Matrix metalloproteinase–activated doxorubicin prodrugs inhibit HT1080 xenograft growth better than doxorubicin with less toxicity

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Abstract

Matrix metalloproteinase (MMP)–activated prodrugs were formed by coupling MMP-cleavable peptides to doxorubicin. The resulting conjugates were excellent in vitro substrates for MMP-2, -9, and -14. HT1080, a fibrosarcoma cell line, was used as a model system to test these prodrugs because these cells, like tumor stromal fibroblasts, expressed several MMPs. In cultured HT1080 cells, simple MMP-cleavable peptides were primarily metabolized by neprilysin, a membrane-bound metalloproteinase. MMP-selective metabolism in cultured HT1080 cells was obtained by designing conjugates that were good MMP substrates but poor neprilysin substrates. To determine how conjugates were metabolized in animals, MMP-selective conjugates were given to mice with HT1080 xenografts and the distribution of doxorubicin was determined. These studies showed that MMP-selective conjugates were preferentially metabolized in HT1080 xenografts, relative to heart and plasma, leading to 10-fold increases in the tumor/heart ratio of doxorubicin. The doxorubicin deposited by a MMP-selective produrg, compound 6, was more effective than doxorubicin at reducing HT1080 xenograft growth. In particular, compound 6 cured 8 of 10 mice with HT1080 xenografts at doses below the maximum tolerated dose, whereas doxorubicin cured 2 of 20 mice at its maximum tolerated dose. Compound 6 was less toxic than doxorubicin at this efficacious dose because mice treated with compound 6 had no detectable changes in body weight or reticulo-cytes, a marker for marrow toxicity. Hence, MMP-activated doxorubicin prodrugs have a much higher therapeutic index than doxorubicin using HT1080 xenografts as a preclinical model. [Mol Cancer Ther 2005;4(5):751–60]

Introduction

Doxorubicin is an anthracycline natural product that is frequently used to treat breast cancer, liver cancer, soft-tissue sarcomas, and non-Hodgkin’s lymphoma (reviewed in ref. 1). Doxorubicin kills tumor cells by causing topoisomerase II–stimulated DNA strand breaks and potentially other mechanisms (reviewed in ref. 2). Like other cytotoxic drugs, doxorubicin doses are limited by unwanted toxicity to nontumor tissues. Myelosuppression is the dose-limiting toxicity for doxorubicin, although stomatitis, mucositis, and alopecia are also frequently observed. In addition to these typical chemotherapeutic toxicities, doxorubicin causes cardiomyopathy that depends on the cumulative dose of doxorubicin.

Matrix metalloproteinases (MMP) are a family of >20 enzymes (reviewed in ref. 3). MMPs are synthesized as inactive proenzymes that become activated by proteolysis. For instance, the secreted enzyme pro-MMP-2 is proteolyzed and activated by the membrane-anchored MMP-14 (4). Once activated, MMPs can cleave a variety of extracellular matrix proteins, such as collagen, laminin, fibronectin, and elastin, which are potentially important for tumor angiogenesis, invasion, and metastasis. Additionally, MMPs cleave a variety of other proteins, such as growth factor receptors and cell adhesion molecules, which may be important for tumor growth and survival (reviewed in refs. 5, 6). MMPs can be inactivated by tissue inhibitors of MMPs, a family of four proteins, which bind tightly to MMPs.
Increasing the therapeutic index of doxorubicin could benefit cancer treatment by allowing greater concentrations of doxorubicin in tumors and thereby potentially greater tumor growth inhibition. Alternatively, increasing the therapeutic index of doxorubicin could allow reduced toxicities and thereby permit the use of other cytotoxics that would be prevented because of overlapping toxicities. One way to improve the therapeutic index of doxorubicin is to create prodrugs that are selectively activated in the tumor environment. MMPs are attractive enzymes to activate prodrugs in the tumor environment because MMPs are intimately connected with tumorigenesis. In particular, preclinical studies implicate MMPs in tumor progression (reviewed in ref. 6). For instance, overexpression of MMP-14 in mouse mammary tissue causes mammary tumors (7), whereas loss of MMP-2 or -9 reduced the lung colonization by B16-LBL6 melanoma cells and Lewis lung carcinoma cells (8, 9). Additionally, MMP expression in human tumors frequently correlates with disease progression (reviewed in ref. 6). For instance, one study found MMP-2 and -14 expression in 90% of breast carcinomas and a strong correlation between tumor cell membrane staining and lymph node metastases (10). Although there are strong connections between MMPs and tumor progression, published studies do not determine the absolute or relative MMP activity in the tumor environment. Such information is clearly critical to the design of MMP-activated prodrugs because the MMP enzyme activity must be sufficient to activate efficacious levels of prodrug.

