Prodrugs of Doxorubicin and Melphalan and Their Activation by a Monoclonal Antibody-Penicillin-G Amidase Conjugate

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The syntheses and cytotoxic activities of substituted *N*-phenylacetamido derivatives of doxorubicin and melphalan are described. The derivatives were designed as prodrugs which could be activated in a site-specific manner by monoclonal antibody-penicillin-G amidase (mAb-PGA) conjugates. *N*-(Phenylacetamido)doxorubicin (2) and *N*-(phenylacetyl)melphalan (6) were found to be 10- and 20-fold less cytotoxic against H2981 lung adenocarcinoma cells than doxorubicin and melphalan, respectively. When incubated with PGA, the cytotoxicity of 2 and 6 increased and became equivalent to that of the corresponding drugs from which they were made. The poor solubility characteristics of 2 in aqueous solutions provided the basis for the development of the more soluble doxorubicin derivatives, *N*-(4-aminophenylacetyl)doxorubicin (3) and *N*-(4-phosphonooxy)phenylacetyl)-doxorubicin (4). In vitro cytotoxicity assays indicated that 3 and 4 were at least 1000-fold less toxic than doxorubicin against H2981 cells. PGA and the mAb conjugate L6-PGA were able to effect the activation of 3 and 6 on H2981 cells (L6-antigen positive). Hydrolysis of the phosphate group of 4 was required prior to activation with PGA or L6-PGA. This was achieved using alkaline phosphatase, or by exposing 4 to phosphatases present in cell culture medium. The activation of 3, 4, and 6 on H2981 cells by L6-PGA occurred in an immunologically specific manner, since activation could be blocked by saturating cell surface antigens with L6 prior to treatment with L6-PGA. These results demonstrate that 3, 4, and 6 are prodrugs that can be specifically activated to release clinically approved anticancer agents by a mAb-PGA conjugate.

The toxicity of anticancer drugs to noncancerous cells is a major limitation in cancer chemotherapy.[1] Because of this, a great deal of research has been directed toward the development of more specific therapeutic strategies such as the use of monoclonal antibody (mAb) conjugates for the delivery of chemotherapeutic agents to tumor cells.[2] A recently described extension of this methodology involves the use of mAb-enzyme conjugates for the activation of anticancer prodrugs. In this approach, a mAb-enzyme conjugate is systemically administered and allowed to localize within tumor masses, and a prodrug is then administered which is converted to a more toxic drug by the targeted enzyme. Several mAb-enzyme/prodrug combinations possessing significant in vitro and in vivo activities have been described.[3,4]

We report the development of a series of prodrugs that can be activated by a mAb-penicillin G amidase (PGA) conjugate. PGA, from *Escherichia coli*, is an inexpensive, readily available enzyme used commercially for the production of semisynthetic penicillins.[5] The enzyme hydrolyzes not only the phenylacetyl group of benzylpenicillin, but also a variety of substituted and unsubstituted phenylacetamides that are unrelated to benzylpenicillin.[6] We reasoned that because of its lack of substrate specificity, PGA could also act on phenylacetyl-substituted anticancer drugs. Thus, amino groups that contribute to the cytotoxic effects of antitumor agents could be chemically modified to form phenylacetamides which would serve as substrates for targeted mAb-PGA conjugates. This report describes the preparation, properties, activities, and immunologically specific activation of phenylacetyl derivatives of doxorubicin (1) and melphalan (5).

Results

Chemical Syntheses. The *N*-phenylacetyl derivatives 2 and 6 were prepared by reacting the respective amines, 1 and 5, with phenylacetyl chloride. The doxorubicin derivative 2 was extremely insoluble in aqueous solutions (solubility $<10^{-5}$ M in phosphate-buffered saline containing 10% DMSO). Because of this, water-soluble doxorubicin derivatives were prepared. Scheme I illustrates the preparation of the 4-aminophenylacetyl derivative, 3. The amino group of 4-aminophenylacetic acid was protected with an Fmoc group, and the crude sodium salt was isolated in quantitative yield. The activated ester 7 was then prepared by reacting the Fmoc derivative with dissuccinimidyl carbonate. Condensation of 7 with 1, followed by removal of the Fmoc group with morpholine and acidic workup, yielded 3 which was isolated as the hydrochloride salt.

