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CATHEPSIN B-SENSITIVE DIPEPTIDE PRODRUGS. 2. MODELS OF ANTICANCER DRUGS PACLITAXEL (TAXOL®), MITOMYCIN C AND DOXORUBICIN

Gene M. Dubowchik,* Kathleen Mosure, Jay O. Knipe and Raymond A. Firestone[1]

Bristol-Myers Squibb Pharmaceutical Research Institute,
5 Research Parkway, P.O. Box 5100, Wallingford, CT 06492-7660, U.S.A.

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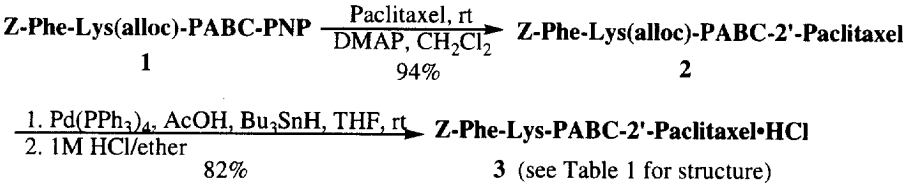
Abstract Substrates containing doxorubicin (DOX), paclitaxel (taxol®), and mitomycin C (MMC) attached to the cathepsin B-sensitive dipeptide Phe-Lys via a self-immolative spacer were prepared as model compounds for internalizing anticancer immunoconjugates. Cathepsin B-mediated release rates of free drug, rat liver lysosomal susceptibility and human plasma stability were measured for each.

Doxorubicin (DOX),[2,4] paclitaxel (taxol®),[3] and mitomycin C (MMC)[4] are three examples of a wide range of cytotoxic anticancer drugs that exert their actions through distinct mechanisms, often possessing very different activities against specific tumor cell types. Therefore, it is advantageous to be able to selectively deliver different classes of drugs, using various targeting vehicles, with a versatile mode of linkage. Such vehicles include monoclonal antibodies, polymers, and enzymatically-cleavable small molecule prodrugs.[5] Acid cleavable hydrazone linkers have been used successfully to target DOX by means of internalizing monoclonal antibodies[6] but are inapplicable to most other classes of drugs. Lysosomally-cleavable tetrapeptides have been employed, but these are generally slow-releasing and hydrophobic.[5] Dipeptides composed of a basic or strongly hydrogen bonding amino acid (Lys, Arg or Cit) at P_1 and a hydrophobic amino acid (i.e., Phe or Val) at P_2 are good substrates for the ubiquitous lysosomal cysteine proteases cathepsins B and L. We have shown that model substrates such as Z-Phe-Lys-PABC-DOX **12** are efficiently cleaved by cathepsin B to release free DOX and are very stable in human plasma.[7] The self-immolative PABC spacer[8] is required for drug release, presumably because of steric interference of substrate binding by DOX in the enzyme active site In this paper we describe the preparation of N-capped Phe-Lys-PABC substrates containing paclitaxel and MMC, their drug release behavior with cathepsin B and in a lysosomal preparation, and their stabilities in human plasma.

Synthesis[9]

The active carbonate intermediate **1** coupled selectively, and in high yield with the 2'-hydroxyl group of paclitaxel using 1.1 equiv of DMAP to give the carbonate **2**. Deprotection of the alloc protecting group proceeded as with the corresponding DOX substrate to give Z-Phe-Lys-PABC-2'-Paclitaxel•HCl **3**.

Scheme 1



Esterase activity in vivo could result in systemic release of free paclitaxel from a 2'-linked immunoconjugate or prodrug. Esters and carbonates at the 7-position are protected to a greater extent than their 2' analogues from esterases and simple hydrolysis.[10] Boc-Phe-Lys-PABC-7-Paclitaxel **8** was prepared as shown in Scheme 2. The N-terminal capping groups Boc and Z are not expected to cause any significant differences in drug release

