Self-Immolative Anthracycline Prodrugs for Suicide Gene Therapy

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Four novel potential prodrugs derived from daunorubicin (**8, 10**) and doxorubicin (**12, 14**) were designed and synthesized. They are self-immolative prodrugs for suicide gene therapy activation by the enzyme carboxypeptidase G2 (CPG2) subsequently releasing the corresponding anthracyclines, by a 1,6-elimination mechanism. A mammary carcinoma cell line (MDA MB 361) was engineered to express CPG2 intracellularly (CPG2*) or extracellularly, tethered to the outer cell membrane (stCPG2(Q)3). The prodrugs derived from doxorubicin showed prodrug/drug cytotoxicity differentials of 21-fold (compound **12**) and 23-fold (compound **14**). Prodrug **12** underwent an 11-fold activation when assayed in the cell line expressing externally surface-tethered CPG2.

Introduction

Gene-directed enzyme prodrug therapy (GDEPT)[1,2] and virally directed enzyme prodrug therapy (VDEPT)[3] are two-step treatments for solid tumors. In the first step, gene delivery technology ideally leads to the expression of a prodrug-activating enzyme only in cells in the tumor. During the second step, a nontoxic prodrug is administered which is converted by the activating enzyme to a potent cytotoxic agent.[4] We have previously described a GDEPT system in which the bacterial enzyme carboxypeptidase G2 (CPG2) is used to activate prodrugs.[5] CPG2 catalyzes the scission of amidic,[6] urethanic, or ureidic[7,8] bonds between a benzene nucleus and L-glutamic acid. This is a versatile system, because CPG2 can be expressed either intracellularly[2] or extracellularly anchored to the outer membrane of tumor cells.[9] The potential advantages of extracellular expression are two-fold. First, it should, theoretically, give an improved bystander effect because the drug will be generated in the interstitial spaces within the tumor, rather than inside the cells as with an intracellularly expressed activating enzyme. Second, the prodrug need not enter cells to become activated, and therefore non-cell-permeable prodrugs can be used. Thus, prodrugs which release drugs with intracellular targets may be rendered nontoxic by preventing their entry into cells. Upon activation, a potent and cell-permeable active moiety is released. This has already been demonstrated to be beneficial for prodrug-impermeable tumor cells.[9] However, the potential for increased toxicity due to the diffusion of the active drug away from the tumor is a potential disadvantage, although this could happen also to active drugs from an intracellularly expressed enzyme.

The anthracyclines, such as doxorubicin and daunorubicin, are antitumor drugs with the widest spectrum of activity in human tumors.[10,11] Their therapeutic efficacy is limited by toxic side effects, mainly cardiotoxicity and myelosuppression. To overcome these toxicities several "self-immolative" and "non-self-immolative" anthracycline prodrugs, obtained mainly by derivatization of the amine functionality of the daunosamine, have been described for activation by a wide range of enzymes in ADEPT strategies.[12−30] However, CPG2 cannot activate any of the reported prodrugs. Furthermore, direct addition of L-glutamyl residues to doxorubicin or daunorubicin does not generate a molecule which is a substrate for CPG2. We therefore describe the synthesis of novel prodrugs from doxorubicin and daunorubicin, based on a similar strategy, which include self-immolative linkers designed to reduce their intrinsic toxicity. These produgs, for use in suicide gene therapy, can be activated by CPG2, releasing the active drugs by a 1,6-elimination mechanism.[31−33]

Chemistry

Linkers cleavable by CPG2 were incorporated into the prodrugs. The linkers **3a** (Z = NH) and **3b** (Z = O) (Scheme 1) were previously synthesized using a five-step and a three-step procedure, respectively.[5] Here, we describe a simple one-pot procedure which allows the concise synthesis of **3a** and **3b** by direct coupling of diallyl L-glutamylisocyanate, **2**, with 4-amino- or 4-hydroxybenzylic alcohol, **1a** and **1b**, respectively, under controlled conditions. The condensation of the 4-aminobenzylic alcohol is catalyzed by triethylamine. *t*-BuOK and 18-crown-6 ether were used for the condensation of the 4-hydroxybenzylic alcohol (Scheme 1).