Preclinical testing of MMP-activated prodrugs is complicated by differences between mouse and human tumors. Many human tumors, including breast cancers, are composed of roughly equal numbers of tumor cells and stromal cells, including stromal fibroblasts, inflammatory cells, and endothelial cells (reviewed in ref. 11). The interplay of these cell types is thought to be crucial for MMP activation in that different cell types express and activate specific MMPs (reviewed in ref. 6). For instance, stromal fibroblasts express MMP-2, yet tumor cells are the source of MMP-14 that convert pro-MMP-2 to active MMP-2. Unfortunately, mouse tumor models do not accurately reflect the cell types observed in human tumors because both xenografts and transgenic tumor models are composed primarily of rapidly growing tumor cells with relatively few stromal cells.

This study discovered MMP-activated doxorubicin prodrugs that had a greater therapeutic index than doxorubicin using HT1080 cells as a model system. In the absence of information about enzyme activity levels, prodrugs were identified that were efficiently cleaved by MMP-2, -9, and -14 because these enzymes were frequently overexpressed in human tumors, affected tumor progression in preclinical models, and were very active in vitro. Because these MMPs may have insufficient activity for prodrug activation in vivo, prodrug design was further constrained so that prodrugs were likely be activated by other MMPs, with the exception of MMP-11 (12), based on the known MMP substrate specificities (13). HT1080 cells were used as a model system because these cells grow as xenografts, are sensitive to doxorubicin, and express several different MMPs (14, 15). Furthermore, HT1080 cells are derived from a fibrosarcoma and were, therefore, hoped to mimic stromal fibroblasts, a major constituent of many human tumors. Using this strategy, prodrugs were identified that were excellent in vitro substrates for MMP-2, -9, and -14. Optimization of conjugate structure led to prodrugs that were selectively metabolized by MMPs in HT1080 cultures and preferentially deposited doxorubicin in HT1080 xenografts relative to heart tissue. The preferential doxorubicin deposition led to an improved therapeutic index, relative to doxorubicin, as shown by improved HT1080 xenograft growth inhibition with reduced toxicity.

Materials and Methods

Compound Synthesis

All peptides were synthesized following the same protocol. An example synthesis of compound 1 is provided. The peptide acid was synthesized on the solid phase starting with commercially available Fmoc-Leu-Wang resin (0.40 g, 0.25 mmol, Advance Chemtech, Louisville, KY). The synthesis was done on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) using standard Fmoc protocols. The completed peptide on resin was N-acetylated with acetic anhydride. The desired peptide was cleaved from the resin with 90% trifluoroacetic acid in water for 2 hours. After solvent removal, the peptide was dissolved in H2O:CH3CN and freeze dried. Product was confirmed by mass spectrometry (ES m/e calculated for C21H36N4O6 [M-H]-, 439.54; found, 439.3). Analytic high-performance liquid chromatography on a Metachem Monochrome C18 reverse-phase column (50 x 4.6 mm, MetaChem Technologies, Torrance, CA) showed crude peptide to be 85% pure. To this intermediate (19.9 mg, 40 µmol) dissolved in dimethylformamide (0.2 mL) in a small amber vial was added benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (20.8 mg, 40 µmol). Doxorubicin hydrochloride (18.6 mg, 32 µmol, Aldrich, Milwaukee, WI) was added as a suspension in dimethylformamide (0.1 mL) followed by diisopropylethylamine (0.0139 mL, 80 µmol). The reaction was stirred for 2 hours. Solvent was removed under vacuo. The sample was dissolved in H2O:CH3CN and purified using a Dynamax C18 reverse-phase column (41.4 x 250 mm, Varioan, Palo Alto, CA) with a linear gradient from 30% to 50% acetonitrile, 0.05% ammonium acetate over 20 minutes with a flow rate of 45 mL/min. Fractions were pooled and freeze dried to afford the purified peptide-doxorubicin conjugate (ES m/e calculated for C48H63N5O16 [M-H]-, 965.06; found, 964.6). Caution: Doxorubicin is a highly toxic compound. Appropriate measures should be taken to avoid exposure to the compound.

Enzyme Assays

Doxorubicin prodrugs were incubated with MMP-2, -9, or -14 or neprilysin to compare the competency of these conjugates as substrates for these enzymes. Recombinant
MMP-2, -9, and -14 were purified from HT1080 fibrosarcoma cells, insect cells, and Escherichia coli, respectively, as described previously (12, 16, 17). MMP-2 and -9 were activated with 4-aminophenylmercuric acetate for 2 hours at 37°C. Unreacted 4-aminophenylmercuric acetate was removed using Bio-Spin 6 columns (Bio-Rad, Hercules, CA). Neprilysin was purified from rat kidney as described (18). Conjugates (1 μmol/L) were incubated with either 10 nmol/L MMP-2, 2 nmol/L MMP-9, or 5 nmol/L MMP-14 in 50 mmol/L HEPES (pH 7.5), 10 mmol/L CaCl2, 0.1% Brij-35 at 37°C. At various times up to 60 minutes, an aliquot of the reaction was removed and injected into a Novapak C18 reverse-phase high-performance liquid chromatography column (Waters, Milford, MA). The substrate neprilysin in 50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L CaCl2, 0.1% Brij-35 and processed as above. The peak area was used to determine the $k_{cat}/K_m$ for each conjugate under first-order conditions (substrate << $K_m$):

$$k_{cat}/K_m = \ln(S_o/S_f)/(t \times (E)),$$

where $S_o$ is the initial substrate concentration, $S_f$ is the substrate concentration at various times, $t$ is time, and $E$ is enzyme concentration (19).

