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Discovery of matrix metalloproteases selective and activated peptide–doxorubicin prodrugs as anti-tumor agents ☆

Zilun Hu *, Xiangjun Jiang, Charles F. Albright, Nilsa Graciani, Eddy Yue, Mingzhu Zhang, Shu-Yun Zhang, Robert Bruckner, Melody Diamond, Randine Dowling, Maria Rafalski, Swamy Yeleswaram, George L. Trainor, Steven P. Seitz, Wei Han

Bristol-Myers Squibb Research and Development, PO Box 5400, Princeton, NJ 08543, USA

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ABSTRACT

To selectively target doxorubicin (Dox) to tumor tissue and thereby improve the therapeutic index and/or efficacy of Dox, matrix metalloproteinases (MMP) activated peptide–Dox prodrugs were designed and synthesized by coupling MMP-cleavable peptides to Dox. Preferred conjugates were good substrates for MMPs, poor substrates for neprilysin, an off-target proteinase, and stable in blood ex vivo. When administered to mice with HT1080 xenografts, conjugates, such as **19**, preferentially released Dox in tumor relative to heart tissue and prevented tumor growth with less marrow toxicity than Dox.

Doxorubicin (Dox) is an anthracycline natural product that is widely used to treat tumors such as breast cancer, liver cancer, soft-tissue sarcomas, and non-Hodgkin's lymphoma. Dox has a complex mechanism of action with some of its activity arising from inhibition of nucleic acid synthesis within cancer cells.[1] Like other cytotoxic drugs, the therapeutic efficacy of Dox is limited by unwanted toxicity to non-tumor tissues, most notably myelosuppression.[2] In addition to these typical chemotherapeutic toxicities, Dox also causes cardiomyopathy which depends on the cumulative dose of drug.

There have been several attempts to develop Dox prodrugs that increase its therapeutic index.[3–5] For example, investigators used the prostate-specific antigen to activate Dox conjugates in mice leading to increased efficacy with reduced toxicity in mouse xenografts.[6] Unfortunately, these results did not effectively target Dox to tumors in humans.[7] It was hoped that this approach could deliver an efficacious concentration of Dox at tumor sites with limited systemic exposure, and hence would significantly increase its therapeutic index.

Matrix metalloproteases (MMPs) are a family of structurally related zinc-containing proteases containing more than 20 members.[8] Under normal conditions, these enzymes play an important role in the maintenance and remodeling of connective tissues. These enzymes are also implicated in several critical events in tumor evolution including tumorigenesis, tumor growth, angiogenesis, generation of reactive stroma and tumor cell metastasis. In fact, elevated level of MMP expression in human tumors was frequently found to correlate with disease progression.[9]

We reasoned that a MMP-activated prodrug of Dox might selectively release Dox at the tumor sites and thereby reduce side effects. We chose MMP-2, -9 and -14 to guide our in vitro structure–activity relationship (SAR) effort because of good expression of these enzymes in tumors.[9]

The desired conjugates of Dox should possess several properties. In particular, they should be good substrates for MMP enzymes to allow efficient activation in the tumor. Conjugates should be poor substrates for other enzymes, including enzymes typically found in the plasma compartment. Of particular concern for MMP-activated prodrugs was neprilysin since this cell-surface protease is expressed outside tumor tissue and may therefore lead to non-tumor activation of the prodrugs.[10] In addition, the prodrugs should not be cytotoxic prior to activation[11] and should have aqueous solubility compatible with intra venous administration. When properly designed, the resulting prodrugs have the potential to efficiently and preferentially deposit Dox in tumor tissue relative to non-tumor tissue leading to an improved therapeutic index and improved tumor growth inhibition.

Based on these considerations, our medicinal chemistry approach was to design and link an MMP peptide substrate, $\cdots P_3P_2P_1\text{–}P'_1P'_2P'_3\cdots$, to Dox to form a peptide–Dox conjugate. The COOH terminus of a peptide was conjugated by an amide bond

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* Corresponding author. Tel.: +1 609 818 5290.
E-mail address: zilun.hu@bms.com (Z. Hu).

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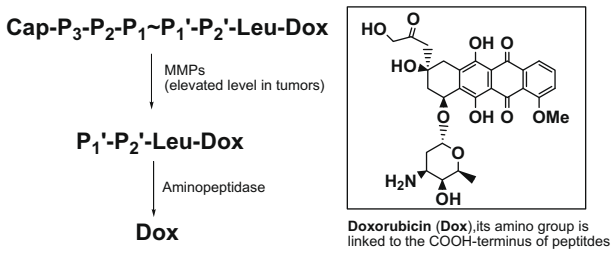
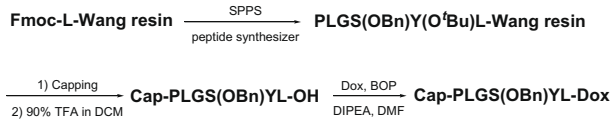


Figure 1. Activation of peptide–Dox prodrug.