The linkers **3a** and **3b** were activated as 4-nitrophenyl carbonates **4a** and **4b**, respectively,[5] and reacted with daunorubicin or doxorubicin in DMF (see Scheme 2). The protected prodrugs **7**, **9**, **11**, and **13** were purified by a two-step preparative TLC procedure and deprotected with tetrakis(triphenylphosphine)Pd(0) and an allyl scavenger (morpholine, sodium toluenesulfinate[34]) to prodrugs **8**, **10**, **12**, and **14**. The desalting of prodrugs **12** and **14** was achieved using a weak acidic ion exchanger (IRC 50). The final purification of these prodrugs was performed by semipreparative HPLC.

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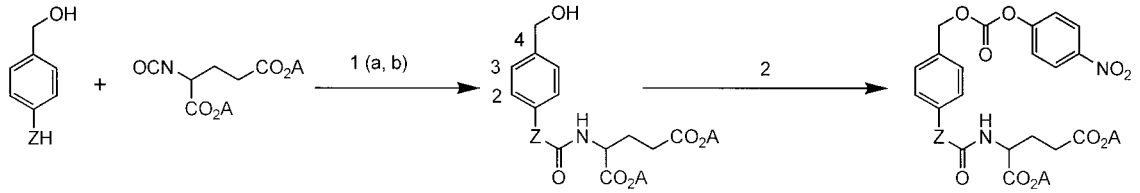
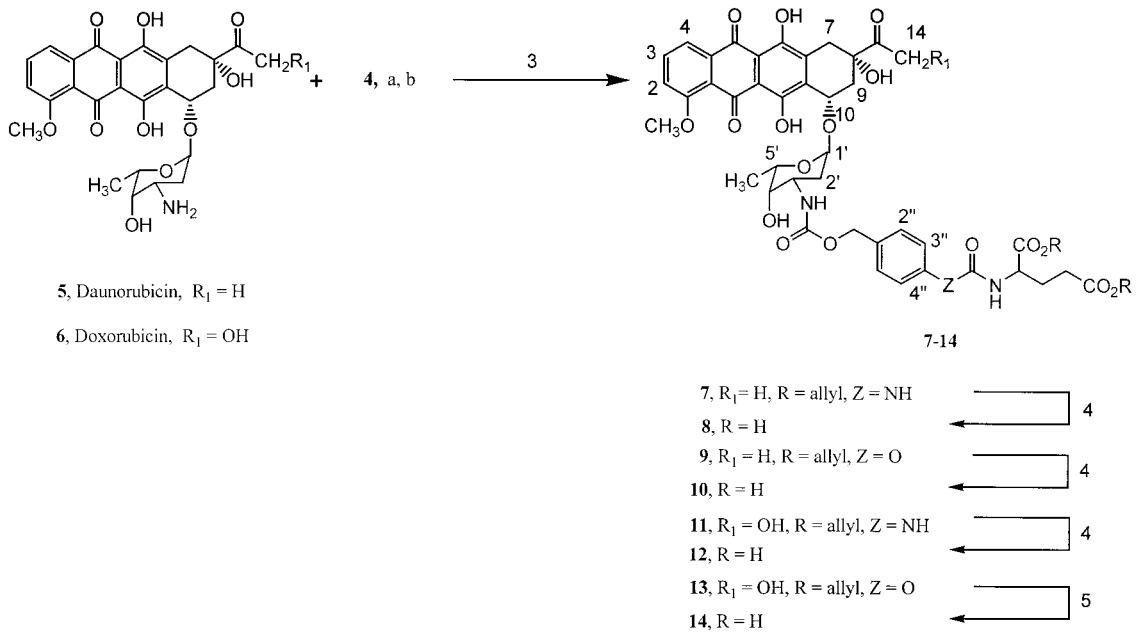
Scheme 1[a]



1, a, Z = NH b, Z = O **2**, A = allyl **3**, a, b **4**, a, b

[a] (1a) Z = NH, THF, NEt_3; (1b) Z = O, THF, *t*-BuOK, 18-crown-6; (2) 4-nitrophenyl chloroformate, NEt_3.

Scheme 2[a]



5, Daunorubicin, R_1 = H

6, Doxorubicin, R_1 = OH

7-14

[a] (3) DMF, NEt_3; (4) Pd(0), morpholine; (5) Pd(0), sodium toluenesulfinate or morpholine.

