to liberation of free paclitaxel extracellularly, but is mediated by membrane translocation of the intact conjugates, which is rapid, followed by intracellular release of bioactive paclitaxel.[9,28]

Examination of cancer cell line cultures showed that induction of cell death was very similar in paclitaxel- and conjugate-treated samples. In both cases, characteristic accumulation of cells in the G2/M-phase was observed and induction of apoptosis (by TUNEL assay) was extensive at 24 h post-treatment.

Furthermore, conjugates **3b** and **3c** were assessed for their ability to affect microtubule polymerisation (Fig. 2). Presumably the observed activity of the conjugates, which was only somewhat lower than that of paclitaxel itself, is a result of liberation of paclitaxel or bioactive metabolites from the conjugates under the physiological conditions of the assay.

In summary, the chemistry for the synthesis of paclitaxel–pAntp constructs and cellular biological properties of the conjugates were described.[32] Paclitaxel–2′-Antp[43–58]-NH_2 **3b** and paclitaxel–2′-Antp[52–58]-

Table 2. Effect of exposure time on antiproliferate activity of paclitaxel **1** and conjugates **3** (IC_{50}, μM)[a]

Compound	1-h treatment		72-h treatment	
	MCF-7[b]	A549[b]	MCF7[c]	A549[c]
3b	0.202	0.618	<0.017	<0.015
3c	0.325	0.043	<0.015	<0.015
Paclitaxel **1**	0.043	0.028	<0.015	<0.015

[a] Concentration of test compound producing 50% growth inhibition using an MTT assay. Cells were treated with test compounds for 72 h. The stated values are means of three independent experiments (na: not active; nt: not tested).
[b] 1 h treatment.
[c] 72 h treatment.

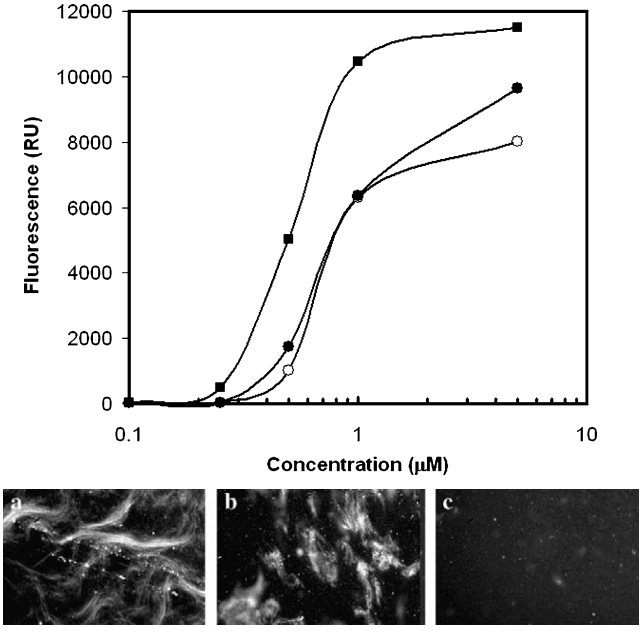


Figure 2. In vitro microtubule stabilization using a rhodamine–tubulin assay.[30,31] Dose–response curves are shown in the upper panel for paclitaxel **1** (■) and the pAntp conjugates **3b** (●) and **3c** (○). Low-level fluorescence micrographs are shown in the lower panel (a, 0.5 μM **1**; b, 0.5 μM **3c** and **c**, negative control).

NH_2 **3c** exhibited promising antitumour activity in vitro. Their pharmaceutical properties, antitumour activity in vivo, as well as the ability to overcome multi-drug resistance, are subjects for future investigations.