Scheme 1. Synthesis of peptide–Dox conjugates.

with the amino group of Dox. In our design of the peptide sequence, Glycine (Gly, G) was chosen as the P_1 group because it was found to be optimal for MMP cleavage from our initial findings and also a literature report.[12] Leucine (Leu, L) was chosen as the COOH terminal residue linked directly to Dox because L-Dox was reported to be more efficiently converted to Dox than other conjugates.[13–15] The N-termini of the conjugates were capped to prevent aminopeptidase degradation before MMP cleavage. Based on these considerations, N-terminus capped peptide conjugates with Gly as P_1 and Leu linked to Dox as shown in Figure 1 were designed and optimized for MMP cleavage and selectivity. Incorporation of polar groups either within the N-terminal caps or within the side chains of amino acid residues was used to improve solubility of the conjugates. A target solubility of 1 mg/mL was chosen to guide compound design.

The synthesis of an example of the peptide–Dox conjugates is outlined in Scheme 1. Compound preparation was performed on a peptide synthesizer following a standard Fmoc solid phase protocol starting from Fmoc-Leu–Wang resin using HBTU as the coupling reagent.[16] The N-terminus of the completed peptide on resin was capped and the peptide was then cleaved from the resin with 90% trifluoroacetic acid in dichloromethane. The resulting peptide was then coupled to Dox to form the conjugate using the BOP coupling reagent.

The initial SAR was based on simple collagen-like peptide conjugates containing the sequence PLG ~ L, which is cleaved by most MMPs.[14] We first determined the preferred peptide length. As we previously reported[17] and show in the set of conjugates **1**–**6** in Table 1, the optimized conjugate length was a hexapeptide with

Table 1
In vitro profiles of peptide–Dox conjugates

No.	Conjugate	Enzyme cleavage k_{cat}/K_m (mM^{-1} s^{-1})				Stability[a] (%)	Solubility[b] (mg/mL)
		MMP-2	MMP-9	MMP-14	Neprilysin		
1	Ac-PLG–L-Dox	<1	<1	—	—	—	—
2	Ac-PLG–LL-Dox	18	>120	4	22	—	—
3	Ac-LG–LL-Dox	<1	<1	<1	5	—	—
4	Ac-LG–LYL-Dox	6	1	24	8	—	—
5	Ac-PLG–LYL-Dox	88	390	>120	22	—	—
6	Ac-PLG–LYAL-Dox	>120	>120	>120	>120	—	—
7	Ac-PLG–S(OMe)[c]YL-Dox	24	79	69	2.1	31	0.04
8	Ac-PLG–S(OBn)[d]YL-Dox	7	34	25	<1	—	—
9	Ac-PLG–Hof[e]AL-Dox	11	<1	19	1	—	—
10	Ac-PLG–HofYL-Dox	>120	34	>120	<1	47	0.01
11	Ac-PLG–HofHoy[f]L-Dox	116	>120	>120	<1	13	0.001
12	Ac-PLG-Hoa[g]YL-dox	21	43	58	<1	59	0.38
13	Ac-PLG–HofGmp[h]L-Dox	55	73	>120	<1	100	0.13
14	Ac-PLG–HofK(NMe_2)L-Dox	31	43	62	<1	90	1.27
15	Cap1[i]-PLG–S(OBn)YL-Dox	20	52	89	<1	100	1.4
16	Cap2[j]-PCit[k] G–S(OBn)YL-Dox	25	38	56	<1	29	>2.3
17	Ac-γE-PQG–S(OBn)YL-Dox	29	120	72	8	70	1.9
18	Ac-γE-PCitG–S(OBn)YL-Dox	21	64	48	<1	83	2.1
19	Ac-γE-PLG–S(OBn)YL-Dox	31	55	83	<1	88	>2.6
20	Ac-γE-PLG–C(SBn)YL-Dox	40	107	97	<1	90	>2.2
21	Ac-γE-PLG–HoyYL-Dox	>120	>120	>120	<1	100	1.5
22	Ac-βD-PLG–S(OBn)YL-Dox	69	>120	>120	<1	95	>3.9
23	Cap3[l]-PQG–S(OBn)YL-Dox	47	68	97	<1	81	>2.2
24	Cap3-PLG–S(Bn)YL-Dox	30	85	76	<1	87	1.8
25	Cap2-PLG–S(OBn)YL-Dox	37	25	111	<1	90	>2.4(2.9)[n]
26	Cap3-PCitG-T(OBn)YL-Dox	59	68	>120	<1	90	(2.9)
27	Cap4[m]-PSG-T(OBn)YL-Dox	114	79	>120	<1	100	(2.5)