Table 1. Half-Lives ($t_{1/2}$) of Doxorubicin and Daunorubicin Prodrugs in the Presence or Absence of CPG2 and Their Cytotoxicity[a] to MDA MB 361 Cells Expressing Control LacZ, CPG2 Internally (CPG2*), or CPG2 Tethered to the Outer Surface of the Cell Membrane (stCPG2(Q)3)

	$t_{1/2}$ (min)		cytotoxicity in MDA MB 361 cell line expressing:					
			LacZ		CPG2*		stCPG2(Q)3	
drug	prodrug in the absence of enzyme	(apparent) prodrug in the presence of enzyme	compound, IC_{50} (nM)[b]	differential between prodrug/drug	compound, IC_{50} (nM)[b]	differential between prodrug, LacZ/CPG2*	compound, IC_{50} (nM)[b]	differential between prodrug, LacZ/stCPG2(Q)3
Doxo			12 ± 2	na	33 ± 12	na	18 ± 1	na
12	948	42.0	256 ± 48	21.3	47 ± 26	5.4	24 ± 6	10.7
14	1702	267.4	279 ± 66	23.3	204 ± 26	1.4	92 ± 22	3.0
Dauno			17 ± 6	na	36 ± 13	na	21 ± 5	na
8	1187	38.7	112 ± 57	6.6	123 ± 66	0.9	29 ± 9	3.9
10	1482	132.4	115 ± 57	6.8	405 ± 304	0.3	177 ± 144	0.7

[a] Cytotoxicity differentials describe either the ratio between prodrug:drug in the control cell line expressing LacZ (LacZ column), the ratio for a given prodrug between the cell line expressing CPG2* compared to that expressing LacZ (CPG2* column), or the ratio for a given prodrug between the stCPG2(Q)3 line compared to the LacZ line (stCPG2(Q)3 column). [b] Data are nM ± SEM; na, not applicable; see Enzymic Activity and Biological Evaluation for the analysis of variance of Table 1 data.

Enzymic Activity and Biological Evaluation

The ability of **8**, **10**, **12**, and **14** to act as substrates for CPG2 was determined by comparing their half-lives in phosphate-buffered saline (pH 7.4, 37 °C) in the presence or absence of CPG2 (50 mU). All four prodrugs were similarly stable, but their half-lives were shortened in the presence of CPG2 indicating that they are substrates, **12** and **8** being more rapidly hydrolyzed than **14** and **10** (Table 1). The extent to which derivatization of daunorubicin and doxorubicin reduced their cytotoxicity was determined by comparing their IC_{50} to that of **8** and **10** or **12** and **14**, respectively, in control MDA MB 361 (LacZ) cells. Two-tailed analysis of variance showed significant differences ($P < 0.0001$) between the cytotoxicity of doxorubicin compared to **12** (21-fold) and **14** (23-fold) and differences ($P < 0.085$) between daunorubicin, **8**, and **10** (7-fold) (Table 1). A P value of <0.1 was selected as indicating significance. The assumptions

of the analysis of variance were shown to be correct in that the residuals were normally distributed about zero with equal variance. The ability of **8**, **10**, **12**, and **14** to act as prodrugs was determined by comparing their IC_{50} in control MDA MB 361 (LacZ) cells with that in cells expressing CPG2 intracellularly (CPG2*) or extracellularly (stCPG2(Q)3). There were highly significant differences ($P < 0.0001$) between the cytotoxicities experienced by all three cell lines following exposure to doxorubicin, **12**, and **14**. Compound **12** gave the higher cytotoxicity differential in both cell lines, commensurate with its more rapid digestion by CPG2 (Table 1). Cells expressing CPG2 extracellularly showed approximately twice the activation of both **12** and **14**, compared with those expressing CPG2 intracellularly, probably reflecting greater access of the prodrug to the enzyme. Statistically significant differences did not exist between the IC_{50} of daunomycin, **8**, and **10** in the three cell lines, although the highest differential of 4-fold seen with **8** in stCPG2(Q)3-expressing cells is consistent with the result seen with its doxorubicin congener (**12**) and, considering that **8** is shown to be a substrate for CPG2, probably reflects a genuine prodrug effect.

