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The design, synthesis, and evaluation of two universal doxorubicin-linkers: Preparation of conjugates that retain topoisomerase II activity

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Abstract—The design, synthesis, and evaluation of two *N*-alkylmaleimide aldehydes have been achieved, which upon reductive alkylation with the C3′-amino group of doxorubicin (DOX) permits the preparation of DOX conjugates via Michael addition of thiol-containing vectors. This method enables the mild, facile, and high-throughput preparation of DOX conjugates that retain the basic C3′-nitrogen, a pre-requisite for topoisomerase II inhibition. Seven DOX–amino acid conjugates were prepared, each displaying similar inhibitory activity as the parent drug.

Anthracycline cytotoxic agents, in particular doxorubicin (DOX) and daunorubicin, have been employed in the area of cancer chemotherapy for more than 30 years. Side effects, such as cardiotoxicity, in conjunction with the development of spontaneous and acquired resistance, however, limit the effectiveness of these drugs. To improve efficacy, various tumor targeting approaches have been explored wherein the anthracycline is attached to a vector.[1]

Examples of anthracycline conjugates incorporating tumor-specific or selective vectors have been reported which contain hydrazone linkages at the C13 carbonyl,[2] an ester linkage at the C14 hydroxyl,[3] and/or amide[4] or alkylamine[5] linkages at the C3′-amine. Most reported C3′-amine conjugates comprise peptides or proteins attached via amide bonds, which are later removed exploiting prodrug strategies such as proteolysis. Such prodrug approaches have been taken in order to release the free drug with a basic anthracycline C3′-nitrogen, known to be a pre-requisite for topoisomerase II activity. Conjugates at the C3′-nitrogen employing alkylamines have also been prepared, which are stable to proteolysis and retain the basic nitrogen. One example of a proteolytically stable conjugate, which does not contain a basic C3′-nitrogen, is a DOX–D-penetratin conjugate prepared as a C3′-amide. This conjugate lost 5-fold of the cytotoxic activity compared to the parent drug in K562 cells, but importantly displayed a 20-fold improvement in MDR cells. Such a toxicity profile however may be ascribed to a difference in bio-distribution.[6]

Alkylamine derivatives at the C3′-nitrogen of DOX have been prepared by reductive alkylation.[4a,b,7] However, the reaction requires an excess (2–3 equiv) of aldehyde as the vector input to ensure complete reaction. Even then only poor, or at best modest, yields are often observed.

To avoid laborious syntheses and to permit parallel attachment of a series of vectors to DOX while retaining the topoisomerase II inhibition, we have designed, synthesized, and evaluated two potentially universal DOX-linkers (Scheme 1), which feature a basic C3′-nitrogen and a maleimide terminus that would permit direct attachment of a wide variety of vectors through formation of a maleimido–thioether linkage. The structures of the universal DOX-linkers (**1a** and **b**) are illustrated in Scheme 1, in conjunction with a general reaction scheme for attachment of thiol-bearing vectors.

Keywords: Anthracycline; Doxorubicin; Conjugate; Topoisomerase II; Maleimide.
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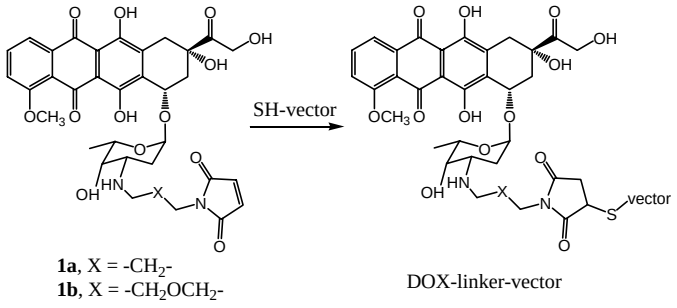
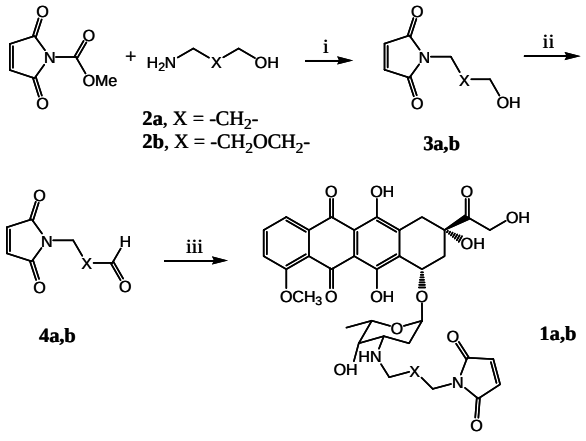