[a] % Remaining after 6 h in blood.
[b] In pH 7.4 buffer solution.
[c] *O*-Methylserine.
[d] *O*-Benzylserine.
[e] Homophenylalanine.
[f] Homotyrosine.
[g] 2-Amino-4-(pyridine-4-yl)butanoic acid.
[h] *N*-Methylpiperazinepropylglycine.
[i] Cap1: succinic acid.
[j] Cap2:2-sulfoacetic acid.
[k] Citrulline.
[l] Cap3:3-sulfobenzoic acid.
[m] Cap4: 3,5-disulfobenzoic acid.
[n] Value in bracket was the solubility in 5% dextrose solution.

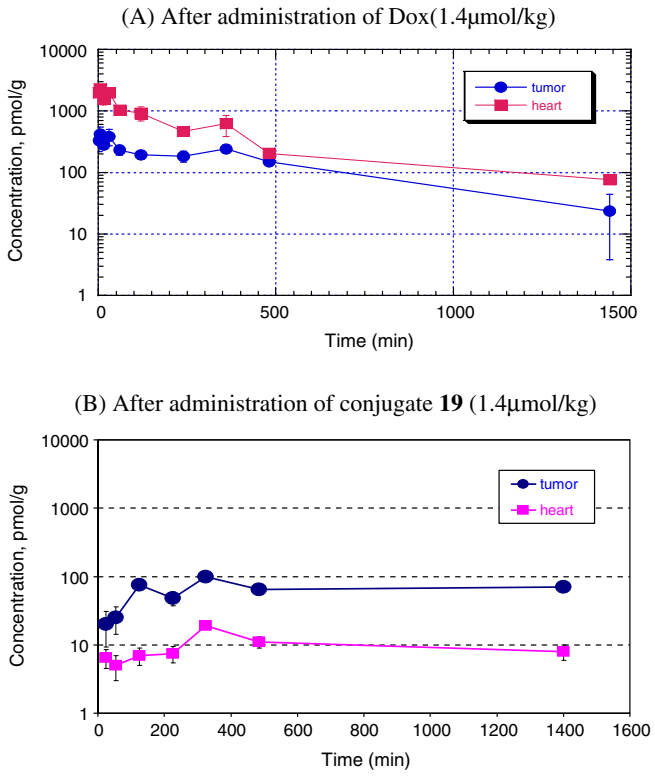


Figure 2. Dox deposition in HT1080 xenografts and heart tissue after administration of Dox (A) and conjugate **19** (B).

three prime side and three non-prime side residues to achieve optimal MMP-2, -9 and -14 cleavage and selectivity over neprilysin.

Further SAR efforts kept P_1 Gly and P'_3 Leu constant and focused on hexapeptide conjugates with three prime side and three non-prime side residues. When P'_1 was changed from Leu and *O*-methyl serine to bulky hydrophobic residues such as homophenylalanine (Hof), *O*-benzyl serine (S(OBn)) and 2-amino-4-(pyridine-4-yl)butanoic acid (Hoa), the selectivity over neprilysin increased (**5**-**12**). Replacing the small P'_2 residue Ala (**9**) with amino acids containing larger side groups (Y in **10** and Hoy in **11**) further increased selectivity and improved MMP cleavage. It was also found that P_2 and P'_2 could tolerate a variety of amino acids (natural and non-natural) of different sizes and properties such as *N*-methyl piperazinepropylglycine (**13**), *N*,*N*-dimethyllysine (**14**), citrulline(**16**, **18**) and glutamine (**17**). This discovery provided an opportunity to incorporate amino acids with polar side groups such as basic amines to make conjugates with increased aqueous solubility. Unfortunately, it was observed that conjugates with positively charged functional groups such as basic amine derivatives incorporated at P'_2, P_2 positions or as capping groups such as in **12**, **13** and **14**, frequently caused acute toxicity when administered to mice.

Capping groups of the conjugates also affected the stability and solubility properties. Simple acetyl (Ac) capped conjugates (**9**–**12**) were quite stable in blood but had limited solubility in pH 7.4 buffer solution. With the introduction of water soluble capping groups

Table 3
Tumor/heart distribution ratio of Dox in *neu* mice 24 h after iv dose

Compound	Dox	17	19	20	23	24
Dox ratio[a] tumor/heart	1	6.7	7.2	11	14	8.2
Tumor Dox concentration (pmol/g)	2600	310	500	104	400	290

[a] Normalized ratio.