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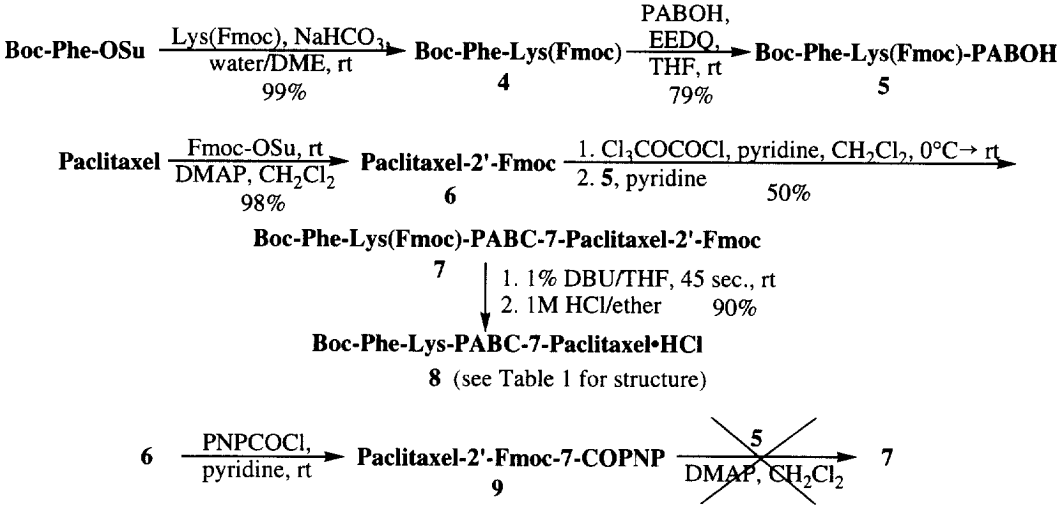
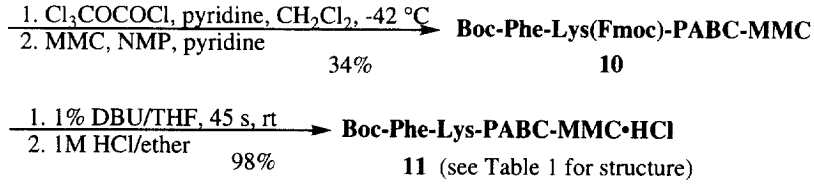
rates by cathepsin B. For convenience we wanted to use the same protecting group on the 2'-hydroxyl of paclitaxel and the Lys amino group. Fmoc was chosen over alloc because of concern that the Pd catalyst might be inhibited by the steric clutter around the 2'-position. Fmoc-OSu was used to selectively protect the 2'-hydroxyl and the product (**6**) was treated with diphosgene/pyridine to give the corresponding 7-chloroformate in situ. This was coupled with Boc-Phe-Lys(Fmoc)-PABOH **5** (prepared as described previously for the alloc analogue) to give the bis-Fmoc substrate **7** in moderate yield. Deblocking of both Fmoc groups proceeded smoothly with brief exposure to 1% DBU/THF followed by quenching with 1 M HCl/ether. A *p*-nitrophenylcarbonate at the 7-position proved to be inert to coupling with **5**, even with equimolar amounts of DMAP.

Scheme 2



The aziridine nitrogen of MMC, being much less nucleophilic than a typical secondary amine, is inert to *p*-nitrophenyl and N-hydroxysuccinimidyl carbonates under normal conditions. We prepared the benzyl chloroformate of **5** in situ using diphosgene/pyridine and coupled it with MMC in NMP in modest yield. At temperature much above -42 °C the chloroformate quickly decomposed to the benzyl chloride. Subsequently, we found that MMC could be coupled efficiently and in high yield with *p*-nitrophenyl carbonates such as **1** by the addition of catalytic (0.2 equiv) HOBt/DIEA in dry DMF at rt.[11] Fmoc removal as above gave the MMC substrate **11** cleanly, and in high yield.