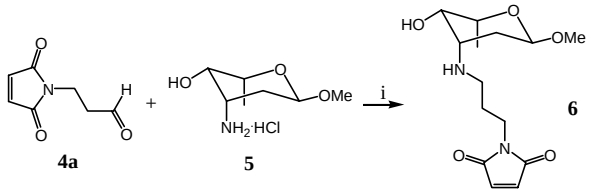
Scheme 1. General reaction scheme for the preparation of DOX-linker–vector conjugates via maleimide–thioether linkage.

The prospective DOX-3′-aminoalkylmaleimide derivatives (**1a** and **b**) were prepared via the route depicted in Scheme 2. The respective maleimide alcohols **3a** and **b** were constructed via the literature protocols,[8] involving the condensation of amino alcohols **2a** and **b** with *N*-methoxycarbonyl maleimide in saturated aqueous $NaHCO_3$. The resultant alcohols were then oxidized with Dess–Martin periodinane[9] to furnish the corresponding aldehydes **4a** and **b**, respectively. Aldehyde **4a** had been reported to be unstable due to rapid polymerization[10] and therefore not readily isolable in pure state. We were however able to alleviate this problem by using **4a** and **b** immediately in the next step after purification by silica gel column chromatography. A small sample of **4a** was diverted for characterization by NMR.[11]

Treating a mixture of DOX and freshly prepared aldehydes **4a** and **b** under reductive alkylation conditions, employing $NaCNBH_3$, provided **1a** and **b** in modest yield (ca. 20–30%).[12] The major side products, observed in both cases, were tentatively assigned to be the corresponding dihydroquinones, based on the observation of parent ions with two higher mass units (LC/MS) than those of the DOX conjugates. To verify that the side reactions were not associated with the aminosugar or with the maleimido aldehydes, daunosaminide **5** was condensed with aldehyde **4a** via reductive alkylation



Scheme 2. Reagents: (i) satd aq $NaHCO_3$, (a) 47%, (b) 61%; (ii) Dess–Martin periodinane, CH_2Cl_2, (a) 43%, (b) 41%; (iii) doxorubicin hydrochloride, 1 M $NaCNBH_3$, cat. AcOH, MeCN/H_2O, (a) 21%, (b) 21%.



Scheme 3. Reagents: (i) $NaCNBH_3$, MeOH, 88%.

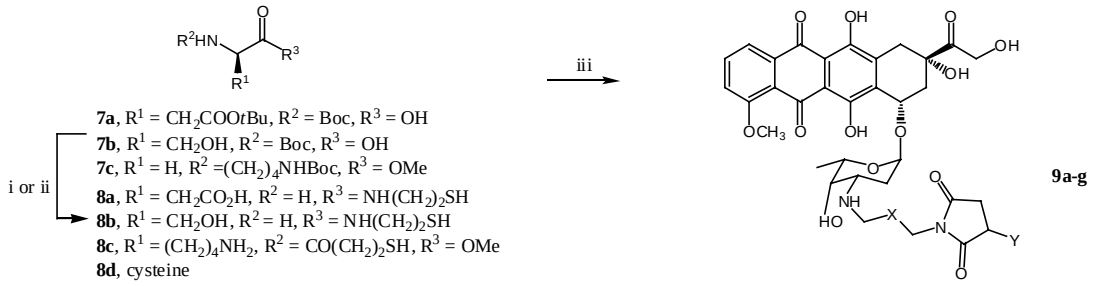
(Scheme 3). As expected the mono-alkylated product **6** was produced in 88% yield after HPLC purification.