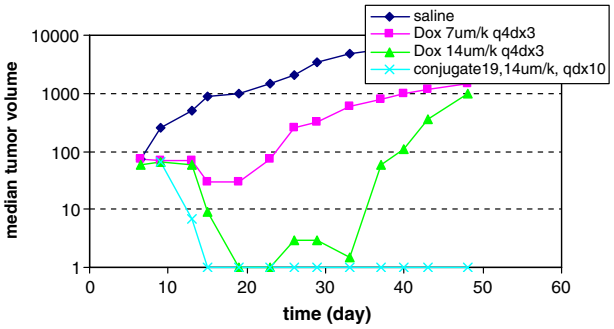


Figure 3. Tumor growth inhibition in HT1080 mice.

such as succinic acid (**15**), 2-sulfoacetic acid (**16** and **25**) or Ac-γ-glutamic acid (**17**–**21**), both the solubility and stability of the conjugates improved. Further exploration found capping groups such as Ac-β-aspartic acid (**22**), 3-sulfobenzoic acid (**23**, **24** and **26**) or 3,5-disulfobenzoic acid (**27**) were tolerated with increased solubility. This SAR exercise resulted in a series of peptide–Dox conjugates with excellent profiles in terms of enzyme selectivity, stability, solubility, and lack of acute toxicity that met our program criteria.

To determine how the peptide–Dox conjugate prodrugs were metabolized in animals, HT1080 cells were used as a model system. MMP-selective conjugates were injected into mice with HT1080 xenografts to determine the tissue distribution of Dox following the procedure described by us previously.[17] As shown in Figure 2A, B and Table 2, when the peptide–Dox conjugates were administered, more Dox was deposited in HT1080 tumor than in heart with tumor to heart Dox ratio ranging from 6.5 to 17, consistent with our previous results. Further metabolic studies showed conjugates disappeared rapidly from the plasma compartment after dosing. Dox levels were below detectable limits in plasma. In heart tissue, L-Dox levels (data not shown) were lower than the levels of conjugates, and heart tissue accumulated Dox presumably by metabolism of L-Dox. Unlike plasma and heart, L-Dox levels in HT1080 tumor were higher than those of conjugates. Since L-Dox did not preferentially accumulate in HT1080 tumor, these findings support the idea that the conjugates were preferentially metabolized in HT1080 tumor relative to heart and plasma. This was further confirmed by injecting a non-MMP cleavable conjugate into mice resulting in no detectable Dox in HT1080 xenografts. Similar preferential deposition of Dox in tumors (tumor/heart ratio 6.7–14) was observed when selected conjugates (Table 3) were administered to *neu* mice but with less Dox exposures than in HT1080 mice.

Table 2
Tumor/heart distribution ratio of Dox in HT1080 mice 24 h after iv dose

Compd	Dox	17	18	19	21	23	24	25	26	27
Dox ratio[a] Tumor/heart	1	16	16	17	10	17	17	16	9.7	13
Dox Conc. in tumor (pmol/g)	1600	993	800	900	1450	870	727	1500	790	600

[a] Normalized ratio.

Table 4
Bone marrow toxicity in mice

Compound	Dose schedule	Reticulocyte% of RBC[a]
Saline		3.7
Dox	2 μmol/kg qdx10	0.4
Dox	4 μmol/kg qdx10	0.1
Dox	6 μmol/kg qdx10	0.3
Dox	14 μmol/kg qdx10	0.2
Conjugate **19**	14 μmol/kg qdx10	3.5

[a] Reticulocytes measured 3 days after last dose.

The efficacies of selected conjugates were determined by measuring tumor growth inhibition in mice implanted with HT1080 and treated with Dox or conjugates. Shown in Figure 3 as a representative example, conjugate **19** was more effective than Dox. At 14 μmol/kg qdx10, **19** reduced tumor size to baseline for up to 48 days. It was superior to Dox at its maximum tolerated doses (14 μmol/kg, q4dx3). It was also observed that mice treated with conjugate **19** did not show signs of toxicity.

To further investigate the toxicity of the conjugates, marrow toxicity was determined by quantification of reticulocytes, the short-lived precursors of red blood cells (RBC). As shown in Table 4, there was a dramatic drop in reticulocyte count for mice treated with Dox even at a dose as low as 2 μmol/kg qdx10. In contrast, mice treated with conjugate **19** at its efficacious dose of 14 μmol/kg qdx10 showed no difference in the levels of reticulocytes (3.5%) from the vehicle-treated mice (3.7%).

In a mouse pharmacokinetic study, conjugate **19** demonstrated a clearance 0.4 L/h/kg, a volume of distribution of 0.4 L/kg and a half life of 0.6 h after iv administration.

In summary, a series of soluble and stable peptide–Dox conjugates was discovered and optimized for selectivity, solubility, efficacy and toxicity profiles. These conjugates were selectively cleaved by MMPs and preferentially delivered Dox to tumor relative to heart tissues. Conjugate **19** was shown to be more effective than Dox with less toxicity in HT1080 mouse model.

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