**Prodrug Metabolism in Cultured HT1080 Cells**

To measure the rate of prodrug metabolism in cell culture, actively growing HT1080 fibrosarcoma cells (American Type Culture Collection, Manassas, VA) were plated at 60% confluence. Two hours after plating, the cell medium (DMEM, 10% fetal bovine serum) was removed and replaced with DMEM containing 0.1% bovine serum albumin and 40 mmol/L neprilysin in 50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L CaCl2, 0.1% Brij-35 and processed as above. The substrate peak area was used to determine the $k_{cat}/K_m$ for each conjugate under first-order conditions (substrate << $K_m$):

$$k_{cat}/K_m = \ln(S_o/S_f)/(t \times (E)),$$

where $S_o$ is the initial substrate concentration, $S_f$ is the substrate concentration at various times, $t$ is time, and $E$ is enzyme concentration (19).

**Prodrug Metabolism in Mice**

HT1080 tumors were transplanted into naive mice using trocar needles. Dry conjugate powders were dissolved in $N,N$-dimethylacetamide and diluted with 5% dextrose to yield a final dosing solution of 10% $N,N$-dimethylacetamide, 4.5% dextrose. Mice were then given the desired dose using 0.1 mL dosing solution. Drugs were given i.v. into the tail vein. At the indicated times, three mice per time point were anesthetized with CO$_2$. Blood (~1 mL) was collected by cardiac puncture in a syringe containing 0.1 mL sodium citrate to prevent coagulation and cells were removed by centrifugation. The resulting plasma was transferred to another tube and frozen in liquid nitrogen. Tumor and heart tissue were removed, frozen in liquid nitrogen, and stored at ~80°C. For analysis, tissues were thawed on ice, weighed, and minced with scissors, and cold, citrated mouse plasma was added (Cocalico Biological, Reamstown, PA). Plasma was added at 5 mL/g for tumor tissue and 10 mL/g for heart tissue. Slurries were homogenized for 1 minute and 0.5 mL was transferred to an Eppendorf tube. Silver nitrate (0.1 mL, 33%) was added to the homogenate to improve the recovery of doxorubicin and the mixture was vortexed briefly. Acetonitrile (0.05 mL) was then added, vortexed briefly, and mixed for 15 minutes. This mixture was centrifuged for 5 minutes. The supernatant was transferred to another tube, dried under a nitrogen stream, and stored at ~80°C. Doxorubicin-containing compounds were separated by reverse-phase high-performance liquid chromatography as above and quantified using a doxorubicin standard curve. Control experiments showed that this method extracted 80% doxorubicin-containing compounds and that metabolism of doxorubicin-containing compounds during analysis was negligible.

**Inhibition of HT1080 Xenograft Growth**

HT1080 tumors were transplanted into naive mice. After 5 to 7 days, tumors had reached a median size of 75 to 100 mm$^3$. Mice were then grouped into cohorts of 10 mice such that each cohort had the same median tumor size and a similar tumor size distribution. Treatment was then initiated as described in Results and tumors were measured every 3 days with calipers.

**Reticulocyte Measurements**

Three mice per condition were anesthetized with CO$_2$ 3 days after the last treatment because control experiments showed that this time corresponded to the nadir of the reticulocyte decrease. Blood (0.5 mL) was collected by cardiac puncture in a syringe containing EDTA to prevent coagulation. RBC and reticulocyte concentrations were
determined the same day using an Advia 120 Hematology Analyzer.

Lethal Dose Determination
Increasing doses of drugs were given i.v. to five mice per dose. Animals were observed for 30 days for signs of severe toxicity and euthanized if required according to Dupont Pharmaceuticals Animal Care and Use Committee guidelines.

