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Antibody-penicillin-V-amidase conjugates kill antigen-positive tumor cells when combined with doxorubicin phenoxyacetamide

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Summary. The two monoclonal antibodies (mAb), L6 (anti-carcinoma), and 1F5 [anti-(B-cell-lymphoma)], were chemically linked to the enzyme penicillin-V amidase (PVA), which hydrolyzes phenoxyacetamides, to explore the potential of using mAb-enzyme conjugates for the localizaton of chemotherapeutic drugs at tumor cells. The phenoxyacetamide derivatives of doxorubicin and melphalan were prepared, yielding the less toxic amides, doxorubicin-*N*-*p*-hydroxyphenoxyacetamide (DPO) and melphalan-*N*-*p*-hydroxyphenoxyacetamide (MelPO). These were hydrolyzed by PVA to doxorubicin and melphalan respectively. *In vitro* studies with the L6-positive lung carcinoma cell line, H2981, and the 1F5-positive B-cell lymphoma line, Daudi, showed that DPO was 80-fold less toxic to H2981 cells and 20-fold less toxic to Daudi cells than doxorubicin, and its toxicity was substantially increased when the H2981 cells were pretreated with L6-PVA or the Daudi cells were pretreated with 1F5-PVA. The cytotoxic effect was antigen-specific, since only the binding mAb-enzyme conjugate increased the cytotoxicity of the prodrug. MelPO was more than 1000-fold less toxic than melphalan to H2981 cells and more than 100-fold less toxic than melphalan to Daudi cells. Pretreatment with the mAb-PVA conjugates did not enhance the toxicity of MelPO in either cell line, because PVA hydrolyzes the phenoxyacetamide bond of MelPO too slowly to generate a toxic level of melphalan.

Introduction

Much work has been done using monoclonal antibodies (mAb) to tumor cell-surface antigens as carriers of antitumor agents, such as chemotherapeutic drugs and toxins [4, 7–9, 11, 21, 22, 24, 26], in order to increase the relative amount of the therapeutic agent at the tumor site compared to normal tissue. Although some promising results with this approach have been obtained [4, 8, 20], several obstacles must be overcome for the treatment of solid tumors.

Solid tumors are poorly vascularized, and a number of physiological barriers prevent large macromolecules, such as mAb-drug or mAb-toxin conjugates, from penetrating efficiently throughout the tumor mass [12]. The heterogeneity of target antigen expression in tumor cells can lead to some cells escaping the cytotoxic effects of the conjugates [8, 23]. Limitations in drug potency [7, 21] can result in a sub-optimal therapeutic effect. Other problems are presented by the necessity of delivering the targeted agent to its precise site of activity within the cell, since many mAb are not efficiently internalized [13], or are directed to cellular compartments where the drug or toxin is not active [4, 16, 24].

We, and another group, have recently developed a strategy for the delivery of cytotoxic agents to tumor cells that involves the use of targeted enzymes for the activation of prodrugs [1, 17, 18]. In this approach, mAb are used to deliver enzymes to the tumor cells, and then relatively non-toxic prodrugs are administered. The prodrugs are converted by the enzyme into cytotoxic agents at the tumor cell surfaces, and are then taken up by the cells via the same mechanisms as when the agents are used in their free form. This approach has been designed to overcome the limitations described earlier.

In the work presented here, we report the use of conjugates of mAb and penicillin-V-amidase (PVA) for prodrug activation. PVA is a readily available enzyme [15] that is used industrially to hydrolyze the phenoxyacetamide group of penicillin-V, forming 6-aminopenicillanic acid. We have prepared the *p*-hydroxyphenoxyacetamide derivatives, DPO and MelPO, from the anticancer drugs doxorubicin and melphalan and tested the ability of the mAb-PVA conjugates to release doxorubicin from DPO and melphalan from MelPO. The cytotoxic effects of mAb-PVA/prodrug combinations against carcinoma and lymphoma cell lines are reported.

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Materials and methods

Materials. The antibodies used were L6 (an IgG_{2a}), which binds to an antigen on human lung carcinomas [10], and 1F5 (an IgG_{2a}), which is specific for the CD-20 antigen on normal and neoplastic B cells [5]. PVA, from *Fusarium oxysporum* [15] (specific activity 3 units/mg for the hydrolysis of penicillin-V, 1 unit being the hydrolysis of 1 μmol substrate to product/min) was supplied by Bristol-Myers (Syracuse, NY). H2981 is a lung adenocarcinoma cell line established at Oncogen [10]. L6 binds to these cells ($K_a \approx 3 \times 10^8$ M^{-1}, saturation at approximately 10 μg/ml), while 1F5 does not bind to H2981 cells. The lymphoma cell line, Daudi, was obtained from ATCC. 1F5 binds to these cells ($K_a = 10^8$ M^{-1}, saturation at approximately 10 μg/ml), while L6 does not.