In conclusion, we show that self-immolative prodrugs can be synthesized, which release cytotoxic anthracyclines when cleaved by CPG2. We show that doxorubicin-based prodrugs are the more deactivated and yield higher cytotoxicity differentials. Prodrugs based on linker **3a** are more rapidly cleaved by CPG2 and give higher cytotoxicity differentials, with the highest differential seen in cells expressing CPG2 extracellularly.

Experimental Section

All starting materials, reagents, and anhydrous solvents were from Aldrich, unless otherwise stated. The diallyl glutamate was obtained from Sigma. Daunorubicin·HCl and doxorubicin·HCl were obtained from Meheco, China. Kieselgel 60 was used in gravity columns (Art. 9385 and 15111, Merck). TLC was performed on precoated sheets of Kieselgel 60 F_{254} (Art. 5735, Merck). Semipreparative HPLC was performed on ThermoQuest equipment using an Apex ODS-WP, column (25 × 0.8 cm) from Jones Chromatography, using an isocratic mobile phase of 35 mM ammonium acetate buffer, pH 5.0/MeOH, 30:70. Melting points were determined on a Kofler hot-stage melting point apparatus (Reichert Thermovar) and are uncorrected. Low-resolution FAB mass spectra were performed on a VG-2AB-SE double-focusing magnetic sector mass spectrometer (Fisons Instruments, Warrington, Manchester, U.K.), operating at a resolution of 1000. High-resolution accurate mass spectra were determined on the same system, but with the resolution set to 8 000−10 000. Reported spectra are by FAB unless otherwise stated. NMR spectra were determined in Me_2SO-d_6 on a Brucker AC250 spectrometer (250 MHz) at 30 °C (303 K) unless otherwise stated. For proton assignment a numbered formula is given in Scheme 2. The assignment of protons is partial for the prodrugs. IR spectra (film) were recorded on a Perkin-Elmer 1720X FTIR spectrometer. Elemental analyses were determined by Butterworth Laboratories Ltd. (Teddington, Middlesex, U.K.) and are within 0.4% of theory except when stated.

Diprop-2-enyl *N*-[(4-{Hydroxymethyl}phenyl)carbamoyl]-L-glutamate (3a). To a solution of diallyl glutamyl isocyanate (**2**)[5] (25.0 mmol) in 100 mL of THF were added 4-aminobenzyl alcohol (3.0 g, 24.3 mmol) and triethylamine (3.41 mL, 24.3 mmol) in 20 mL of toluene, dropwise, over 10 min, at room temperature. The reaction was complete within 15 min. The reaction mixture was filtered and evaporated to dryness; the residue was dissolved in 20 mL of EtOAc, washed with water (2 × 20 mL), and dried ($MgSO_4$), before evaporating again. A yellow oil resulted (10.25 g); 2.7 g of the obtained product was submitted to purification by preparative HPLC (CH_2Cl_2:EtOAc, 1:1) which yielded 1.52 g (63%) of pure **3a**: ν_{max} cm^{-1} (film) 3354 (NH-, OH, broad), 1737 (C═O, ester), 1659 (C═O, urea); ^1H NMR δ_H 1.85−1.93 (m, 1H, CH_2CH-(NH)-), 2.00−2.05 (m, 1H, -CH_2CH(NH)-), 2.46 (t, 2H, CH_2-CO_2, $J = 5.4$ Hz), 4.26−4.35 (m, 1H, -CH(NH)-), 4.40 (d, 2H, CH_2−Ph, $J = 5.6$ Hz), 4.55 (d, 2H, CH_2O-allyl, $J = 5.3$ Hz), 4.61 (d, 2H, CH_2O-allyl), 4.98 (t, 1H, OH), 5.17−5.37 (m, 4H, CH_2═allyl), 5.85−5.94 (m, 2H, CH═allyl), 6.56 (d, 1H, NH-G, $J = 8.0$ Hz), 7.17 (d, 2H, H_{2+6}, $J = 8.5$ Hz), 7.32 (d, 2H, H_{3+5}), 8.52 (s, 1H, NH−Ph); MS *m*/*z* 399 ($M^+ + 23$, 35), 377 ($M^+ + 1$, 100), 359 ($M^+ - H_2O$, 34). Anal. ($C_{19}H_{24}N_2O_6$) C, H, N.