The synthesis of phosphate derivative 4 was carried out as shown in Scheme II. The known *N*-hydroxysuccinimidate ester of 4-hydroxyphenylacetic acid[7] was phosphorylated with diphenyl phosphorochloridate. Catalytic hydrogenation of 8 gave the phenylphosphoric acid derivative bearing an *N*-hydroxysuccinimidate ester residue. Condensation of 1 with this active ester gave 4 in 71% yield. As expected, compounds 3 and 4 were much more soluble in aqueous solutions than was 2. It was possible to prepare 10 mM solutions of 3 and 4 in 5% aqueous dextrose.

Enzymatic Hydrolysis and Cytotoxic Activities of Drug Derivatives. The ability of PGA to hydrolyze 3, 4, and 6 was established using reversed-phase HPLC. Analytical conditions allowing the separation of the modified drug from the drug were developed to monitor the enzymatic hydrolysis at various intervals of time. The drug derivatives (0.1 mM) were treated with PGA (final concentration 10 μg/mL for 3 and 4 and 1 μg/mL for 6),

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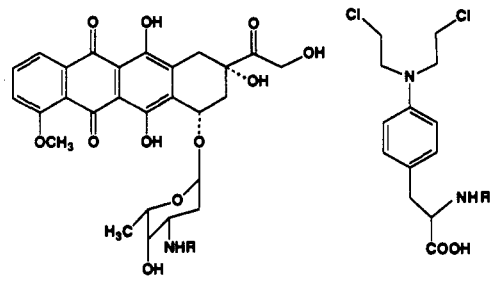
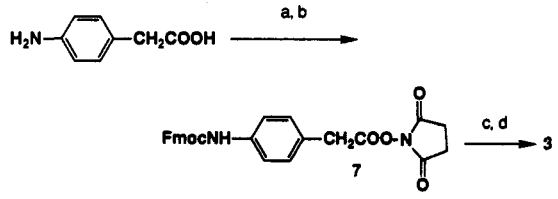
Chart I



1 R = H
2 R = $COCH_2C_6H_5$
3 R = $COCH_2C_6H_4$-p-NH_2
4 R = $COCH_2C_6H_4$-p-PO_4H_2

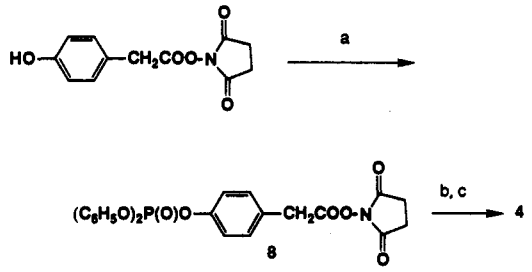
5 R = H
6 R = $COCH_2C_6H_5$

Scheme I



a. Fmoc-Cl / Aq. Na_2CO_3
b. Disuccinimidyl carbonate / DMF
c. 1 / $(i\text{-}Pr)_2EtN$
d. Morpholine

Scheme II



a. $(C_6H_5O)_2P(O)Cl$ / EtOAc / Pyridine
b. H_2 · Pt / i-PrOH
c. 1 / DMF / Et_3N

Table I. Enzymatic Hydrolysis of Prodrugs

compound	specific activity (nmol/min per mg PGA)	compound	specific activity (nmol/min per mg PGA)
3	12.7	4 + AP[a]	35.8
4	<0.05	6	6720

[a] Calf intestinal alkaline phosphatase (AP, 10 μg/mL) was added along with PGA.

and HPLC was used to determine the extent of hydrolysis. Specific activities were determined with each of these drug derivatives and are listed in Table I. PGA effected the hydrolysis of 3, and HPLC analysis confirmed that 1 was formed. In contrast, no apparent reaction took place when 4 was similarly treated. The presence of the phosphate group was evidently responsible for this lack of reactivity, since 1 was formed when 4 was treated with the combination of alkaline phosphatase and PGA. It was not possible to subject 2 to similar kinetic analyses due to its insolubility in aqueous solutions. However, qualitative

Table II. Cytotoxic Effects of Drugs and Prodrugs on H2981 Cells

compound	IC_{50} (μM)[a] without PGA	IC_{50} (μM)[a] with PGA
1	0.7	nd[b]
2[c]	2.0	0.2
3	>1000	10
4	>1000	4.0
4 + AP[d]	>1000	0.7
5	5.0	nd[b]
6	100	5.0

[a] Drug concentration resulting in 50% inhibition of [^{3}H]thymidine incorporation. [b] Not determined. [c] In this experiment, the IC_{50} value for doxorubicin was 0.2 μM. [d] 10 μg/mL calf intestinal alkaline phosphatase (AP) added to the incubation mixture.