Succinimidyl 4-(*N*-meleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and 2-iminothiolane hydrochloride were obtained from Pierce (Northridge, Ill). Doxorubicin was obtained from Bristol-Myers, melphalan from Sigma (St. Louis, Mo), *N*-hydroxysuccinimide, 4-hydroxyphenoxyacetic acid, and dicyclohexylcarbodiimide from Aldrich (Milwaukee, Wis), and [^{3}H]thymidine from New England Nuclear (Boston, Mass). The Iscove's modified Dulbecco's medium (IMDM) was from Irvine Scientific (Santa Ana, Calif), fetal bovine serum from Hyclone Laboratories (Logan, Utah), and the 96-well microtiter plates from Becton-Dickinson (Lincoln Park, NJ).

Preparation of mAb-PVA conjugates. L6 and 1F5 were derivatized with 2-iminothiolane hydrochloride as previously described [13]. The number of sulfhydryl groups introduced was determined to be one to two. PVA was dissolved at 9 mg/ml in phosphate-buffered saline (PBS) (0.01 M phosphate, 0.14 M NaCl), to which was added SMCC (100 mM in dimethylformamide) at a final concentration of 5 mM. After reacting for 30 min at 30° C, excess SMCC was removed on a Sephadex G-25 column and the modified enzyme was added to the thiolated mAb in a 3 : 1 molar ratio. The number of maleimides per PVA was three to four. The solution was purged with nitrogen and left at room temperature for 3 h, followed by an additional 18 h at 4° C. At this time, unreacted maleimides were quenched with 2-aminoethanethiol (1 mM final concentration), and the buffer was exchanged on a Sephadex G-25 column, which was eluted with 20 mM TRIS, 50 mM NaCl, pH 7.2. Unreacted mAb was separated from conjugates and unconjugated enzyme by ion-exchange chromatography on a DEAE-Sephadex column. The unconjugated mAb was eluted from the column with 20 mM TRIS, 50 mM NaCl, pH 7.2, after which the conjugates and unreacted PVA were eluted with 20 mM TRIS, 0.5 M NaCl, pH 7.2. Fractions containing PVA and conjugate were then concentrated and applied to a Sephacryl S-300 column. Fractions containing 1 : 1 mAb : PVA adducts, as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (4%–12% gradient gel), were pooled.

Characterization of conjugates. The enzyme activities of the conjugates were determined using penicillin-V as a substrate [2]. Competitive antigen-binding activity was measured against fluorescein-isothiocyanate (FITC)-labeled L6 on H2981 cells and against FITC-labeled 1F5 on Daudi cells using a Coulter Epics-C flourescence analyzer. H2981 or Daudi cells were suspended in 0.5 ml IMDM with 10% fetal bovine serum (2.5×10^5 cells/tube). To each tube of H2981 cells was added L6-FITC to a final concentration of 40, 32, 24, 16, 8, or 0 μg/ml and one of the conjugates to be tested to a final concentration of 0, 8, 16, 24, 32, or 40 μg/ml. Each tube of Daudi cells was treated the same except that 1F5-FITC was used. The total amount of mAb was maintained at 40 μg/ml. The cells were incubated at 4° C for 30 min and washed with IMDM with 10% fetal bovine serum. L6-FITC or 1F5-FITC binding was then measured and the percentage binding was calculated and compared to L6 and 1F5.