Diprop-2-enyl *N*-[(4-{Hydroxymethyl}phenoxy)carbonyl]-L-glutamate (3b). 4-Hydroxybenzyl alcohol (1.00 g, 8.10 mmol) was dissolved in THF (dry) (10 mL) and 18-crown-6 ether (0.20 mL) and potassium *tert*-butoxide (0.16 g, 1.4 mmol) were added. To this mixture was added a clear solution of diallyl L-glutamylisocyanate (**2**)[5] (8.20 mmol) in 50 mL of THF, dropwise at room temperature with vigorous stirring. The reaction was monitored by IR (disappearance of the ν_{NCO} 2252 cm^{-1} peak) and stopped by addition of AcOH (0.5 mL). The reaction mixture was filtered and the solvent removed under vacuum. The residue was dissolved in EtOAc (20 mL), and the solution was washed sequentially with aqueous 4% NaOH (20 mL) and water (2 × 20 mL), dried, and evaporated to dryness. Compound **3b** (1.62 g, 53%) resulted and was purified by preparative HPLC (eluent cyclohexane:EtOAc, 1:1) which resulted in a clear oil (0.80 g, 26.5%): ν_{max} cm^{-1} (film) 3343 (NH-, OH, broad), 1734 (C═O, ester); ^1H NMR δ_H 1.90−2.01 (m, 1H, CH_2CH(NH)-), 2.05−2.09 (m, 1H, -CH_2CH(NH)-), 2.50−2.56 (m, 2H, CH_2CO_2), 3.96−4.05 (m, 1H, -CH(NH)-), 4.48 (d, 2H, $PhCH_2$, $J = 5.7$ Hz), 4.58 (d, 2H, CH_2O-allyl, $J = 5.3$ Hz), 4.63 (d, 2H, CH_2O-allyl), 5.16 (t, 1H, OH), 5.19−5.38 (m, 4H, CH_2═allyl), 5.95−5.99 (m, 2H, CH═allyl), 7.04 (d, 2H, H_{3+5}, $J = 8.5$ Hz), 7.32 (d, 2H, H_{2+6}), 8.24 (d, 1H, NH-G, $J = 7.8$ Hz); MS *m*/*z* 400 ($M^+ + 23$, 82), 378 ($M^+ + 1$, 3); mass ($C_{19}H_{23}NO_7Na$) calcd 400.1372, found 400.1376. Anal. ($C_{19}H_{23}NO_7$) H, N; C: calcd, 60.47; found, 60.01.

***N*-[4-(Dipropen-2-yl-L-glutamylcarbonylamino)benzyloxycarbonyl]daunorubicin (7).** To a solution of 60 mg (0.10 mmol) of daunorubicin hydrochloride in 4 mL and DMF were added 54 mg (0.10 mmol) of diallyl 4-[[(4′-nitrophenyloxycarbonyl)oxymethylphenyl]carbamoyl]-L-glutamate, **4a**,[5] and 70 μL (0.50 mmol) of triethylamine with stirring at room temperature. The reaction was complete after 3.5 h. The reaction mixture was evaporated under vacuum, EtOAc (5 × 5 mL) and CH_2Cl_2 (2 × 5 mL) were added, and the solution was evaporated again. A dark-red solid resulted which was purified by preparative TLC using first THF as eluent (5−6-cm migration) followed by EtOAc:cyclohexane (2:1) (for another 10 cm). After extraction of the compound in THF, 73 mg (74.1%) of a red solid resulted: mp 142−4 °C; ν_{max} cm^{-1} (film) 3358 (NH, broad), 1736 (C═O, ester), 1717 (C═O, ketone); ^1H NMR δ_H 1.12 (d, 3H, $(CH_3)_{5'}$, $J = 6.4$ Hz), 2.15 (m, 2H, H_9), 2.26 (s, 3H, $(CH_3)_{14}$), 2.43 (m, 2H, CH_2CO_2A), 2.88 (s, 2H, $H_{7,}$), 3.28−3.30 (m, 1H, $H_{4'}$), 3.99 (s, 3H, OCH_3), 4.27−4.35 (m, 1H, -CH(NH)-), 4.54 (d, 2H, CH_2O-allyl, $J = 5.3$ Hz), 4.59 (d, 2H, CH_2O-allyl), 4.86 (s, 2H, CH_2−Ph), 4.96 (t, 1H, H_{10}), 5.13−5.34 (m, 4H, CH_2═allyl), 5.82−5.99 (m, 2H, CH═allyl), 6.58 (d, 1H, NH-G, $J = 8.1$ Hz), 6.84 (d, 1H, NH-dauno, $J = 8.0$ Hz), 7.17 (d, 2H, $H_{3''+5''}$, $J = 8.3$ Hz), 7.32 (d, 2H, $H_{2''+6''}$), 7.63−7.67 (m, 1H, H_3), 7.91−7.95 (m, 2H, H_2+H_4), 8.58 (s, 1H, NH−Ph); MS *m*/*z* 930 ($M^+ + 1$, 2), 952 ($M^+ + 23$, 62); mass ($C_{47}H_{51}N_3O_{17}Na$) calcd 952.3116, found 952.3140.