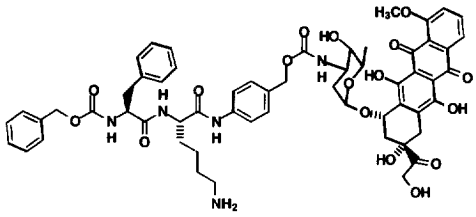
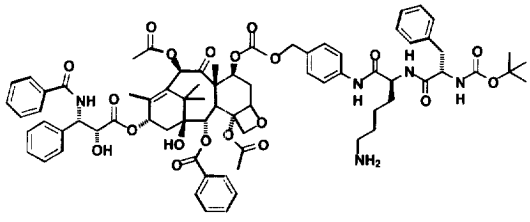
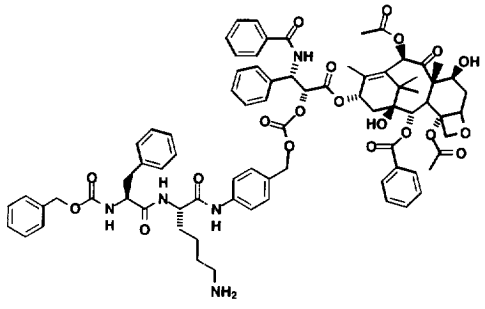
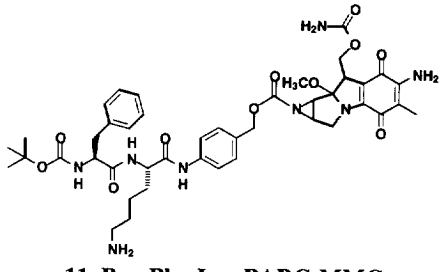
Scheme 3



Cathepsin B Assays

Assay conditions (enzyme concentration, pH, etc.) were chosen to approximate the lysosomal medium as a

Table 1. Half-lives of Peptide Model Substrates of Various Anticancer Drugs

Compound	Cathepsin B	Rat Liver Lysosomes	Human Plasma
 **12 Z-Phe-Lys-PABC-DOX**	8 min	29 min[a]	NH[b]
 **8 Boc-Phe-Lys-PABC-7-Paclitaxel**	40 min	66 min	NH
 **3 Z-Phe-Lys-PABC-2'-Paclitaxel**	9.0 h	19 min	NH
 **11 Boc-Phe-Lys-PABC-MMC**	10 min	24 min	149 h

a DOX release was completely inhibited by the cysteine protease inhibitor E-64.

b NH = not hydrolyzed (no observed change over 6 - 7 h).

model for intracellular drug release. Bovine spleen cathepsin B (Sigma) was activated at rt with a solution of 30 mM DTT/15 mM EDTA for 15 min, and then diluted with 25 mM acetate/1 mM EDTA buffer (pH 5.0, preincubated at 37 °C),[12] followed immediately by the substrate in methanol giving the following initial concentrations: [cathepsin B] = ca. 40 nM, [substrate] = 0.04 mM. The mixture was incubated at 37 °C and aliquots were periodically removed and injected into the HPLC (15 cm C-18 column, 8:2 methanol/50 mM Et_3N-formate buffer (pH 2.8) at 1 mL/min, λ = 495 nm (DOX), 365 nm (MMC), and 280 nm (Paclitaxel)). The time between injections varied depending on speed of drug release. The ratios of drug/substrate peak areas were used to calculate half-lives ($t_{1/2}$) of release for DOX and MMC. For paclitaxel, half-lives were determined by comparison with a standard curve of free paclitaxel. Half-lives are shown in Table 1.

Rat Liver Lysosomal Assay

Rat liver lysosomes were isolated by differential centrifugation according to the method of Lardeux and coworkers.[13] The protein concentration in the lysosomal pellets was determined by the Bradford assay (Bio-Rad Protein Assay Kit II, Bio-Rad, Hercules, CA). For a typical incubation, a known concentration of linker was added to a pH 5.5 sucrose/phosphate buffer mixture containing 0.1% Triton-X and 6 mg lysosomal protein. However, in the case of paclitaxel linkers where the detergent would interfere with UV-vis detection of the drug, the lysosome/buffer mixture was gently sonicated on ice to rupture the lysosomal membrane prior to the addition of substrates. The mixtures were incubated at 37 °C and at specific time points aliquots were removed and the protein was precipitated. The supernatant solution was then extracted and analyzed for the released cytotoxic agent by HPLC. In some experiments, the DOX substrate **12** was incubated with the potent cathepsin B, H and L inhibitor, E-64 (L-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; final concentration, 10 μg/mL).[14]

Human Plasma Stability Assays

A solution of the substrate (40 μM) in freshly-drawn human plasma was incubated at 37 °C. Aliquots (50 μL) were periodically removed and diluted with cold methanol (100 μL). The samples were centrifuged for 10 min and the supernatants injected into the HPLC. Half-lives (Table 1) were calculated as described above for the cathepsin B assays.