Synthesis of DPO and MelPO. *p*-Hydroxyphenoxyacetic acid (84 mg, 0.5 mmol), *N*-hydroxysuccinimide (57 mg, 0.5 mmol), and dicyclohexylcarbodimide (100 mg, 0.5 mmol) were mixed in 5 ml tetrahydrofuran. This was stirred for 1 h, filtered, and the filtrate was added to 200 mg (0.35 mmol) doxorubicin hydrochloride and 73 mg (0.7 mmol) triethylamine. After 4 h, the reaction mixture was filtered, concentrated, and purified on a SiO_2 column, which was eluted with 95:5 dichloromethane: methanol to yield 140 mg DPO as a red solid (58% yield). The mass spectrum calculated for $C_{35}H_{36}NO_{14}$ was 694.2136 (observed 694.2125). The ^{1}H NMR (dimethylsulfoxide-d_6) was consistent with the proposed structure. *p*-Hydroxyphenoxyacetic acid (250 mg, 1.5 mmol), *N*-hydroxysuccinimide, (170 mg, 1.5 mmol), and dicyclohexylcarbodiimide, (280 mg, 1.5 mmol), were mixed in 10 ml tetrahydrofuran. This was stirred for 1 h, filtered, and the filtrate was added to 300 mg (1.0 mmol) melphalan hydrochloride and 300 mg (2.0 mmol) triethylamine. After 10 h, the reaction mixture was filtered, concentrated, and purified on a SiO_2 column, which was eluted with 9:1 dichloromethane: methanol with 0.1% acetic acid to yield 260 mg MelPO as a white solid (68% yield). The mass spectrum calculated for $C_{18}H_{20}Cl_2N_2O_3$ was 383.0931 (observed 383.0937). The ^{1}H NMR (dimethylsulfoxide-d_6) was consistent with the proposed structure.

Reaction of PVA and mAb-PVA conjugates with DPO and MelPO. Solutions of DPO (10 mM in dimethylformamide) were diluted to 0.1 mM with PBS and to these were added PVA, L6-PVA, or 1F5-PVA (final concentration of PVA 10 μg/ml). The hydrolysis was monitored by HPLC using a C-18 column (3 μm, 4.5 × 100 mm) and a 15-min gradient elution of 20%–60% buffer A (tetrahydrofuran, 0.1% concentrated H_3PO_4) in buffer B (H_2O, 0.1% concentrated H_3PO_4) (1.0 ml/min, monitored at 495 nm). Under these conditions the doxorubicin eluted at 8.9 min and the DPO eluted at 12.2 min. Solutions of MelPO were prepared the same as for DPO, and PVA, L6-PVA, and 1F5-PVA were added (final concentration of PVA 100 μg/ml). The hydrolysis was monitored by HPLC using the same column and a 20-min gradient elution of 40%–100% buffer A (methanol, 0.1% trifluoroacetic acid) in buffer B (H_2O, 0.1% trifluoroacetic acid) (1.0 ml/min, monitored at 254 nm). Under these conditions melphalan eluted at 13.0 min and MelPO eluted at 13.9 min. The hydrolysis rates of DPO and MelPO in PBS and mouse serum were also tested. In addition, the hydrolysis of DPO in human plasma was also determined. Prior to HPLC analysis, the serum and plasma proteins were precipitated from these samples by adding an equal volume of methanol; the samples were centrifuged and supernatant was collected.

In-vitro cytotoxicity assays. H2981 and Daudi cells were plated into 96-well microtiter plates in IMDM with 10% fetal bovine serum (10000 cells/well). Since H2981 cells are adherent, they were allowed to attach for 18 h at 37° C and the nonadherent Daudi cells were used immediately. The mAb conjugates were then added at a concentration of 10 μg/ml mAb in IMDM and incubated for 30 min at 4° C. The wells were washed four times with IMDM and the compounds were added at specified concentrations in IMDM. After 2 h for DPO and 4 h for MelPO, the wells were washed again, IMDM was added, and the cells were incubated at 37° C. After a total of 18 h, [^{3}H]thymidine was added (1 μCi/well, 1 μCi = 37 kBq), and 6 h later the Daudi cells were harvested onto glass-fiber filters. The plates with H2981 cells were frozen at –70° C to halt incorporation of [^{3}H]thymidine and to detach the cells. The H2981 cells were then thawed and harvested onto glass-fiber filters. The incorporation of [^{3}H]-thymidine was measured in a Beckman 3801 scintillation counter and compared to that of untreated cells.