Topoisomerase II catalyzes the formation of relaxed conformations of DNA from the super-coiled plasmid.[13] The mechanism of action of DOX is believed to involve inhibition of topoisomerase II activity, which leads to the eventual breakage of genomic DNA.

With the prospective universal DOX–maleimide linkers in hand, we prepared a small set of DOX–amino acid conjugates to evaluate their ability to inhibit topoisomerase II activity. We selected amino acid conjugates of Asp, Lys, Ser, and Cys based on the following considerations. First, given that many targeting vectors will be peptides, a prodrug approach using the DOX-linkers described herein will likely result in an amino acid conjugate. Second, by employing amino acids with common and representative peptide functionalities, such as amino, carboxyl, and hydroxyl groups, we would at the same time examine the compatibility of the Michael addition between thiols and maleimides in the presence of these common functional groups.

Seven DOX–amino acid conjugates were prepared in a parallel fashion by mixing, under neutral conditions, the DOX–maleimides **1a** or **b** with cysteine or related amino acids pre-equipped with a thiol functional group (Scheme 4). The coupling reactions in general proved to be fast (completed within 30–60 min), with high conversion rates. Amino, carboxyl, and hydroxyl groups do not appear to interfere with the reaction. Yields after HPLC purifications are listed in Table 1.

The DOX derivatives **9a**–**g** were then directly compared to DOX for their inhibitory effect in a standard topoisomerase II activity assay, at 10, 3, 1, 0.3, 0.1, and 0.03 μmol concentrations. All compounds revealed first inhibition effects at 1–10 μmol levels, similar to that of the parent drug.



Scheme 4. Reagents: (i) for **8a** and **b**, $HS(CH_2)_2NH_2{\cdot}HCl$, BOP, DIEA, DMSO; (ii) for **8c**, $HS(CH_2)_2CO_2H$, BOP, DIEA, DMSO; and (iii) **1a** or **b**, DMSO.

Table 1. Examples of DOX–amino acid conjugates prepared via **1a**,**b**

Compound	X	Y	Conversion (%)[b]	Yield (HPLC, %)	First effective concentration for topo II inhibition (μmol)
DOX					1–3
9a	$-CH_2-$	H-Asp-$NHCH_2CH_2S$-	>95	46	1–3
9b	$-CH_2-$	$-SCH_2CH_2CO$-Lys-OH	>95	38	1–3
9c	$-CH_2-$	H-Cys(−)-OH[a]	80[c]	19	3–10
9d	$-CH_2OCH_2-$	H-Asp-$NHCH_2CH_2S-$	>95	44	3–10
9e	$-CH_2OCH_2-$	$-SCH_2CH_2CO$-Lys-OH	>95	43	1–3
9f	$-CH_2OCH_2-$	H-Ser-$NHCH_2CH_2S-$	>95	49	1–3
9g	$-CH_2OCH_2-$	H-Cys(−)-OH[a]	85[c]	26	3–10

[a] The cysteine conjugates are linked through the side-chain thiol.
[b] A mixture of **1a** or **b** and **9a**,**b**,**d**–**f** was stirred in DMSO for 30 min before LC/MS determination of the conversion rate and HPLC purification.
[c] Cysteine was suspended in DMSO with 5% H_2O and stirred with **1a** or **b** for 1 h to ensure complete consumption of the latter compounds.

In summary, two new DOX-3′-aminoalkylmaleimide derivatives designed to be versatile DOX-linkers for the preparation of stable conjugates and/or prodrugs have been designed and synthesized. When conjugated with a series of amino acids pre-equipped with thiol groups, the conjugates retained the parent drug's activity toward topoisomerase II. The availability of this methodology holds the promise for high-throughput synthesis of DOX conjugates, as well as conjugates of other anthracycline drugs.

