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An Acid-Cleavable Linker Stable at Neutral pH that Releases Doxorubicin at Lysosomal pH

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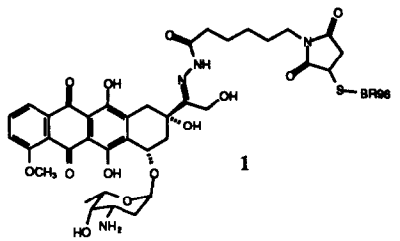
Abstract: A Kemp's triacid amide of doxorubicin (DOX), esterified at a second carboxyl, releases DOX at lysosomal pH at a reasonable rate but is extremely stable at neutral pH.

It is often desirable to deliver a drug to its target cell in a deactivated form, to be unmasked at the desired site of therapeutic response, because in this way undesirable side effects elsewhere in the body are minimized. Anticancer cytotoxic compounds have especially severe side effects that, with most tumors, prohibit administration of curative doses because they are lethal. Targeting antitumor drugs into cancer cells so that they are unmasked intracellularly, as via endocytosis (termed "piggyback endocytosis"[1]) is particularly efficacious,[2] and can be done by carriers such as specifically created monoclonal antibodies[2] (MAbs), natural substances whose receptors abound on some tumor types (e.g., LDL[3] or transferrin[4]), or small peptides.[5]

Endocytosis brings substances from outside the cell directly into endosomes and thence to lysosomes, without contacting the cytosol. Lysosomes are acidic, so it is desirable to join the drug to the carrier by a link that is stable at blood pH (ca. 7.4) but unstable at lysosomal pH[6] (ca. 4.8[7]). In our antitumor targeting program we have been using the internalizing MAb BR96[8] as carrier, which recognizes the Le^y antigen present on many breast, lung, colon and ovarian carcinomas, joined to doxorubicin (DOX) via a hydrazone that is acid-labile.[9] We measure hydrolysis rates at 37°C in pH 5 (acetate) and 7 (phosphate buffered saline) buffers to be conservative, using HPLC to monitor release of DOX,[10] at which $t_{1/2}$ for **1** = 3.2h and >158 h respectively.



1

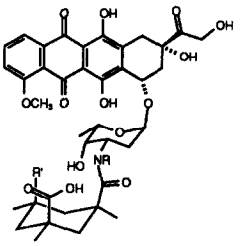
However, this type of link cannot be used with drugs that lack a suitable carbonyl group, and even those that have one often give hydrazones with unsuitable hydrolysis kinetics.[11] Therefore there is great interest in other acid-cleavable linkers.

Although it might seem that one must have $t_{1/2}$ very long at pH 7 and very short at pH 5, this is not necessarily the case with an internalizing MAb such as BR96. What is required is that $t_{1/2}$ at pH 7 be long enough to prevent significant loss of free DOX during localization to the tumor, which takes about 24 h, and short enough at pH 5 to release substantially all the DOX while the conjugate is still retained in the tumor. After administration of a single dose of the BR96-DOX conjugate of ref. 9 to tumor-bearing mice, the tumor retains BR96 for at least 72 h, and intratumoral DOX levels remain steady for days, with $t_{1/2}$ for decline ≥ 4 days[11]. Thus while long and short half-lives at pH 7 and 5 respectively are certainly desirable, what is actually needed is $\geq$ ca. 50 h at pH 7, and $\leq$ ca. 50 h at pH 5.

Using DOX as the model drug, we tested several acid-labile linkers both from the literature and of our own invention (to be described in a full paper). Of these, only one met the above criteria, and we wish to describe its chemistry.