Results

Preparation and characterization of conjugates and drug derivatives

PVA (70 kDa) was linked to the mAb, L6 and 1F5, via a thioether bond. This was done by modifying the mAb with 2-iminothiolane hydrochloride (Traut's reagent) and PVA with SMCC [13]. A two-stage purification procedure yielded the desired conjugates (ratio of 1 : 1 mAb : PVA), which were free of aggregates, unreacted mAb, or unre-

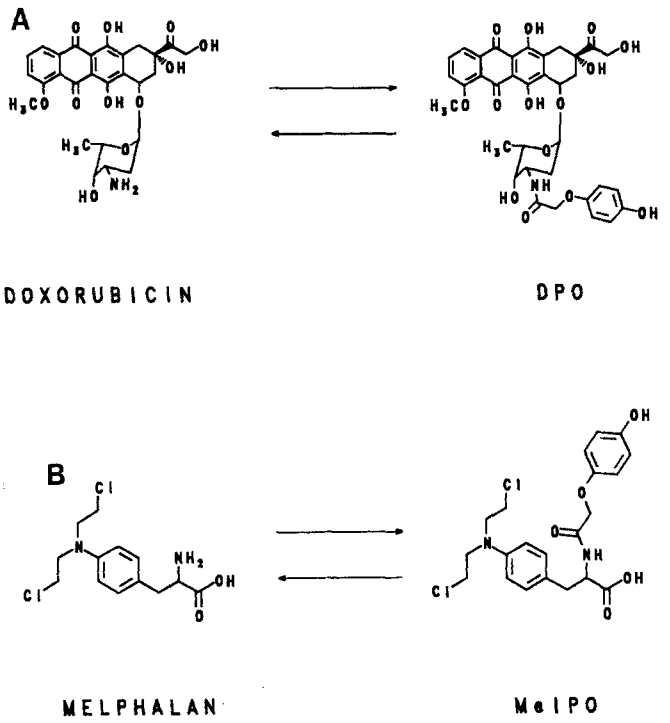


Fig. 1. A Reaction of doxorubicin with *p*-hydroxyphenoxyacetyl-*N*-hydroxysuccinimide, and the hydrolysis of doxorubicin-*N*-hydroxyphenoxy-acetamide (*DPO*) catalyzed by penicillin-V amidase (PVA). B Reaction of melphalan with *p*-hydroxyphenoxyacetyl-*N*-hydroxysuccinimide, and the hydrolysis of melphalan-*N*-*p*-hydroxyphenoxyacetamide (*MelPO*) catalyzed by PVA

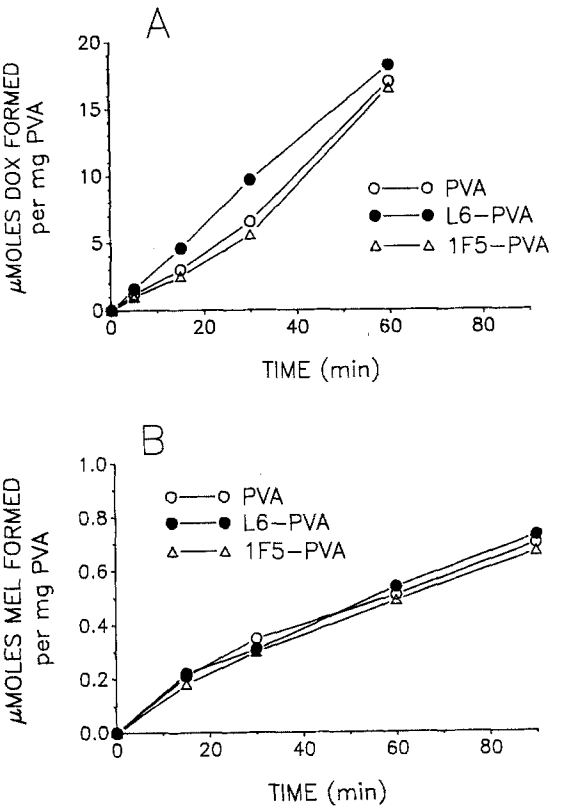


Fig. 2 A, B. Reaction of DPO and MelPO with PVA or the mAb-PVA conjugates. A DPO was treated with PVA, L6-PVA, or 1F5-PVA at PVA concentrations of 10 μg/ml. B MelPO was treated with PVA, L6-PVA, or 1F5-PVA at PVA concentrations of 100 μg/ml. These reactions were monitored by HPLC, as outlined in Materials and methods

acted PVA. The yield of conjugates was approximately 20%, based on mAb. Fluorescence-activated cell sorting analysis indicated that L6 and L6-PVA bound equally well to H2981 cells, and that 1F5 and 1F5-PVA bound equally well to Daudi cells. There was no apparent binding of 1F5 or 1F5-PVA to H2981 cells or of L6 or L6-PVA to Daudi cells. The conjugates retained all enzymatic activity, as established by their ability to hydrolyze penicillin-V to 6-aminopenicillanic acid at the same rate as unmodified PVA (3 units/mg PVA).