Results and Discussion

The release rates of free drug from Phe-Lys-PABC-Drug substrates by cathepsin B and in a rat liver lysosomal preparation are shown in table 1. Both Z-Phe-Lys-PABC-DOX **12** and Boc-Phe-Lys-PABC-MMC **11** quickly release free drug in both assays. The release of DOX from **12** was completely inhibited by the addition of the cathepsin inhibitor E-64 (final conc. 10 μg/mL) to the lysosomal preparation, implying that activity is restricted to cathepsins B, L, and H. As we have already shown,[7] Z-Phe-Lys-DOX **13**, which lacks the self-immolative PABC spacer, is not cleaved by cathepsin B. This substrate was also tested in the lysosomal assay and was found to be similarly inert. A MMC substrate lacking the PABC spacer, Boc-Phe-Lys-MMC **14** (prepared by DCC/HOBt coupling of MMC with **4**, followed by Fmoc deprotection), was also prepared. Like **13**, it was completely resistant to cathepsin B. There was no indication, in the lysosomal assay, of unexpected cleavage products with any of the substrates tested. Furthermore, MMC, which is known to be acid-labile,[15] was found to be quite stable at pH 5 for at least 7 h, indicating that it should remain effective if delivered intralysosomally. DOX substrate **12** was unchanged in human plasma over 7 h, reflecting the stability of the dipeptide-PABC unit

to plasma enzymes as well as simple hydrolysis (Table 1). The reactivity of the aziridine carbamate of **11** is more like a carbonate or ester, making it susceptible to slow, plasma-induced hydrolysis ($t_{1/2}$ = 149 h).

Paclitaxel substrates **3** and **8** show more divergent behavior in the two proteolysis assays. As already discussed, we attached the R-Phe-Lys-PABC fragment to two different hydroxyl groups on paclitaxel that have different steric environments. The 2'-hydroxyl is most easily modified but an ester or carbonate incorporating it is also more likely to be a target for plasma esterases or simple hydrolysis than one at the 7-position.[10] It might be expected that a substrate that contains paclitaxel, being a much larger and bulkier drug than either DOX or MMC, would be less readily processed by cathepsin B. This is borne out for both **3** and **8** (Table 1). The cathepsin B-mediated half-life for the 7-paclitaxel substrate **8** (40 min) is significantly longer than those for the DOX (**12**, 8 min) or MMC (**11**, 10 min) compounds, while that for the 2'-paclitaxel substrate **3** is even longer (9 h). The reason for the added resistance to proteolysis seen for **3** in comparison with **8** may be due to steric crowding in the wider area around the site of attachment. The 7-position offers greater steric protection to the acyl carbonyl group, but the region around it appears less cluttered (Figure 1). In contrast, the acyl carbonyl at 2' appears more vulnerable but the larger area contains bulky paclitaxel side chain phenyl groups close to the PABC spacer that is to be bound in the enzyme active site. In the lysosome assay, however, **3** releases free paclitaxel much more quickly than with cathepsin B alone and even faster than **8**. This may indicate the participation of other proteases that have different active site demands (perhaps cathepsin L[16]) or esterases that attack the carbonate directly. Both **3** and **8** were stable in human plasma.

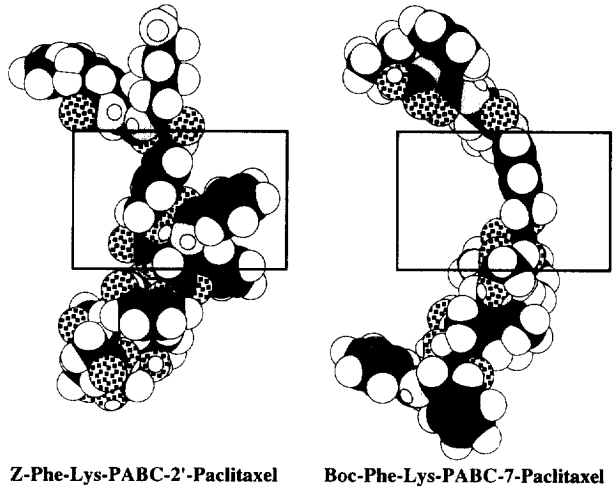


Figure 1. Space-filling models of Z-Phe-Lys-PABC-2'-Paclitaxel **3** and Boc-Phe-Lys-PABC-7-Paclitaxel **8**. Both structures are oriented with the dipeptide on top and paclitaxel at the bottom. The area around the self-immolative PABC spacer is highlighted to show steric crowding by the side chain phenyl groups of paclitaxel in **3** that might inhibit binding in the enzyme active site.