TLC analysis showed that it also was a substrate for PGA (data not shown). The melphalan derivative 6, was hydrolyzed at a much faster rate than the doxorubicin derivatives.

The cytotoxic activities of 1, 5, and the phenylacetyl derivatives were determined on H2981 human lung adenocarcinoma cells. The cells were exposed to the drugs in the presence or absence of added enzyme(s) for 4 h, washed, and then plated out into 96-well plates. The cytotoxic effects were determined by measuring the incorporation of [^{3}H]thymidine into DNA. Table II shows that all of the phenylacetyl drug derivatives were significantly less toxic than the corresponding anticancer drugs. In the doxorubicin series, cytotoxicity appeared to be related to water solubility, since the water-soluble derivatives, 3 and 4, were much less cytotoxic than 2. These compounds were at least 1000 times less cytotoxic than the parent drug 1. Treatment of 2–4 and 6 with PGA resulted in greatly increased levels of cytotoxic activities relative to the prodrugs alone. The increased cytotoxic activity of 4 in the presence of PGA is most likely dependent on phosphatases in the tissue culture media, since it was not hydrolyzed to a significant extent without phosphate hydrolysis (Table I). The combination of alkaline phosphatase, PGA, and 4 resulted in cytotoxic activity equivalent to that of the parent drug 1 (IC_{50} 0.7 μM). Compounds 2 and 6 were also activated by PGA and were equitoxic with the corresponding parent drugs. The combination of 3 and PGA was less active than 1. This might be due to kinetic factors, since the specific activity of PGA with 3 as substrate was low compared to 4 + AP and 6 as substrates (Table I). These results establish that 2–4 and 6 are prodrugs that can be enzymatically activated to form active anticancer agents.

Activation of Prodrugs by L6–PGA. PGA was covalently conjugated to the anticarcinoma antibody L6[8] as previously described.[9] Briefly, PGA was derivatized with the heterobifunctional cross-linking reagent succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), introducing 1–2 maleimide groups. L6 was selectively reduced with dithiothreitol, cleaving 1–2 disulfide bonds. The modified proteins were combined, allowed to react, and purified by gel filtration. Analysis of SDS-polyacrylamide gel electrophoresis indicated that the purified conjugate consisted primarily of one molecule of L6 linked to one molecule of PGA. It was also shown that enzymatic and antigen binding activities were preserved.

The ability of L6–PGA to activate 3, 4, and 6 on H2981 cells was tested by incubating the cells with L6–PGA prior to treatment with varying concentrations of the prodrugs.