Menger and Ladika[12] reported that Kemp's triacid,[13] with one carboxyl in the form of a pyrrolidine amide, releases pyrrolidine in a pH-dependent manner. Only one of the two free carboxyls is necessary. Although the rate is very high at pD = 1, 21°C ($t_{1/2}$ = 1.9 min), as pD rises it does not fall fast enough for our purpose, being only fourfold slower at pD = 7.05 ($t_{1/2}$ = 7.7 min). This is because the pK_a of the carboxyl is 6.9, so that the break in the pH-rate curve comes at too high a pH. However, two aspects were interesting: (1) when an amide and an ester are both present, competing for the last remaining free COOH, it is the amide, not the ester, that hydrolyzes; (2) an NH-bearing amide (as DOX is) reacts ten times slower.

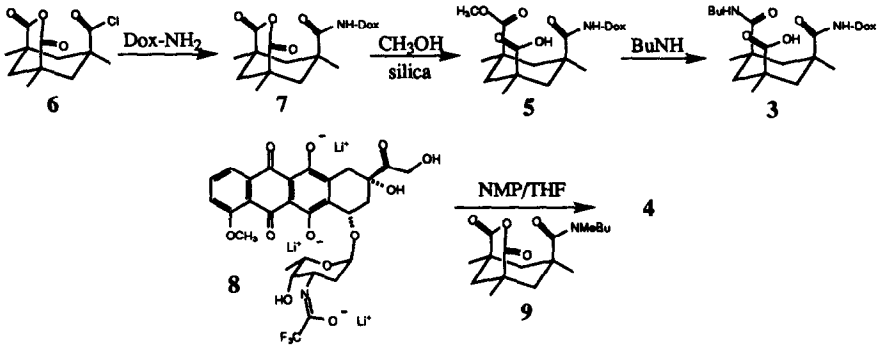
We reasoned that with a DOX amide at one carboxyl, and an ester or an amide at the other as a surrogate for the link to the MAb, hydrolysis at neutral pH might fall to an acceptably low rate owing to the electron-withdrawing ester or amide group's lowering the pK_a of the remaining carboxyl, which should move the pH-rate break to lower pH, and also to the presence of the amide proton. At the same time, at lysosomal pH even a tenfold retardation would leave an acceptably high rate of DOX release. Although this line of reasoning was not completely borne out by experiment, it was ultimately successful. DOX release data for compounds **2-5** are shown in the table below.



Compound	2	3	4	5
R	H	H	$COCF_3$	H
R'	COOH	CONHBu	CONMeBu	COOMe
$t_{1/2}$, pH 5, 37°C	30h	90h	>174h	40h
$t_{1/2}$, pH 7, 37°C	--	>160 days	>174h	>100 days

Compound **2**, the diacid amide, released DOX at a useful rate at pH 5 but at pH 7 it decomposed rapidly, forming a substance whose structure was not established but which did not release DOX, an unacceptable circumstance since this would inevitably occur before reaching the tumor. Cpd. **3**, with a butylamide as a surrogate for a linker to the antibody, had excellent pH 7 stability and did release DOX at pH 5, but at a borderline slow rate. We thought that replacement of the butyl and DOX NHs with methyl and trifluoroacetyl[14] respectively might speed up release by (1) removing intramolecular H-bonding of the free carboxyl which might have interfered with direct activation of the amide carbonyl, and (2) increasing the leaving ability of DOX. Instead, hydrolysis ceased at both pHs, perhaps because the trifluoroacetyl group decreases the basicity of the target carbonyl so that it cannot be activated by the free carboxyl. Finally, replacement of the butyramide of **3** with a methyl ester (**5**) indeed led to a satisfactory acceleration of (quantitative) DOX release at lysosomal pH, in preference to ester hydrolysis, while at pH 7 high stability was retained. Thus **5** fulfills the criteria for an acid-cleavable linker suitable for delivering a drug bearing a primary amine via endocytosis to lysosomes.

Compounds **2**-**5**[15] were prepared as shown below. Kemp's acid chloride-anhydride[12] was coupled to DOX and then chromatographed on silica, eluting with CH_2Cl_2/CH_3OH. This gave mostly methyl ester **5** along with small amounts of anhydride **7**. Diacid **2** resulted from in situ hydrolysis of **7** in pH 5 or 7 buffer as evidenced by a single new peak in the HPLC. Slow aminolysis of **5** with butylamine gave butylamide **3**. Preparation of **4** was carried out by acylation of trianion **8** with methylbutylamide **9**.