Results
Design of MMP-Activated Doxorubicin Prodrugs
Doxorubicin prodrugs were formed by linking the COOH terminus of a peptide to the amino group of doxorubicin (Fig. 1). The resulting prodrug is not cytotoxic because the peptide prevents the prodrug from entering cells (22). MMP cleavage occurs in the middle of the illustrated hexapeptide generating a tripeptide doxorubicin conjugate (Fig. 1). The tripeptide doxorubicin conjugate must be further cleaved by extracellular proteases to form Leu-Dox before doxorubicin-containing compounds can efficiently penetrate cells. Leu-Dox may be converted to doxorubicin either inside or outside cells. Leucine was chosen as the COOH-terminal residue because Leu-Dox was more efficiently converted to doxorubicin than other amino acid doxorubicin conjugates (23) and leucine allowed efficient MMP-2, -9, and -14 cleavage (data not shown). The peptide was capped to prevent aminopeptidase degradation and increase solubility. Sufficient aqueous solubility was required to allow the i.v. administration of compounds to humans. Although the necessary solubility would depend on clinical usage, a target solubility of 1 mg/mL was chosen to guide compound design.

Prodrug Cleavage by MMP-2, -9, and -14 in vitro
To determine the effect of prime-side length on MMP cleavage rate, peptides of various lengths were attached to doxorubicin and the catalytic efficiencies for cleavage by MMP-2, -9, and -14 were determined (Table 1). For this analysis, simple collagen-like peptide sequences (PLG-L) were used because most MMPs cleave peptides containing this sequence (reviewed in ref. 13). A conjugate with the one prime-side residue, compound 1, was not detectably cleaved by MMP-2 or -9. A conjugate with two prime-side residues, compound 2, was efficiently cleaved by MMP-9, modestly cleaved by MMP-2, and poorly cleaved by MMP-14. Conjugates with three prime-side residues, compound 3, or four prime-side residues, compound 4, were cleaved efficiently by MMP-2, -9, and -14. The absolute \( k_{cat}/K_m \) values for efficiently cleaved conjugates, such as compounds 3 and 4, were close to values for the most efficient MMP substrates. In particular, \( k_{cat}/K_m \) values for the best reported fluorogenic peptide substrates for MMP-2, -9, and -14 are 629, 87, and 1,590 mmol/L⁻¹ s⁻¹, respectively (24–26).

Metabolism of Conjugates in Cultured HT1080 Cells
The metabolism rate of conjugates with two or more prime-side residues in cultured HT1080 cells was determined because \textit{in vitro} enzymatic digestions showed that these conjugates were substrates for MMP-2, -9, or -14. For this analysis, conjugates were incubated with cultured HT1080 cells, aliquots of culture supernatant were removed after various times, and the composition of doxorubicin-containing compounds was determined. Using compound 2 as an example, the parental conjugates disappeared linearly with time for several hours and the major metabolite was L-Dox (Fig. 2A). Other metabolites, such as LL-Dox, which would be formed by MMP cleavage of compound 2, were only detected in trace amounts. To determine why these expected metabolites were not detected at higher levels, compounds were cleaved by MMP-2 \textit{in vitro} and then added to cultured HT1080 cells. Analysis of these culture supernatants showed that post-MMP cleavage metabolites were rapidly converted to L-Dox (data not shown). The kinetics of conversion of these post-MMP cleavage metabolites to L-Dox were consistent with the trace amounts of these metabolites detected following incubation of the parental compounds. To allow comparisons between compounds, the fraction processed was defined as the fraction of compounds present in the culture supernatant that could result from MMP cleavage. The fraction processed per hour was calculated by fitting a straight line to the fraction processed values measured during the first 6 hours after incubation of the compound with cultured HT1080 cells. Although this method is considered the most rigorous, very similar values would be obtained by simply considering the formation of L-Dox. Analysis of compounds 2 to 4 showed a dramatic effect of prime-side

![Figure 1](http://example.com/f1.png)

**Figure 1.** Structure of doxorubicin (A) and compound 6, a representative prodrug, with expected enzymatic cleavage products (B). The MMP cleavage site is indicated by ~. Residues on the NH₂-terminal side of the cleavage site are designated P1, P2, etc., whereas residues on the COOH-terminal side of the cleavage site are designated P₁', P₂', etc. See Results for further description.
length on processing in HT1080 cultures; the fraction processed per hour was 0.02, 0.065, and 0.3 for these compounds that contained 2, 3, or 4 prime-side residues, respectively (Fig. 2B). None of the compounds in Fig. 2B were detectably degraded in the absence of cultured HT1080 cells (data not shown).

To determine the amount of the metabolism that resulted from MMP cleavage, reactions were conducted with marimastat, a broad-spectrum MMP inhibitor. This analysis showed that most of the cleavage of compounds 2 and 4 were not due to MMPs (Fig. 2B). In the case of compound 3, approximately one half of the metabolism resulted from MMPs. Similar results were obtained with other small-molecule MMP inhibitors (data not shown).