The syntheses and enzymatic hydrolyses of DPO and MelPO are shown in Fig. 1. These compounds were prepared in moderate yield and ^{1}H-NMR and high-resolution mass spectrometry were used to confirm their structures.

Reactivity of DPO and MelPO

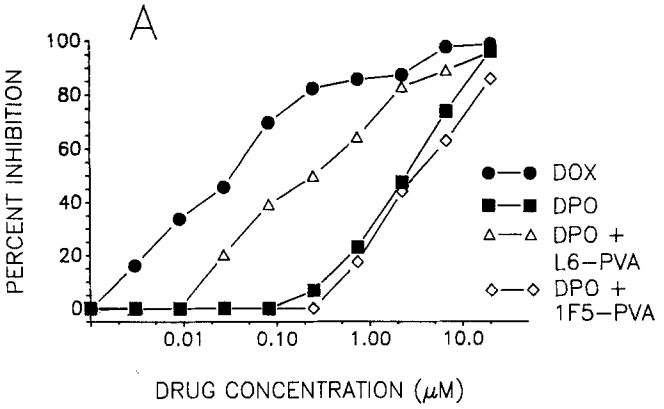
HPLC analysis indicated that DPO was hydrolyzed by PVA, producing doxorubicin. PVA and the mAb-PVA conjugates, at 10 μg/ml enzyme, were able to hydrolyze 0.1 mM solutions of DPO at comparable rates (Fig. 2A). PVA catalyzed the hydrolysis of DPO to doxorubicin at a rate of 0.25 units/mg enzyme (8% of the rate with penicillin-V as the substrate).

MelPO was also hydrolyzed by PVA, yielding melphalan. PVA and the mAb-PVA conjugates, at 100 μg/ml enzyme, hydrolyzed 0.1 mM solutions of MelPO to melphalan (Fig. 2B). In this case the activity of the enzyme was only 0.01 unit/mg enzyme (0.3% of the rate with penicillin-V as the substrate).

DPO and MelPO were tested for their stabilities in PBS and mouse serum (also in human plasma for DPO). There was no detectable hydrolysis of DPO or MelPO after 2 h and 6 h, respectively. Thus, these derivatives were stable in the absence of PVA and the mAb-PVA conjugates were able to effect their hydrolyses.

In vitro cytotoxicity

The cytotoxic effects of DPO, alone, or with mAb-PVA, on H2981 cells (L6 positive, 1F5 negative) and Daudi cells (1F5 positive, L6 negative) was determined by measuring the incorporation of [^{3}H]thymidine into DNA, Doxorobucin (IC_{50} = 30 nM) was 80-fold more toxic to H2981 cells than DPO (IC_{50} = 2.5 μM) (Fig. 3A). Pretreatment of H2981 cells with saturating amounts of L6-PVA, followed by DPO, enhanced the toxicity 12.5-fold to an IC_{50} of 200 nM, while pretreatment of the H2981 cells with an equivalent amount of 1F5-PVA, prior to DPO, did not affect the toxicity. Doxorubicin was also more toxic to Daudi cells (IC_{50} = 70 nM) than DPO (IC_{50} = 1.5 μM) (Fig. 3B). Pretreatment of the Daudi cells with saturating



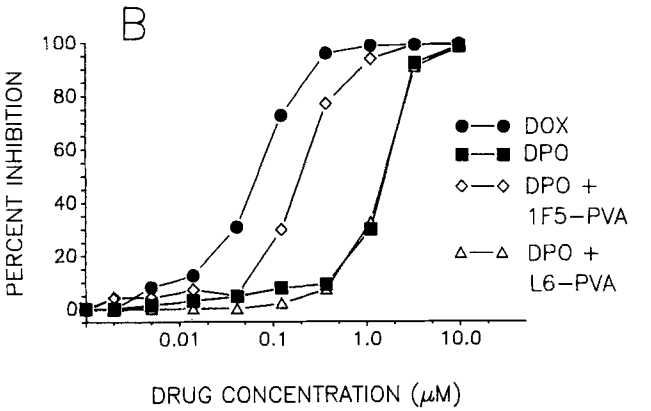
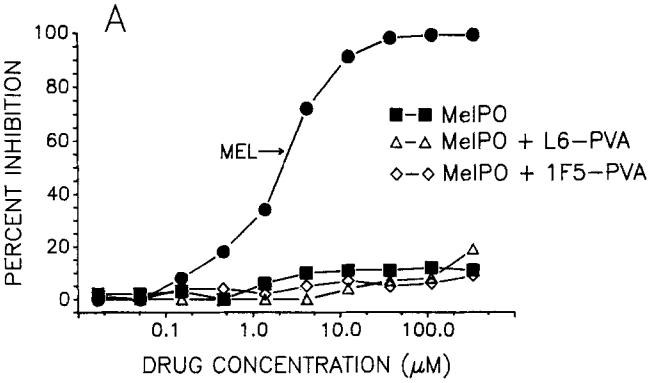