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10. The formation of DOX and disappearence of substrate were monitored directly using a C-18 column and a mobile phase consisting of methanol/triethylammonium formate buffer (50 mM)
11. Unpublished experiments from these Laboratories by H.D. King, P.A. Trail and J. Knipe.
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15. For **2**: ^{1}H-NMR (DMSO-d_6) δ 0.97, 1.06 & 1.09 (each 3H, s), 1.12 (3H, m), 1.27 (4H, m), 1.91 (2H, m), 2.08 (4H, m), 2.47 (2H, m), 2.81 (2H, ABq), 3.04 (1H, q), 3.33 (1H, brs), 3.89 (3H, s), 4.12 (1H, q), 4.33 (1H, d), 4.56 (2H, d), 4.78 (1H, brt), 4.89 (1H, t), 5.15 (1H, brd), 7.09 (1H, d), 7.48 (1H, d), 7.71 (2H, m), MS (ion spray) 784.4 $(MH)^+$, 801.2 $(M+NH_4)^+$, 806.0 $(M+Na)^+$, (FAB$^-$) 782 $(M-H)^-$.
For **3**: ^{1}H-NMR (DMSO-d_6) δ 0.71 (3H, t), 0.95 (2H, m), 1.02, 1.03 & 1.09 (each 3H, s), 1.12 (3H, d), 1.21 (2H, m), 1.36 (2H, ABq), 1.88 (2H, m), 2.15 (4H, m), 2.50 (2H, ABq), 2.68 (1H, m), 2.89 (4H, m), 3.42 (1H, brs), 3.78 (1H, m), 3.96 (3H, s), 4.10 (1H, q), 4.54 (2H, d), 4.90 (1H, t), 5.20 (1H, d), 7.51 (1H, brd), 7.62, 7.87 & 7.99 (each 1H, m), MS (FAB) 839.2 $(MH)^+$, 856.4 $(M+NH_4)^+$, 861.2 $(M+Na)^+$; Accurate mass calc. for $C_{43}H_{54}N_2O_{15}Na$: 861.3422, Found: 861.3395.
For **4**: ^{1}H-NMR (CD_3OD) δ 0.72 (3H, t), 0.80-1.20 (12H, m), 1.29 (2H, m), 1.41 (3H, m), 1.86 & 2.32 (3H, m), 2.60 (3H, s), 2.88 (2H, ABq), 2.92 (2H, t), 3.59 (1H, brs), 3.89 (3H, brs), 4.22 (1H, m), 4.33 (1H, m), 5.29 (1H, brs), 7.32, 7.63 & 7.75 (each 1H, m); MS (FAB) 973 $(M+Na)^+$, 989 $(M+K)^+$, (FAB$^-$) 948.6 $(M-H)^-$.
For **5**: ^{1}H-NMR ($CDCl_3$) δ 1.08, 1.14 & 1.19 (each 3H, s), 1.23 (3H, d), 1.39 & 1.71 (each 1H, m), 2.10 (2H, m), 2.68 (4H, brt), 2.94 (2H, ABq), 3.59 (3H, s), 3.84 (1H, m), 3.90 (1H, brs), 3.99 (3H, s), 4.07 (1H, m), 4.72 (2H, brs), 5.19 (1H, brs), 5.49 (1H, brs), 7.29 (1H, d), 7.70 (1H, t), 7.88 (1H, d); MS (FAB): 798.0 $(MH)^+$, 815.2 $(M+NH_4)^+$, 820.0 $(M+Na)^+$, (FAB$^-$) 796 $(M-H)^-$, Accurate mass calc. for $C_{40}H_{48}NO_{16}$: 798.2973, Found: 798.2956.