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11. Synthetic procedure and proton NMR for **4a**: 1-(3-hydroxypropyl)-1*H*-pyrrole-2,5-dione (**3a**, 200 mg, 1.29 mmol) was dissolved in 5 mL CH_2Cl_2. DMP (15% wt in CH_2Cl_2, 4 mL, 1.93 mmol) was added in one portion and the mixture was stirred for 2 h. 2-Propanol (3 mL) was added and the solution was stirred for 30 min. The resulting solution was filtered through a silica gel pad eluted with EtOAc and the filtrate was concentrated. The crude product was purified by silica gel chromatography eluting with EtOAc–hexane (2/1) providing 3-(2,5-dioxo-2,5-dihydro-pyrrole-1-yl)-propionaldehyde (**4a**, 110.0 mg, 0.72 mmol, 55.7% yield), which was used immediately. ^1H NMR ($CDCl_3$, 300 MHz) δ 9.74 (t, J = 1.2 Hz, 1H), 6.69 (s, 2H), 3.84 (t, J = 6.9 Hz, 2H), 2.77 (dt, J = 1.2, 6.9 Hz,

2H). Although NMR provided a clean spectrum, we observed the formation of precipitate 15 min after the sample was dissolved in $CDCl_3$ at a concentration of 30 mg/mL.

12. Synthetic procedure and selected spectroscopic data for **1a**: to a stirred solution of doxorubicin hydrochloride (100 mg, 0.172 mmol), **4a** (68.2 mg, 0.446 mmol), and glacial AcOH (20 μL, 195 mol %) in CH_3CN–H_2O (2/1, 5 mL) was added a 1 M solution of $NaCNBH_3$ in THF (57 μL, 0.33 mol %). The mixture was stirred under nitrogen atmosphere in the dark at rt for 1 h. The solution was then concentrated in vacuo to give a residue, which was diluted with an aqueous 5% $NaHCO_3$ solution and extracted with CH_2Cl_2. Concentration of the organic solution and purification of the resulting residue by silica gel chromatography eluting with CH_2Cl_2–CH_3OH (20/1) provided 26.0 mg of *N*-3-maleimidopropyl Doxorubicin **1a** (21.4% yield). ^1H NMR ($CDCl_3$, 300 MHz) δ 8.03 (d, $J = 8.4$ Hz, 1H), 7.79 (t, $J = 8.4$ Hz, 1H), 7.41 (d, $J = 8.4$ Hz, 1H), 6.68 (s, 2H), 5.51 (m, 1H), 5.32 (m, 1H), 4.82–4.76 (m, 2H), 4.09 (s, 3H), 3.96 (m, 1H), 3.58 (m, 3H), 3.32–2.98 (m, 2H), 2.76 (m, 1H), 2.54 (m, 2H), 2.37 (m, 1H), 2.15 (m, 1H), 1.85–1.54 (m, 4H), 1.37 (d, $J = 7.0$ Hz, 3H). Electrospray (ESI) *m*/*z* 681.2 ($M+H^+$, $C_{34}H_{36}N_2O_{13}$ required 681.2).
13. All DOX conjugates were assayed for their ability to inhibit topoisomerase II (topo II) using the topo II Drug Screening Kit (TopoGEN Inc, Columbus, OH). Specifically the kit was used to assay whether DOX derivatives alter the ability of topo II to catalyze the formation of relaxed conformation DNA from a super-coiled plasmid. The substrate DNA is a plasmid called pRYG that contains a 54 base pair series of alternating purine/pyrimidine DNA specifically acted on by topo II. In the absence of any drug topo II produces a relaxed conformation DNA from super-coiled starting DNA. The two forms of DNA can be distinguished on an agarose gel: the super-coiled DNA appearing as a single band and the relaxed DNA as a series of usually three bands close together. DOX usually inhibited this process at about 1–3 μmol. DOX derivatives were compared directly to DOX at 10, 3, 1, 0.3, 0.1, and 0.03 μmol concentrations.