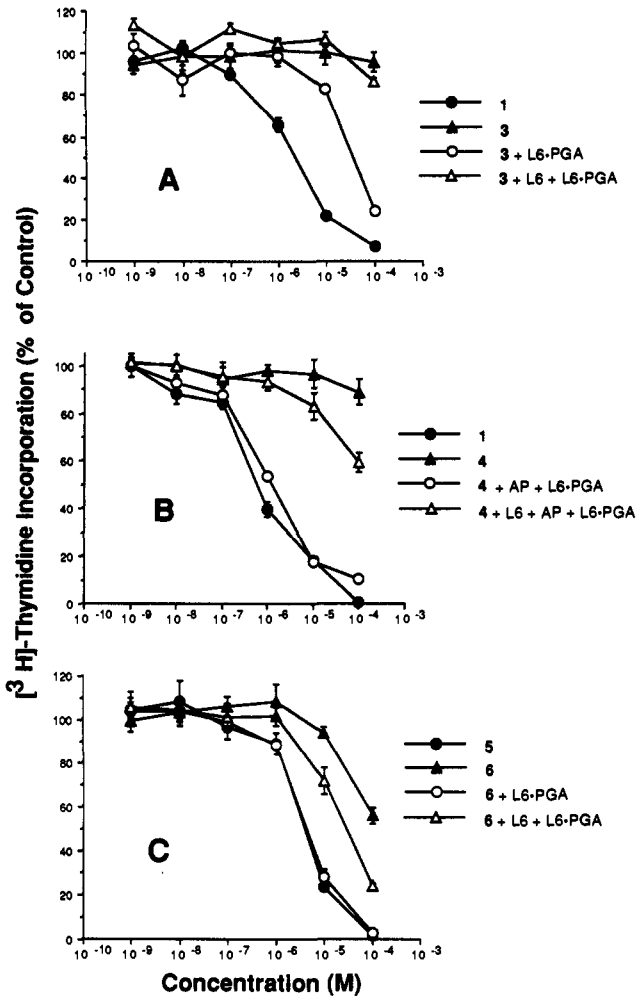


Figure 1. In vitro cytotoxicity of prodrugs and their respective drugs on H2981 lung adenocarcinoma cells (L6 antigen positive). Cells were treated with either media or the L6 antibody (1 mg/mL) prior to treatment with L6–PGA (0.1 mg/mL). The prodrugs were added to the cells after unbound material was washed off.

A significant degree of prodrug activation was observed on cells that were previously treated with the L6–PGA conjugate (Figure 1). The cytotoxic activities displayed in **4** in the presence of alkaline phosphatase (IC_{50} 0.7 μM), and **6** (IC_{50} 5.0 μM) on L6–PGA-treated cells were identical to **1** and **5**, respectively (Figure 1B,C). This is an indication of efficient prodrug activation by antigen-bound conjugate. The finding that **3** was only partially activated (IC_{50} 50 μM) by L6–PGA (Figure 1A) is consistent with both the kinetic data (Table I) and the partial activation of **3** catalyzed by unconjugated PGA (Table II).

The prodrug activation observed with the conjugate was dependent on the ability of the conjugate to bind to cell surface antigens, since little (Figure 1A and 1C) or no (Figure 1B) increase in prodrug cytotoxic activity was obtained on cells that were saturated with unmodified L6 prior to treatment with L6–PGA. This demonstrates that the prodrugs **3**, **4**, and **6** can be activated in an immunologically specific manner by a mAb–PGA conjugate.

Discussion

Many amine-containing anticancer drugs lose a considerable amount of cytotoxic activity as a result of amino group modification. This is particularly true for doxorubicin (**1**) and melphalan (**5**), in which a number of studies have demonstrated reduced activities for their respective amide derivatives. For doxorubicin (**1**), this may be due to the importance of the interaction of the positively charged amino group of the daunosamine with the negatiely charged phosphodiester backbone of DNA in the stabilization of the anthracyline–DNA complex.[10] Melphalan (**5**), a phenylalanine derivative, is taken into cells by active transport via an amino acid transport system.[11] Modification of the amino groups of these drugs was therefore expected to lead to reduced cytotoxic activities. Indeed, *N*-acetylmelphalan has been reported to be 75 times less toxic than melphalan on tumor cells,[12] and previous results from our laboratory have demonstrated reduction of cytotoxicity for phenoxyacetyl derivatives of **1** and **5**.[13] A similar trend was therefore expected with the phenylacetyl derivatives of **1** and **5** described in this paper. These derivatives were designed to be activated by mAb–PGA conjugates bound to antigens on tumor cells.

Our initial work focused on the phenylacetyl amides **2** and **6**. In vitro studies on H2981 lung adenocarcinoma cells demonstrated that **6** was 20-fold less cytotoxic than the parent drug **5** and could be fully activated when treated with PGA (Table II). The modest differential in cytotoxicities between **5** and **6** may be due to the conditions used in determining cytotoxic activity or because the phenylacetyl group in **6** does not completely block the alkylating activity of the nitrogen mustard. A 10-fold differential in cytotoxic activity was found between the doxorubicin derivative **2** and the parent drug. In addition, this derivative was practically insoluble in water, which could pose significant obstacles for its eventual use in vivo. Both the problems of poor solubility and relatively high cytotoxic activity of the doxorubicin prodrug could be circumvented by developing more hydrophilic analogues. The fact that both the amino and phosphorylated doxorubicin derivatives **3** and **4** had less than 1/1000 the potency of doxorubicin (Table II) may be the result of having made the prodrugs more water soluble, since such agents would be expected to have impaired abilities in traversing cellular membranes.