To identify the putative non-MMP-metabolizing enzyme, the effect of other protease inhibitors on compound 4 metabolism was tested. These studies revealed that 100 nmol/L phosphoramidon greatly reduced the metabolism of compound 4 (Fig. 2C). At this concentration, phosphoramidon is specific for neprilysin, a cell surface protease (27). Other data supported the hypothesis that neprilysin was the major non-MMP-metabolizing enzyme for conjugates in cultured HT1080 cells. First, HT1080 cells expressed neprilysin based on Western blotting (data not shown). Second, the non-MMP metabolism rate of conjugates correlated well with the in vitro catalytic efficiency of neprilysin cleavage (Table 1; Fig. 2B; data not shown). Third, other cell lines that expressed neprilysin had non-MMP metabolism rates that correlated with their levels of neprilysin expression (data not shown).

Because neprilysin is expressed outside tumor tissue with high levels in kidneys (27) and would, therefore, lead to nontumor activation of prodrugs, compounds were identified that were good substrates for MMP-2, -9, and -14 but were poor substrates for neprilysin. The data for two such compounds, compounds 5 and 6, are summarized in Table 1. The fraction of metabolism due to MMPs was determined in reactions with 150 nmol/L marimastat. Consistent with the neprilysin hypothesis, the MMP-selective compounds, compounds 5 and 6, were primarily metabolized by MMPs in cultured HT1080 cells (Fig. 2B).

### Table 1. *In vitro* properties of selected prodrugs

<table>
<thead>
<tr>
<th>Code</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}/K_{m}$ (mmol/L$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td></td>
<td>MMP-2</td>
</tr>
<tr>
<td>1</td>
<td>Ac-PLG-L-Dox</td>
</tr>
<tr>
<td>2</td>
<td>Ac-PLG-LL-Dox</td>
</tr>
<tr>
<td>3</td>
<td>Peg-PLG-LYL-Dox</td>
</tr>
<tr>
<td>4</td>
<td>Ac-PLG-LYAL-Dox</td>
</tr>
<tr>
<td>5</td>
<td>Ac-PLG-HofOrnLi-Dox</td>
</tr>
<tr>
<td>6</td>
<td>Ac-E-PCitG-HofYL-Dox</td>
</tr>
</tbody>
</table>

Abbreviations: Peg, polyethylene glycol; Hof, homophenylalanine; Orn, ornithine; Cit, citrulline; ND, not determined.

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**Figure 2.** Metabolism rates of selected prodrugs by cultured HT1080 cells. **A,** amount of L-Dox and doxorubicin formed from compound 2 in cultured HT1080 cells. Amounts are represented as the fraction of all doxorubicin-containing compounds detected. The total amount of doxorubicin-containing compounds differed by <5% at different times. **B,** non-MMP and MMP processing of several conjugates in cultured HT1080 cells. The fraction of metabolism due to MMPs was determined in reactions with 150 nmol/L marimastat. **C,** effect of phosphoramidon (Phos.) and marimastat on compound 4 processing in cultured HT1080 cells.
Analysis of conjugate metabolism in cultured HT1080 cells also showed that there was little correlation between the enzymatic cleavage of conjugates by MMP-2, -9, or -14 and the cleavage of conjugates by cultured HT1080 cells (Table 1; Fig. 2B; data not shown). Potential reasons for this lack of correlation will be discussed later.

**Compound 5 Preferentially Accumulates Doxorubicin in Tumor Xenografts Relative to Heart Tissue**

To determine if the metabolism of conjugates by cultured HT1080 cells was sufficient to lead to the preferential accumulation of doxorubicin in tumor tissue, mice with HT1080 xenografts were injected with 1.4 μmol/kg compound 5, L-Dox, or doxorubicin. At various times after injection, tumor and heart tissue were analyzed for doxorubicin levels. Heart tissue was chosen as a reference tissue because there were published data comparing tumor and heart levels of doxorubicin and L-Dox. Both doxorubicin and L-Dox administration resulted in more doxorubicin in heart tissue than HT1080 xenografts (Fig. 3A and B), consistent with published results (28–31). In contrast, compound 5 administration led to more doxorubicin in HT080 tissue than heart (Fig. 3C). One way to quantify these differences is to integrate the area under the doxorubicin time-concentration curves. This integration showed that compound 5 had a 15-fold better tumor/heart doxorubicin ratio than doxorubicin (Table 2). This analysis also showed that the tumor doxorubicin levels from compound 5 were approximately one third those from doxorubicin at equimolar doses.