The same procedure was used to obtain ***N*-[4-(dipropen-2-yl-L-glutamylcarbonyloxy)benzyloxycarbonyl]daunorubicin (9)** from **4b**:[5] yield 92%; mp 99−101 °C; ν_{max} cm^{-1} (film) 3349 (NH, broad), 1736 (C═O, ester), 1719 (C═O, ketone); ^1H NMR δ_H 1.13 (d, 3H, $(CH_3)_{5'}$, $J = 6.4$ Hz), 1.90−2.00 (m, 1H, CH_2CH(NH)-), 2.15 (m, 2H, H_9), 2.26 (s, 3H, $(CH_3)_{14}$), 2.47−2.50 (m, 2H, CH_2CO_2A), 2.96 (s, 2H, H_7). 3.26−

3.29 (m 1H, $H_{4'}$), 3.99 (s, 3H, OCH_3), 4.15−4.20 (m, 1H, -*CH*(NH)-), 4.55 (d, 2H, CH_2O-allyl, J = 5.2 Hz), 4.60 (d, 2H, CH_2O-allyl), 4.95 (s, 3H, CH_2−Ph+H_{10}), 5.18−5.34 (m, 4H, CH_2=allyl), 5.82−5.99 (m, 2H, CH=allyl), 6.84 (d, 1H, NH-dauno, J = 8.0 Hz), 7.05 (d, 2H, $H_{3''+5''}$, J = 8.2 Hz), 7.32 (d, 2H, $H_{2''+6''}$), 7.64−7.68 (m, 1H, H_3), 7.91−7.94 (m, 2H, H_2+H_4), 8.25 (d, 1H, NH-G, J = 8.0 Hz); MS *m*/*z* 953 (M^+ + 23, 100); mass ($C_{47}H_{50}N_2O_{18}Na$) calcd 953.2956, found 953.2940.

***N*-[4-(Dipropen-2-yl-L-glutamylcarbamoylamino)benzyloxycarbonyl]doxorubicin (11):** yield 91%; mp 145−6 °C; ^{1}H NMR δ_H 1.12 (d, 3H, $(CH_3)_{5'}$, J = 6.4 Hz), 1.89−2.03 (m, 2H, CH_2CH(NH)-), 2.18 (d, 2H, H_9), 2.44 (t, 2H, CH_2CO_2A, J = 8.2 Hz), 3.45 (m, 1H, $H_{4'}$), 3.99 (s, 3H, OCH_3), 4.53−4.56 (m, 6H, $(CH_2)_{14}$+CH_2O-allyl), 4.86 (s, 2H, CH_2−Ph), 4.96 (t, 1H, H_{10}), 5.19−5.36 (m, 4H, CH_2=allyl), 5.88−6.00 (m, 2H, CH=allyl), 6.59 (d, 1H, NH-G, J = 9.4 Hz), 6.84 (d, 1H, NH-doxo, J = 8.0 Hz), 7.18 (d, 2H, $H_{3''+5''}$, J = 8.4 Hz), 7.32 (d, 2H, $H_{2''+6''}$), 7.63−7.67 (m, 1H, H_3), 7.91−7.95 (m, 2H, H_2+H_4), 8.58 (s, 1H, NH−Ph); MS *m*/*z* 947 (M^+ + 1, 5), 969 (M^+ + 23, 32); mass ($C_{47}H_{51}N_3O_{18}Na$) calcd 968.3065, found 968.3100.