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32. Peptides were synthesised using an ABI 433A Peptide Synthesizer (Perkin-Elmer Applied Biosystems). RP-HPLC was conducted using Vydac 218TP54 and 218TP1022 columns for analytical and preparative purposes, respectively. Gradient elution (25 °C) was performed using increasing amounts of MeCN in water (containing a constant concentration of 0.1% TFA) over 20 min (analytical) or 40 min (preparative). Flow rates were 1 mL/min for analytical and 9 mL/min for preparative runs. The purity of all new compounds was checked by RP-HPLC and DE MALDI-TOF mass spectrometer (Dynamo, Thermo Bio-Analysis, Hemel Hempstead, England). NMR spectra were recorded on a Brucker DPX300 instrument.
Compound **2**: white solid (76% yield); ^{1}H NMR ($CDCl_3$): δ 1.13, 1.22, 1.68, 1.91 (s, each 3H, CH_3), 2.23, 2.47 (s, each 3H, CH_3), 2.35 (m, 2H), 2.78 (t, 4H, J = 5.40 Hz), 2.84 (m, 2H), 3.81 (m, 2H), 3.87 (m, 1H), 4.26 (m, 2H), 4.44 (dd, 1H, J = 10.87, 4.25 Hz), 4.98 (d, 1H, J = 7.69 Hz), 5.47 (d, 1H, J = 3.45 Hz), 5.68 (d, 1H, J = 7.09 Hz), 6.05 (dd, 1H, J = 9.28, 5.86 Hz), 6.28 (s, 1H), 6.18 (t, 1H, J = 8.77 Hz), 6.49 (s, 2H), 8.16–7.34 (m, 15H, Ph-H). ^{13}C NMR ($CDCl_3$): δ 10.01, 15.20, 21.22, 22.54, 23.09, 27.18, 32.90, 33.71, 35.90, 43.54, 45.96, 52.86, 58.89, 72.18, 72.53, 74.86, 75.51, 76.02, 79.52, 81.42, 84.89, 126.94, 127.91, 128.94, 128.94, 129.14, 129.45, 129.59, 130.65, 132.39, 133.11, 133.85, 134.09, 134.46, 137.17, 143.25, 167.45, 168.01. 168.10, 169.77, 170.29, 171.10, 171.69 and 204.24.
Compound **3a**: white solid (62% yield); Anal. RP-HPLC t_R = 17.4 min (0–60% MeCN, purity >97%); MS *m/z* 3355.9 $[M+H]^+$ ($C_{161}H_{229}N_{37}O_{38}S_2$requires 3354.9). Compound **3b**: white solid (53% yield); Anal. RP- HPLC t_R = 18.5 min (0–60% MeCN, purity >97%); MS *m/z* 3353.6 $[M+H]^+$ ($C_{161}H_{230}N_{38}O_{37}S_2$ requires 3353.9). Compound **3c**: white solid (53% yield); Anal. RP-HPLC t_R = 17.2 min (0–60% MeCN, purity >97%); MS *m/z* 2211.7 $[M+H]^+$ ($C_{106}H_{148}N_{22}O_{26}S_2$ requires 2210.6).
Compound **4**: white solid (76% yield); Anal. RP-HPLC t_R = 21.8 min (10–70% MeCN, purity >98%); ^{1}H NMR ($CDCl_3$): δ 1.15, 1.20, 1.79, 1.96 (s, each 3H, CH_3), 2.20, 2.45 (s, each 3H, CH_3), 2.34 (m, 2H), 2.63 (m, 4H), 3.40 (s, 3H, OCH_3), 3.73–3.94 (m, 3H), 4.16–4.21 (m, 2H), 4.97 (d, 1H, J = 8.06 Hz),5.54–5.69 (m, 3H), 5.98 (m, 1H), 6.22 (s, 1H), 6.24 (m, 1H), 6.68 (s, 2H), 7.12–8.13 (m, 15H, Ph-H).
Compound **5**: white solid (49% yield); ^{1}H NMR ($CDCl_3$): δ 1.18, 1.12, 1.76, 1.96 (s, each 3H, CH_3), 2.17, 2.26 (s, each 3H, CH_3), 2.10, 2.34 (m, 2H), 2.62 (m, 4H), 3.75 (s, 3H, OCH_3), 3.73-3.79 (m, 3H), 4.06 (m, 2H), 4.61 (d, 1H, J = 3.47 Hz), 4.76 (d, 1H, J = 9.52 Hz), 5.53 (m, 1H), 5.60 (d, 1H, J = 6.98 Hz), 5.71 (m, 1H), 6.14 (s, 1H), 6.60 (m, 3H), 6.75–7.79 (m, 29H, Ph-H).
Compound **6a**: white solid (48% yield); Anal. RP-HPLC t_R = 14.3 min (10–70% MeCN, purity >97%); MS *m/z* 3355.7 $[M+H]^+$ ($C_{161}H_{229}N_{37}O_{38}S_2$ requires 3354.9).