To gain further insight into compound 5 metabolism, the concentration-time profiles for compound 5 and its metabolites in plasma, tumor, and heart were determined. As in cultured HT1080 cells, the major doxorubicin-containing compounds in the tissues following administration of compound 5 were compound 5, L-Dox, and doxorubicin (Fig. 4). Compound 5 disappeared rapidly from the plasma compartment. Whereas L-Dox levels were measurable in plasma, doxorubicin levels in plasma were below detectable limits. In heart tissue, L-Dox levels were also less than the levels of compound 5 (Fig. 4B). Unlike plasma, heart tissue accumulated doxorubicin presumably by metabolism of L-Dox. Unlike plasma and heart, L-Dox levels in HT1080 tissue exceeded compound 5 levels. Because neither compound 5 nor L-Dox preferentially accumulated in HT1080 tissue (Fig. 4; data not shown), these findings provide strong evidence that compound 5 was preferentially metabolized in HT1080 tissue relative to heart and plasma. As a final test for tumor-specific metabolism, conjugates that could not be cleaved by MMP-2, -9, or -14 were injected into mice. These conjugates did not lead to detectable levels of doxorubicin in HT1080 xenografts (data not shown).

**Some Conjugates Cause Acute Toxicity with Administration at High Doses**

When compound 5 was given at higher doses that were compatible with xenograft efficacy studies, such as 14 mmol/kg, an unexpected acute toxicity was observed. In particular, mice became flushed due to redistribution of blood to the extremities. This redistribution also led to a rapid decrease in blood pressure and death within minutes after compound injection. Administration of an equivalent amount of doxorubicin did not cause detectable flushing or acute toxicity.

Several factors supported the hypothesis that conjugates caused mast cell degranulation leading to the release of histamine and other vasoactive peptides. First, the flushing and reduced blood pressure were consistent with mast cell degranulation. Second, preadministration of terfenadine, an antihistamine, partially prevented acute toxicity. Third, acute toxicity was prevented by administration of a nonlethal dose of compound 5 showing...
desensitization consistent with mast cell degranulation. Fourth, doxorubicin was reported to cause mast cell degranulation in mice, rats, and dogs when given at very high doses (32–34). Based on these data, we hypothesize that some non-doxorubicin structures cooperate with doxorubicin to cause mast cell degranulation.

Identification of Soluble, Selective Conjugates without Acute Toxicity

Based on these findings, compounds were screened for those that were specific for MMP cleavage in vitro and cultured cells yet lacked the acute toxicity observed with compound 5. This screening effort revealed that all compounds with positively charged residues caused acute toxicity at 14 μmol/kg. In contrast, compounds, such as compound 6, did not cause acute toxicity at doses up to 112 μmol/kg, the maximum dose that could be given because of compound solubility limitations. Like compound 5, compound 6 was selectively cleaved by MMP-2, -9, and -14, relative to neprilysin (Table 1), was primarily metabolized by MMPs in cultured HT1080 cells (Fig. 2B), and had good aqueous solubility (Table 1). Furthermore, compound 6 led to the selective deposition of doxorubicin in HT1080 xenografts, relative to heart tissue, with a tumor/heart doxorubicin ratio of 12 and doxorubicin HT1080 levels approximately one half those from an equimolar dose of doxorubicin at 14 μmol/kg.

Compound 6 Is More Efficacious Than Doxorubicin with Less Toxicity

To determine if the doxorubicin deposited by conjugates was effective at reducing tumor growth, mice were implanted with HT1080 xenografts, grouped into cohorts of 10 mice with a similar median tumor size and tumor size distribution, and treated with doxorubicin or compound 6. These studies showed that 14 μmol/kg compound 6 q4dx3 was slightly less effective than 7 μmol/kg doxorubicin q4dx3, whereas 14 μmol/kg compound 6 qdx10 was more effective than the maximum tolerated levels of doxorubicin (Fig. 5). In particular, 8 of 10 mice treated with the higher dose of compound 6 were cured of their HT1080 xenografts as defined by lack of detectable tumors 75 days after implantation. In contrast, only 2 of 10 and 0 of 10 mice treated with doxorubicin at 14 μmol/kg q4dx3 and 6 μmol/kg qdx10, respectively, were cured. At these doxorubicin doses, mice lost an average of 10% body weight, which was operationally defined as the maximum tolerated dose. Mice treated with compound 6 had no detectable weight loss.

The observed tumor growth inhibition of compound 6 was in reasonable agreement with that expected from the levels of doxorubicin deposited in tumors. In particular, 14 μmol/kg compound 6 deposited approximately one half the doxorubicin concentration in tumors as 14 μmol/kg doxorubicin. If the doxorubicin deposited by compound 6 was equipotent with that deposited by doxorubicin, then 14 μmol/kg compound 6 q4dx3 should cause a similar growth inhibition to 7 μmol/kg doxorubicin q4dx3 and 14 μmol/kg compound 6 qdx10 should be more effective than 14 μmol/kg doxorubicin q4dx3. As shown in Fig. 5, tumor

| Table 2. Doxorubicin deposition in HT1080 xenografts and heart tissue |
|-----------------|-----------------|-----------------|
| Compound        | Doxorubicin AUC₀₋₂₄ | Tumor/heart ratio |
|                 | (nmol g⁻¹ h⁻¹) | Absolute | Normalized |
| Doxorubicin     | 3.1            | 8.2      | 0.38     | 1          |
| L-Dox           | 0.58           | 1.1      | 0.53     | 1.4        |
| 5               | 1.3            | 0.24     | 5.6      | 15         |

Abbreviation: Doxorubicin AUC₀₋₂₄, area of the doxorubicin concentration-time curve from 0 to 24 hours.
growth inhibition by compound 6 is consistent with these predictions.