***N*-[4-(Dipropen-2-yl-L-glutamylcarbonyloxy)benzyloxycarbonyl]doxorubicin (13):** yield 84%; mp 114−6 °C; ν_{max} cm^{-1} (film) 3371 (NH, OH, broad), 1728 (C=O, ester); ^{1}H NMR δ_H 1.13 (d, 3H, $(CH_3)_{5'}$, J = 6.4 Hz), 1.76−2.14 (m, 2H, CH_2CH(NH)-), 2.47−2.52 (m, 2H, CH_2CO_2A), 3.99 (s, 3H, OCH_3), 4.49−4.61 (m, 6H, $(CH_2)_{14}$+ CH_2O-allyl), 4.95 (sb, 3H, CH_2−Ph+H_{10}), 5.18−5.37 (m, 4H, CH_2=allyl), 5.88−6.00 (m, 2H, CH=allyl), 6.85 (d, 1H, NH-doxo, J = 9.0 Hz), 7.05 (d, 2H, $H_{3''+5''}$, J = 8.4 Hz), 7.32 (d, 2H, $H_{2''+6''}$), 7.65−7.68 (m, 1H, H_3), 7.91−7.93 (m, 2H, H_2+H_4), 8.58 (s, 1H, NH-G, J = 7.8 Hz); MS *m*/*z* 929 (M^+ + 1, 25); mass ($C_{47}H_{50}N_2O_{19}Na$) calcd 969.2905, found 969.2940.

***N*-[4-(L-Glutamylcarbonylamino)benzyloxycarbonyl]daunorubicin (8).** Compound **7** (65 mg, 0.07 mmol) was dissolved in 5 mL of CH_2Cl_2 at room temperature, and 8.1 mg (0.007 mmol) of tetrakis(triphenylphosphine)Pd(0) and 12 μL (0.14 mmol) of morpholine were added with stirring. After 3 h a red solid precipitated. Filtration, washing (2−3 mL of CH_2Cl_2), and drying gave 26 mg (40%) of a red solid: mp 176−8 °C; ^{1}H NMR δ_H 1.13 (d, 3H, $(CH_3)_{5'}$, J = 6.5 Hz), 2.26 (s, 3H, $(CH_3)_{14}$), 3.99 (s, 3H, OCH_3), 4.16 (q, 1H, $H_{5'}$, J = 6.1 Hz), 4.84 (s, 2H, CH_2−Ph), 4.96 (t, 1H, H_{10}), 6.45 (d, 1H, NH-G, J = 6.7 Hz), 6.74 (d, 1H, NH-dauno, J = 7.9 Hz), 7.15 (d, 2H, $H_{3''+5''}$, J = 8.5 Hz), 7.32 (d, 2H, $H_{2''+6''}$), 7.63−7.67 (m, 1H, H_3+NH-G), 7.90−7.93 (m, 2H, H_2+H_4), 8.86 (s, 1H, NH−Ph); MS *m*/*z* 872 (M^+ + 23, 70); mass ($C_{41}H_{43}N_3O_{17}Na$) calcd 872.2485, found 872.2490; purity (HPLC) 98.7% (280 nm), 100% (495 nm).

The following compounds were obtained according to the same route.

***N*-[4-(L-Glutamylcarbonyloxy)benzyloxycarbonyl]daunorubicin (10):** yield 57%; mp 135−7 °C; ^{1}H NMR δ_H 1.13 (d, 3H, $(CH_3)_{5'}$, J = 6.4 Hz), 2.26 (s, 3H, $(CH_3)_{14}$), 2.97 (s, 2H, H_7), 3.99 (s, 3H, OCH_3), 4.94 (s, 3H, CH_2−Ph+H_{10}), 6.86 (d, 1H, NH-dauno, J = 7.9 Hz), 7.05 (d, 2H, $H_{3''+5''}$, J = 8.3 Hz), 7.31 (d, 2H, $H_{2''+6''}$), 7.63−7.67 (m, 2H, H_3+NH-G), 7.90−7.93 (m, 2H, H_2+H_4); MS *m*/*z* 873 (M^+ + 23, 100); mass ($C_{41}H_{42}N_2O_{18}Na$) calcd 873.2330, found 873.2350; purity (HPLC) 94.9% (280 nm), 96.9% (495 nm).