The phosphate derivative **4**, in contrast to **3**, was apparently not a substrate for PGA (Table I). Under the vitro conditions used for measuring cytotoxic activity, a considerable amount of phosphate hydrolysis must have occurred, since a 250-fold enhancement in cytotoxic activity was obtained through the combination of **4** with PGA (Table II). This compound could be further activated to be equitoxic with **1**, by treatment with both PGA and alkaline phosphatase. It is expected that when administered in vivo, **4** would behave as a pro-prodrug, undergoing hydrolysis by endogenous phosphatases[14] prior to hydrolysis by targeted PGA.

We have previously demonstrated that L6–PGA can activate a prodrug of the highly toxic marine toxin palytoxin.[9] The results reported in this paper complement this finding, since prodrugs of the clinically approved agents, doxorubicin and melphalan, were activated in an immunologically specific manner by a mAb–PGA conjugate. Thus, as with conjugates of alkaline phosphatase,[15] β-lactamase,[6] β-glucuronidase,[17] and penicillin-V amidase,[13] mAb–PGA conjugates have the ability to generate a variety of mechanistically dissimilar anticancer drugs. One advantage that PGA has over penicllin-V amidase is that the enzyme is not glycolysated and would most likely not undergo accelerated clearance in the body. Future

studies will concern the in vivo activities of mAb–PGA conjugates in combination with the prodrugs described here.

Experimental Section

General. Commercially available starting materials and reagents of highest available purity were used. Doxorubicin hydrochloride (1) was obtained from Bristol-Myers Squibb Co. Melphalan (5) was purchased from Sigma. PGA and alkaline phosphatase were obtained from Boehringer-Mannheim. All other chemicals were supplied by Aldrich Chemical Co. Elemental analyses were performed in the analytical research division of Bristol-Myers Squibb Co. Fast atom bombardment (FAB) mass spectra were acquired on a Kratos MS 25 mass spectrometer, and high-resolution FAB accurate mass measurements were obtianed on a Kratos MS 50 mass spectrometer. Both are equipped with a saddle-field FAB gun (Ion Tech) with xenon as the primary particle source and *m*-nitrobenzyl alcohol as the matrix. NMR spectra (300 MHz) were run on Bruker AC-300 or AM-300 instruments. Chemical shifts are reported in ppm downfield from $(CH_3)_4Si$. Analytical TLC was performed on Whatman 60 F 254 glass-backed plates with the solvents indicated. HPLC analyses at a flow rate of 1 mL/min were performed on a Beckman chromatograph equipped with a multisolvent delivery system using either a Supelco or an IBM 5-μm analytical RP-18 column. All in vitro cytotoxicity assays reported are representative of experiments performed at least three times. Within any assay the standard deviation of the results was less than 10% in any test group. Solvents are abbreviated as EtOAc for ethyl acetate, DMF for dimethylformamide, MeOH for methanol, EtOH for ethanol, and AcOH for acetic acid.

***N*-(Phenylacetyl)doxorubicin (2).** A suspension of 1 (74 mg, 0.13 mmol) in ice-cold 0.1 M bicarbonate buffer (pH 9, 10 mL) was treated with phenylacetyl chloride (0.04 mL, 0.3 mmol). After 1 h, TLC ($CHCl_3$–MeOH–H_2O–HCOOH, 130:60:10:4) indicated completion of the reaction with the formation of a single less polar compound. The red suspension was filtered through Celite and washed with H_2O (20 mL). The Celite cake was stirred with MeOH (50 mL) and filtered. After evaporating the MeOH from the filtrate, the residue was taken up in $CHCl_3$, washed with H_2O (50 mL), dried (Na_2SO_4), and evaporated to give 100 mg of 2. Crystallization of 75 mg of 2 from $CHCl_3$–*i*-PrOH (20 mL, 2:1) gave 25 mg (37% yield) of 2 as a red crystalline solid: MS, M + K^+ = 700.1796 (calcd), 700.1781 (found); ^1H NMR (DMSO-d_6) δ 13.97 (s, 1 H, OH), 13.20 (s, 1 H, OH), 7.84 (m, 3 H, H-1, H-3, and OH), 7.59 (m, 1 H, H-2), 7.16 (m, 5 H, Ar-H), 5.21 (d, 1 H, H-1′; J = 3.1 Hz), 4.88 (m, 1 H, H-7), 4.83 (m, 2 H, H-3′ and H-4′), 4.55 (d, 2 H, H-14, J = 5.9 Hz), 4.28 (m, 1 H, H-5′), 3.95 (s, 3 H, $ArOCH_3$), 3.37 (AB q, 2 H, $ArCH_2CO$, J_{AB} = 13.8 Hz), 2.91 (AB q, 2 H, H-10, J_{AB} = 18.3 Hz), 2.10 (m, 2 H, H-8), 1.85 (m, 2 H, H-2′ A), 1.40 (m, 1 H, H-2′ B), 1.1 (d, 3 H, H-6′, J = 6.5 Hz). Anal. ($C_{35}H_{36}NO_{12}$·0.5$CHCl_3$) C, H, N.