Mice treated with compound 6 did not show any overt signs of toxicity, including weight loss or changes in coat texture. To investigate compound 6 toxicity in detail, mice were given increasing doses of either compound 6 or doxorubicin. This experiment showed that the LD50 for doxorubicin was ~42 μmol/kg, whereas administration of compound 6 at 112 μmol/kg did not cause any detectable adverse effects. As a measure of marrow toxicity, reticulocytes were quantified. Reticulocytes are short-lived precursors of RBC in blood that are easily quantified (35). Doxorubicin-treated mice showed a dramatic decrease in reticulocytes with an ED50 of 2.5 μmol/kg (Fig. 6). In contrast, mice given 14 μmol/kg compound 6 qdx10 had reticulocyte levels (average, 5.6% RBC; SD, 3.3% RBC) that were not statistically different from vehicle-treated mice (average, 3.0% RBC; SD, 1.2% RBC). Taken together, these results show that compound 6 is more effective than doxorubicin at reducing the growth of HT1080 xenografts with significantly less toxicity than doxorubicin.

Discussion

In this study, MMP-activated doxorubicin prodrugs were discovered that had a higher therapeutic index than doxorubicin when HT1080 cells were used as a preclinical model. To achieve this increased therapeutic index, prodrugs were identified that were efficiently cleaved in vitro by MMP-2, -9, and -14, poorly cleaved by nephrilysin, an off-target metalloprotease, and lacked positively charged residues to avoid acute toxicity. Such conjugates were specifically metabolized by MMPs in cultured HT1080 cells and preferentially deposited doxorubicin in HT1080 xenografts relative to heart. The doxorubicin deposited by compound 6 was effective at reducing the growth of HT1080 xenografts, whereas the reduced doxorubicin deposition in nontumor tissues led to an increased maximum tolerated dose and reduced marrow toxicity.

The results of this study with MMP-activated doxorubicin prodrugs are similar to those from previous studies with extracellular enzyme-activated doxorubicin prodrugs. In particular, doxorubicin prodrugs activated by prostate-specific antigen (36, 37), nephrilysin (38), and plasmin (39, 40) have been studied in mice. For the prostate-specific antigen–activated prodrug L-377,202, the doxorubicin concentration in a prostate cell xenograft was increased 2-fold and the heart doxorubicin concentration was reduced 2-fold leading to a 4-fold increased tumor/heart doxorubicin ratio (41). For the nephrilysin-activated prodrug CPI-0004Na, tumor doxorubicin concentrations were increased 2-fold and heart doxorubicin concentrations were decreased 10-fold, leading to a 20-fold increase in the doxorubicin tumor/heart ratio (38). In both of these cases, the prodrugs had a higher maximum tolerated dose than doxorubicin and inhibited xenograft growth better than doxorubicin (37–39). Very similar results with respect to improvements in the maximum tolerated dose and inhibition of xenograft growth were obtained with a different prostate-specific antigen–activated prodrug (36) and two plasmin-activated prodrugs (40).

Although preclinical studies with tumor-activated prodrugs show that it is possible to increase the therapeutic index of doxorubicin in mice, it is unclear whether the benefits observed in mice will translate to humans. The first human trial with L-377,202 were recently completed and suggest that some of the improvements observed in mice will be translated to humans (42). In the case of MMP-activated prodrugs, this issue is particularly difficult.

Figure 5. Compound 6 inhibits the growth of HT1080 xenografts better than doxorubicin. See legend for doses and schedules. Day 0, time of tumor implantation. Arrows, beginning and end of treatment. A tumor volume of 1 mm³ was assigned to tumors that were undetectable.

Figure 6. Effect of doxorubicin dose on reticulocyte counts. Mice were given the indicated dose of doxorubicin daily for 10 d. Three days after the last dose, reticulocyte counts were determined. Bars, SD.
to address because mouse tumors are poor models for human tumors with respect to MMP expression and activity. In particular, MMPs are frequently expressed and activated by different cell types within human tumors. For instance, MMP-2 is primarily expressed by stromal fibroblasts, yet pro-MMP-2 is activated by MMP-14 that is present on tumor cells. Because xenografts and mouse transgenic tumor models contain relatively few stromal fibroblasts, it is difficult to mimic the MMP activity of human tumors in mice. To deal with this complexity, our studies used HT1080 cells, a fibrosarcoma cell line. By choosing a fibroblast cell tumor line that expressed MMP-1, -2, -3, -7, -9, -14, -15, -16, and -18 (14, 15), we hoped to better represent the MMP composition in human tumors than with more conventional epithelial tumor cell lines. Although HT1080 cells are a rational choice, inhibition of HT1080 growth does not remove many of the unknowns in translating these preclinical findings into the clinic.