In summary, we have prepared cathepsin B-sensitive substrates containing DOX, paclitaxel and MMC linked to N-terminal-capped dipeptides (Phe-Lys) via a self-immolative PABC spacer. These compounds release free drug in the presence of cathepsin B and in a rat liver lysosome preparation with half-lives that, in combination with their excellent human plasma stability, make them good candidates to be used as extracellularly-stable drug-linker combinations for targeted drug delivery.

References

‡ Abbreviations used: Alloc, allyloxycarbonyl; Arg, L-arginine; Boc, t-butyloxycarbonyl; Cit, L-citrulline; DIEA, diisopropylethylamine; DMAP, N,N-dimethylaminopyridine; DME, dimethoxyethane; DMF,

dimethylformamide; DOX, doxorubicin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, N-hydroxybenzotriazole; HOSu, N-hydroxysuccinimide; HPLC, high performance liquid chromatography; Lys, L-lysine; MMC, mitomycin C; NMP, N-methylpyrrolidinone; PABC, p-aminobenzylcarbonyl; PABOH, p-aminobenzyl alcohol; Phe, phenylalanine; PNP, *p*-nitrophenyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Ts, *p*-toluenesulfonyl; Val, L-valine; Z, benzyloxycarbonyl.

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9. All new compounds gave satisfactory NMR, mass-spectral, microanalytical and/or high-resolution mass spectral results. Representative analytical data: For **3**: ^{1}H-NMR ($CDCl_3$/CD_3OD)(selected peaks) δ 1.12, 1.27 and 1.72 (each 3H, s), 2.20 and 2.48 (each 3H, s), 2.98 (2H, m), 4.27 (2H, AB q), 4.39 (1H, m), 5.02 (2H, m), 5.09 (2H, m), 7.06-8.20 (29H, m). HPLC: (C-18, 15 cm column, 6:4 CH_3OH/50 mM Et_3N-HCO_2H buffer (pH 2.8), 1 mL/min, λ = 280 nm): ret. time 9.6 min. MS (ESI) 1413.2 $(MH)^+$. HRMS: calcd for $C_{78}H_{86}N_5O_{20}$: 1412.5866. Found: 1412.5883. For **8**: ^{1}H-NMR ($CDCl_3$/CD_3OD) δ 1.13, 1.19 and 1.78 (each 3H, s), 1.37 (9H, s), 1.10-1.90 (6H, m), 1.86 and 2. 54 (each 1H, m), 2.05 (3H, s), 2.16 and 2.38 (each 3H, s), 2.97 (2H, m), 3.12 (2H, m), 3.90 (1H, d,) 4.24 (2H, m), 4.45 and 4.68 (each 1H, m), 4.83 (1H, brs), 4.91 (1H, d), 5.12 (2H, m), 5.48 (1H, m), 5.67 (1H, d), 5.78 (1H, d), 6.12 (1H, m), 6.33 (1H, s), 7.08-8.12 (24H, m). HPLC: (C-18, 15 cm column, 8:2 CH_3OH/50 mM Et_3N-HCO_2H buffer (pH 2.8), 1 mL/min, λ = 280 nm): ret. time: 7.1-7.3 min. MS (ESI): 1379.2 $(MH)^+$. HRMS: calcd for $C_{75}H_{88}N_5O_{20}$: 1378.6023. Found: 1378.6043. For **11**: ^{1}H-NMR ($CDCl_3$/CD_3OD) δ 1.30 (9H, s), 1.20-1.90 (6H, m), 1.94 (3H, s), 2.83 (2H, m), 2.98 (2H, m), 3.13 (3H, s), 3.20-3.70 (4H, m and ABq), 4.14 and 4.82 (each 1H, ABq), 4.25-4.52 (3H, m), 4.97 (2H, m), 7.12 (5H, brs), 7.23 and 7.50 (each 2H, m). HPLC: (C-18, 15 cm column, 65:35 CH_3OH/50 mM Et_3N-HCO_2H buffer (pH 2.8), 1 mL/min, λ = 365 nm): ret. time: 4.1-4.3 min. MS (FAB): 859 $(MH)^+$, 881 $(M+Na)^+$, 897 $(M+K)^+$. HRMS: calcd for $C_{43}H_{55}N_8O_{11}$: 859.3990. Found: 859.3980.
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