4-((9-Fluorenylmethoxy)carboxamido)phenylacetic Acid *N*-Hydroxysuccinimidate (7). An ice-cold creamy suspension obtained by the addition in portions of 9-fluorenylmethyl chloroformate (2.6 g, 1 equiv) to a solution of 4-aminophenylacetic acid (1.51 g, 10 mmol) in 10% solution of sodium carbonate in H_2O (26.5 mL) was stirred for 4 h and then allowed to warm to ambient temperature. After being stirred for another 12 h, the reaction mixture was filtered and the white cake was washed with H_2O (50 mL) and dried in vacuo over P_2O_5 to give 3.94 g (quantitative yield) of the sodium salt of Fmoc derivative. A portion of the sodium salt (372 mg, 1 mmol) was used to prepare the *N*-hydroxysuccinimidate ester through the reaction with *N*,*N*-disuccinimidyl carbonate (307 mg, 1.2 equiv) in ice-cold DMF (1 mL). After 1 h, TLC with MeOH–CH_2Cl_2 (3:97) indicated completion of the reaction with the formation of a major less polar product. The residue obtained after evaporating DMF was dissolved in EtOAc (50 mL) and washed with H_2O (3 × 50 mL). The EtOAc solution was dried (Na_2SO_4) and evaporated, and the white crystalline material obtained by treatment of the residual gum with ether (30 mL) was separated by filtration to give 270 mg (57% yield) of the active ester 7: MS, M + H = 471; ^1H NMR (DMSO-d_6) δ 7.91–7.21 (m, 12 H, Ar-H), 4.49 (d, 2 H, Fmoc CH_2, J = 6.6 Hz), 4.30 (t, 1 H, Fmoc CH, J = 6.6), 4.01 (s, 2 H, $PhCH_2$), 3.34 (s, 4 H, NHS CH_2). Anal. ($C_{27}H_{22}N_2O_6$) C, H, N.

***N*-(4-Aminophenylacetyl)doxorubicin (3).** A red suspension of 1 (324 mg, 0.559 mmol) and 7 (320 mg, 1.2 equiv) in anhydrous DMF (19 mL) was treated with diisopropylethylamine (0.1 mL). After 18 h, TLC ($CHCl_3$–MeOH–H_2O–HCOOH, 130:60:10:4) revealed completion of the reaction with the formation of a single less polar product. The solution was cooled on an ice bath, morpholine (1.9 mL) was added, and the ice bath was removed. After 3 h, TLC (as above) indicated complete conversion to a more polar product. The reaction mixture was concentrated under reduced pressure to a small volume and diluted with 30% MeOH in $CHCl_3$ (30 mL), and silica gel (10 g, gravity grade) was added. The suspension was dried under reduced pressure, and the resulting red powder was layered on a silica gel column equilibrated with EtOAc. The column was eluted with EtOAc (200 mL) and then with MeOH–EtOAc (1:9, 900 mL). Fractions containing 3 were combined and evaporated in vacuo. A solution of the residue in 10% MeOH in $CHCl_3$ (39 mL) was treated with 4 M HCl in dioxane (0.1 mL) and diluted to 120 mL with $CHCl_3$. The red precipitate was filtered and dried over P_2O_5 to give 146 mg of 3 as the HCl salt (39% yield): MS M–H = 675; ^1H NMR (DMSO-d_6) δ 13.96 (s, 1 H, ArOH), 13.23 (s, 1 H, ArOH), 10.1 (s, 1 H, OH), 7.89 (m, 2 H, H-1 and OH), 7.76 (t, 1 H, H-2, J = 8.0 Hz), 7.60 (d, 1 H, H-3, J = 8.0 Hz), 7.24 (AB, 4 H, *p*-C_6H_4, J_{AB} = 8.7 Hz), 5.29 (d, 1 H, H-1′, J = 3.1 Hz), 5.02 (m, 1 H, H-7), 4.59 (s, 2 H, H-14), 4.11 (m, 1 H, H-5′), 3.98 (s, 3 H, OCH_3), 2.96 (AB, 2 H, H-10, J_{AB} = 18.6 Hz), 2.10 (m, 2 H, H-8), 1.86 (m, 1 H, H-2′A), 1.51 (m, 1 H, H-2′B), 1.14 (d, 3 H, H-6′, J = 6.6 Hz). Anal. ($C_{35}H_{36}N_2O_{12}$·HCl·3H_2O) C, N; H: calcd, 5.65; found, 4.95.