Fig. 3 A, B. Cytotoxic effects of doxorubicin (*DOX*), DPO, and DPO plus the conjugates as determined by the inhibition of [^{3}H]thymidine incorporation into DNA. Details of the experiment are in Materials and methods. **A** H2981 cells. **B** Daudi cells



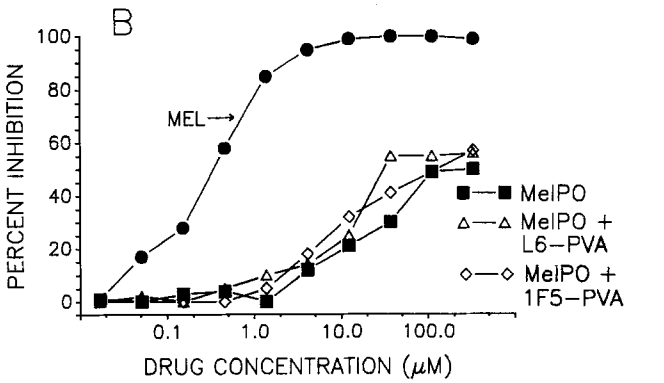


Fig. 4 A, B. Cytotoxic effects of melphalan (*MEL*), MelPO, and MelPO plus the conjugates as determined by the inhibition of [^{3}H]thymidine incorporation into DNA. Details of the experiment are in Materials and methods. **A** H2981 cells. **B** Daudi cells

amounts of 1F5-PVA, prior to treatment with DPO, resulted in an IC_{50} of 200 nM, while pretreatment with an equivalent amount of L6-PVA had no effect.

Melphalan had an IC_{50} of 2 μM and 0.3 μM on H2981 cells and Daudi cells, respectively, while MelPO showed a much reduced level of cytotoxicity at the concentrations tested (Fig. 4). Pretreatment of the cells with the conjugates did not enhance the toxicity of MelPO.

Discussion

Doxorubicin and melphalan are widely used antitumor agents that have activities against a variety of neoplasias [6, 25]. The efficacies of the agents are limited by side-effects that include cardiotoxicity, myelosuppression, and gastrointestinal toxicity.

Amide derivatives of doxorubicin [3, 14] and melphalan [19] have much reduced cytotoxicity in vitro and are less active than the parent drugs against in vivo tumor models. The phenoxyacetamide derivatives, DPO and MelPO, described in this study were therefore expected to be much less toxic than doxorubicin and melphalan, respectively. Hydrolysis by the targeted enzyme, PVA, was anticipated to lead to the formation of the active drugs.

In vitro cytotoxicity studies showed that DPO and MelPO were much less toxic than their corresponding parent drugs (Figs. 3 and 4). Treatment of cultured tumor cells with mAb-PVA conjugates that could bind to antigens on the cell surfaces, prior to treatment with DPO, resulted in a significant increase in cytotoxicity, compared to DPO alone. As shown in Fig. 2, the hydrolysis, of DPO by PVA-containing conjugates liberates doxorubicin over a period of time, so that the cells are exposed to increasing levels of doxorubicin during the incubation with the prodrug. This might explain why the cytotoxic effect is slightly less than that observed for doxorubicin alone. Treatment with the non-binding mAb-PVA conjugate did not enhance the toxicity.

The mAb-PVA conjugates did not increase the toxicity of MelPO, owing possibly to the fact that the rate of which PVA hydrolyzes MelPO to melphalan is too slow to generate a cytotoxic concentration of this drug. Thus, we have indications that, depending on the inherent toxicity of the drug, there is a minimum specific activity requirement for an enzyme to make it useful for the targeting strategy described here. In vivo experiments to test the efficacy of mAb-PVA/prodrug combinations will be the subject of future investigations.

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