***N*-[4-(L-Glutamylcarbonylamino)benzyloxycarbonyl]doxorubicin (12):** yield 50%; mp 182−4 °C; ^{1}H NMR δ_H 1.12 (d, 3H, $(CH_3)_{5'}$, J = 6.4 Hz), 2.18 (m, 2H, H_9), 2.99 (s, 2H, H_7), 3.99 (s, 3H, OCH_3), 4.57 (s, 2H, $(CH_2)_{14}$), 4.84 (s, 2H, CH_2−Ph), 4.96 (t, 1H, H_{10}), 6.46 (d, 1H, NH-G, J = 6.9 Hz), 6.76 (d, 1H, NH-doxo, J = 8.2 Hz), 7.15 (d, 2H, $H_{3''+5''}$, J = 8.6 Hz), 7.31 (d, 2H, $H_{2''+6''}$), 7.63−7.67 (m, 1H, H_3+NH-G), 7.91−7.93 (m, 2H, H_2+H_4), 8.80 (s, 1H, NH−Ph); MS *m*/*z* 888 (M^+ + 23, 15); mass ($C_{41}H_{43}N_3O_{18}Na$) calcd 888.2439, found 888.2410; purity (HPLC): 90.1% (280 nm); 94.2% (495 nm).

The same procedure as described above was used to prepare ***N*-[4-(L-glutamylcarbonyloxy)benzylmethyloxycarbonyl]doxorubicin (14),** with the difference that sodium toluenesulfinate (22.6 mg, 0.13 mmol) instead of morpholine was added to compound **13** (40 mg, 0.04 mmol): yield 22%; ^{1}H NMR δ_H 1.12 (d, 3H, $(CH_3)_{5'}$, J = 6.4 Hz), 2.18 (m, 2H, H_9), 2.98 (s, 2H, H_7), 3.99 (s, 3H, OCH_3), 4.94 (s, 3H, CH_2−Ph+H_{10}), 6.85 (d, 1H, NH-doxo, J = 7.9 Hz), 7.05 (d, 2H, $H_{3''+5''}$, J = 8.3 Hz), 7.31 (d, 2H, $H_{2''+6''}$), 7.63−7.67 (m, 2H, H_3+NH-G), 7.90−7.93 (m, 2H, H_2+H_4); MS *m*/*z* 889 (M^+ + 23, 25), ESI, *m*/*z* 865 (M^- − H, 58); mass ($C_{41}H_{42}N_2O_{19}Na$) calcd 889.2279, found 889.2287; purity (HPLC) 91.8% (280 nm); 94.6% (495 nm).

Determination of Half-Lives. The half-lives of the prodrugs were determined by HPLC in 100 μM solutions in phosphate-buffered saline at 37 °C in the presence or absence of 50 mU of CPG2. Samples of solutions (10 μM) were injected at timed intervals onto a reverse-phase HPLC column (Partisphere ODS, 5 mm, 4.6 × 250 mm), eluted isocratically with 75% methanol:15% water:10% 10 mM NaH_2PO_4 (pH 5.0) at 1 mL·min^{-1} and the eluate continuously monitored at 500 nm. The chemical half-lives were determined from the slope of semilog plots. In the presence of the enzyme, straight-line plots were obtained on linear axes, and the apparent half-life was calculated as the time it took for the concentration to fall to one-half its starting value.

Preparation of Histidine-Tagged CPG2*. Polyhistidine-tagged CPG2* was expressed in Sf9 insect cells, and the protein was purified by nickle-agarose (Quingen) affinity chromatography, as described.[2]

Biological Methods: Cytotoxicity Assays. MDA MB 361 cells stably expressing either surface-tethered CPG2 (stCPG2-(Q)3) or an intracellularly located CPG2 (CPG2*) were used; control cells expressed β-galactosidase (LacZ).[9]

The compounds were dissolved in DMSO at 10 mM immediately prior to treatment, and the cytotoxicity assays were performed as described,[2] except that after the treatment cells were harvested and reseeded in quadruplicate in 96-well plates at ∼2 × 10^3 cells/well. When the control plates has reached confluence, the cells were fixed and stained with sulforhodamine-B.[35] The absorbance at 590 nm was determined, and the results are expressed as percentage of control growth, with IC_{50} (concentration required to give half-maximal cytotoxicity) values being determined by interpolation. Each determination was performed at least three times.

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