Phosphotriester 8. To a stirred solution of 4-hydroxyphenylacetic acid *N*-hydroxysuccinimidate ester[7] (1.88 g, 7.55 mmol) in EtOAc (75 mL) containing 2 equiv of pyridine was added diphenyl phosphorochloridate and type 3A molecular sieves (~5 g). The mixture was stirred at room temperature under N_2 for 24 h. More phosphorylating reagent (0.85 mL) and pyridine (0.85 mL) were added, and the mixture was stirred for a further 18 h at which point TLC (EtOAc–CH_2Cl_2, 3:7) of the supernatant indicated complete conversion to a less polar compound. The reaction mixture was filtered, and the filtrate was washed with 1 N HCl, H_2O, and dried (Na_2SO_4). After evaporation of EtOAc, the residue was purified by flash chromatography with EtOAc–CH_2Cl_2 to give 2.35 g (65%) of phosphotriester 8: MS M + H = 482; ^1H NMR ($CDCl_3$) δ 7.36–7.03 (m, 14 H, ArH), 3.90 (s, 2 H, $ArCH_2$), 2.81 (s, 4 H, $CO(CH_2)_2CO$). Anal. ($C_{24}H_{20}NO_8P$) C, H, N.

***N*-(4-(Phosphonooxy)phenylacetyl)doxorubicin (4).** Compound 8 (2.3 g, 4.78 mmol) in THF–*i*-PrOH (25:67) was reduced with H_2 and 130 mg of PtO_2 at atmospheric pressure for 6 h, and the black suspension was then filtered through Celite. After the solvent was evaporated under reduced pressure, a portion of the residue (380 mg, 1.16 mmol, 1.3 equiv) was dissolved in DMF (8 mL). This was added to a red solution of 1 (525 mg, 0.90 mmol) in DMF (9 mL) containing 1.2 equiv of Et_3N. The reaction was complete according to TLC ($CHCl_3$–MeOH–H_2O–HCOOH, 130:60:10:4) after 6 h in the dark. The solvent was evaporated, and the residue was triturated with EtOAc (100 mL). The EtOAc solution was decanted, and a solution of the residual red gum in MeOH–H_2O (1:4) was treated with NaI (270 mg, 2 equiv). The cloudy mixture was filtered, and the filtrate was subjected to chromatography on C-18 silica gel. A sintered-glass funnel was packed with C-18 silica gel (100 g), and the bed of C-18 was washed under aspirator pressure first with MeOH and then with H_2O. The filtrate was applied to the C-18, and the column was washed under aspirator vacuum with H_2O (150 mL). The desired product (4) was obtained by eluting the column with MeOH–H_2O (3:7, 4 × 100-mL fractions) and MeOH–H_2O (1:1, 2 × 100-mL fractions). The solvent was evaporated, and the residue obtained was redissolved in H_2O and lyophilized to give 4 (568 mg) as the monosodium triethylammonium salt: MS (M – Na^+) = 778; ^1H NMR (DMSO-d_6) δ 13.97 (s, 2 H, ArOH), 7.83 (m, 2 H, H-1 and OH or NH), 7.74 (d, 1 H, H-3, J = 8.0 Hz), 7.58 (m, 1 H, H-2), 7.00 (AB, 4 H, *p*-C_6H_4, J_{AB} = 8.5 Hz), 5.21 (br s, 1 H, H-1′), 4.90 (m, 1 H, H-7′), 4.55 (s, 2 H, H-14), 4.13 (m, 1 H, H-5′),

3.94 (s, 3 H, $ArOCH_3$), 2.91 (m, 8 H, CH_2 of Et_3N and H-10), 2.13 (m, 2 H, H-8), 1.85 (m, 1 H, H-2'A), 1.42 (m, 1 H, H-2'B), 1.08 (m, 12 H, CH_3 of Et_3N and H-6'). Anal. ($C_{35}H_{35}NO_{16}P\cdot(C_2H_5)_3N\cdot Na\cdot H_2O$) C, H, N.