If MMP-activated doxorubicin prodrugs have an increased therapeutic index relative to doxorubicin in humans, then there are two major ways to exploit this increased therapeutic index. Ideally, one would like to increase the efficacy of doxorubicin by increasing the tumor concentration of doxorubicin with prodrug administration. Although such a strategy clearly works in HT1080 xenografts, it is unclear whether this strategy will be successful in human tumors. In particular, several studies have investigated the use of high-dose chemotherapy where autologous hematopoietic progenitor cell transplantation is used to allow chemotherapeutic doses that would otherwise be myeloablative. Whereas overall survival was similar between high-dose chemotherapy and conventional chemotherapy in four trials for patients with metastatic breast cancer, six of seven trials found increased disease-free survival using high-dose chemotherapy (reviewed in ref. 43). Although the apparent lack of increase survival is discouraging, these studies may not have had sufficient patients to detect meaningful differences. For this and other reasons, high-dose chemotherapy trials are ongoing. Alternatively, an increased therapeutic index of doxorubicin prodrugs could be used to decrease the toxicity from doxorubicin therapy. Such decreased toxicity could allow the use of other therapies that would not have been possible due to toxicities from doxorubicin.

The reason for the lack of correlation between the in vitro enzymatic efficiencies for conjugate cleavage and the metabolism rates in cultured HT1080 cells is unclear. Possibly, the major metabolizing MMP in cultured HT1080 cells is not MMP-2, -9, or -14. Consistent with this hypothesis, HT1080 cells express several MMPs other than MMP-2, -9, and -14, including MMP-1, -3, -7, -15, -16, and -18 (14, 15). Alternately, the conditions used for in vitro assays may not accurately reflect the in vivo activities of the MMPs. The enzymatic assays use soluble MMPs in detergent-containing solutions with substrate concentrations well below the $K_m$ and the cell-based assays use membrane-bound MMPs with conjugates dissolved in 0.1% bovine serum albumin, DMEM. Although conjugate concentrations were not saturating for metabolism in HT1080 cultures (data not shown), differences in the assay conditions between the in vitro assays and the cell-based assays could yield divergent results. In this case, the differences would be specific for MMPs because the neprilysin in vitro and in vivo rates were well correlated. Clearly, additional work will be needed to resolve this issue.

An acute toxicity similar to that with compound 5 was reported with $\beta$-Ala-Leu-Ala-Leu-Dox (44). Interestingly, capping of the positively charged NH$_2$ terminus in this conjugate with a succinic group also eliminated the acute toxicity of this conjugate. These investigators, however, attributed this improvement to decreased aggregation of the prodrugs.

The use of MMPs to activate prodrugs should be applicable to other cytotoxics. For instance, other investigators have made paclitaxel and vinblastine prodrugs. Although the peptide sequence identified with the doxorubicin prodrug is a good starting point for these other cytotoxics, it is likely that additional optimization will be required because the cytotoxic moiety affects the enzymatic utilization of the resulting conjugate. It is also likely that other cytotoxic prodrugs will not have the acute toxicity due to mast cell degranulation that was observed with the doxorubicin prodrugs. In particular, the positively charged peptides apparently increase the amount of histamine release that can result from doxorubicin alone under some circumstances.

In a prodrug that requires multiple steps for activation, like the MMP-activated doxorubicin prodrugs, the initial activation step should have the slowest rate and subsequent steps should be rapid compared with the initial step for optimal prodrug performance. The MMP-activated prodrugs described here do not possess this optimal property. In particular, the removal of the prime-side residues following MMP cleavage appears rapid, relative to MMP cleavage, except for the conversion of L-Dox to doxorubicin. This conclusion is supported by the low concentrations of intermediates with multiple prime-side residues and the high concentration of L-Dox observed in cultured cells and tumor tissue. Additionally, L-Dox concentrations in HT1080 xenografts at times soon after injection exceeded doxorubicin concentrations at later times. Taken together, these data show that a significant fraction of the L-Dox that is formed in the HT1080 xenografts diffuses outside the tumor before conversion to doxorubicin. If this loss could be reduced, then the potency, and potentially therapeutic index, of prodrugs would be increased. Because the enzymatic conversion of L-Dox to doxorubicin appears relatively slow, it would be interesting to investigate nonenzymatic mechanisms to eliminate the final residue, such as the self-immolating linkers described previously (45).

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MMP-Activated Doxorubicin Prodrugs

References

Matrix metalloproteinase–activated doxorubicin prodrugs inhibit HT1080 xenograft growth better than doxorubicin with less toxicity

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