***N*-(Phenylacetyl)melphalan (6).** To an ice-cold stirred suspension of melphalan hydrate (49.4 mg, 152 mmol) in H_2O (1 mL) was added $NaHCO_3$ (21 mg) and acetone (1 mL). The solution was then treated with phenylacetyl chloride (1.3 equiv) in portions until the reaction was complete as indicated by TLC (EtOAc–MeOH–AcOH, 8:1:1). At the end of the reaction, 1.2 N HCl (0.3 mL) was added. After centrifugation, the precipitate was washed with H_2O (2 × 4 mL). Crystallization from ethanol gave 26 mg (40% yield) of 6 as white lustrous crystals: MS M + K^+ = 461; ^1H NMR (DMSO-d_6) δ 8.38 (d, 1 H, NH, J = 8.2 Hz), 7.18 (m, 5 H, C_6H_5), 6.80 (dd, 4 H, p-C_6H_4, J = 8.7 Hz), 4.34 (m, 1 H, CH), 3.71 (s, 8 H, $N(CH_2CH_2Cl)_2$), 3.40 (AB, 2 H, CH_2CO, J_{AB} = 13.9 Hz), 2.90 (m, 2 H, CH_2CHCO). Anal. ($C_{21}H_{24}N_2O_3Cl_2$), C, H, N.

Kinetic Analyses. A 0.1 mM solution of the prodrug in 10% DMSO, 90% phosphate buffered saline pH 7.2 (consisting of 0.14 M NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 8 mM Na_2HPO_4) and 0.1 mg/mL of bovine serum albumin at 37 °C was treated with PGA (final concentration 10 μg/mL for 3 and 4 and 1 μg/mL for 6). In one experiment, calf intestinal alkaline phosphatase (final concentration 10 μg/ml) was added to 4 along with PGA. Aliquots at various times were diluted with equal volumes of acetonitrile and centrifuged, and the supernatants (25 μL) were then analyzed by reversed-phase HPLC using a C-18 column. Doxorubicin derivatives were estimated by absorbance at 495 nm (AUFS = 0.2). For 5 and 6, a detector wavelength of 280 nm (AUFS = 0.5) was used. Compound 3 was eluted with CH_3CN–8.5 mM $HCOONH_4$ (3:7, pH 4.0). Under these conditions the retention times (t_R) of 1 and 3 were 6.0 and 3.4 min, respectively. A linear gradient at 1 mL/min consisting of 80–100% solvent B against solvent A was used for the analysis of 4 (solvent A = 0.025 M $(n\text{-}C_4H_9)_4NHSO_4$ and 0.05 M each of KH_2PO_4 and K_2HPO_4 (pH = 7), solvent B = 50% CH_3CN in solvent A, t_R for 1 = 3.4 min, t_R for 4 = 5.3 min). Solvent B alone was used for the analysis of compound 6 (t_R for 5 = 4.2 min, t_R for 6 = 6.6 min).

Cytotoxicity Assays. H2981 cells (10^7 cells/0.2 mL) were incubated at 4 °C in IMDM (Iscove's modified Dulbeco's medium with 10% fetal bovine serum v/v) or in IMDM containing L6–PGA at 0.1 mg/mL. After 30 min, the cells were washed with IMDM (3 × 2 mL), treated with prodrug, drug, or medium (0.2 mL of IMDM), and incubated at 37 °C for 4 h. The cells were washed again with medium (3 × 1 mL) and counted, and triplicate samples were added to a 96-well plate (0.1 mL, 10^5 cells/mL). Following incubation at 37 °C for 24 h, the cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 24 h. The cells were harvested after detachment by trypsin/EDTA using an LKB WALLAC 1295-001 cell harvestor. Incorporation of [^{3}H]thymidine was quantified with an LKB WALLAC 1205 liquid scintillation counter. In experiments to demonstrate specificity, cells were treated with L6 (0.1 mL, 1 mg/mL) at 4 °C for 15 min prior to conjugate addition.

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