Synthesis and Stability of Four Maleimide Derivatives of the Anticancer Drug Doxorubicin for the Preparation of Chemoimmunoconjugates

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By attaching maleimide groups to anticancer drugs, derivatives are obtained which bind selectively to thiolated carrier proteins. Four maleimide derivatives of the anticancer drug doxorubicin were prepared in which 3-maleimidobenzoic acid or 4-maleimidophenylacetic acid was bound to the 3′-amino position of doxorubicin through a benzoyl or phenylacetyl amide bond (1 or 2) or in which 3-maleimidobenzoic acid hydrazide or 4-maleimidophenylacetic acid hydrazide was bound to the 13-keto position through a benzoyl or phenylacetyl hydrazone bond (3 or 4). The maleimide derivatives of doxorubicin were characterized by means of ^{13}C-NMR spectroscopy, elemental analysis and mass spectrometry. In addition, the stability of the maleimide derivatives 1—4 at pH values of 5.0 and 7.4 was investigated with the aid of HPLC. The amide or hydrazone bond of 1—4 is stable at pH 7.4 whereas the hydrazone bond is acid-sensitive.

Key words doxorubicin; maleimide derivative; stability; chemoimmunoconjugate

Doxorubicin is a widely used antineoplastic agent in the treatment of leukemia, breast carcinoma, and other solid tumors.[1] The clinical application of this anthracycline drug is, however, limited by its toxic dose-related side effects, such as cumulative cardiotoxicity, myelosuppression, nephrotoxicity and extravasation.[2,3] One approach to overcome the toxicity of anticancer drugs to normal tissue—thereby increasing the therapeutic index of these agents—is to attach cytotoxic drugs to carrier proteins which exhibit a significant uptake in tumor tissue. An effective method of preparing such chemoimmunoconjugates is to introduce a maleimide group into the drug, which is then able to bind selectively to sulfhydryl groups of carrier proteins through its carbon double bond.[4] Recently, we have developed a number of maleimide compounds for this purpose.[5]

When preparing our maleimide derivatives, we wished to vary the site and stability of the chemical link between the drug and the maleimide spacer group. The stability of the bond between the anticancer drug and the carrier protein is an important parameter for the therapeutic efficacy of chemoimmunoconjugates because carrier proteins such as certain monoclonal antibodies, transferrin, or epidermal growth factor are taken up by the cell through receptor-mediated endocytosis.[6] During this process the pH value is reduced from 7.4 to 5.0—5.5 so that the drug can be released if the crosslinking to the carrier is acid-labile. Although a hexoyl hydrazone link with acid-labile properties has been reported for doxorubicin,[7] it has been noted that this chemical link lacks stability under physiological conditions.[8] Thus, we prepared four maleimide derivatives of doxorubicin which differed in the site (3′-amino or 13-keto position) and stability (amide or acylhydrazone bond) of the chemical link between the drug and spacer group. We introduced an aromatic moiety in the linker arm which would allow a subsequent variation of stability parameters by introducing suitable substituents into the aromatic ring.

The method of preparing the maleimide amide derivatives of doxorubicin **1** and **2** is depicted in Fig. 1. The benzoyl or phenylacetyl amides of doxorubicin were synthesized by reacting doxorubicin·HCl with 3-maleimidobenzoic acid chloride or 4-maleimidophenylacetic acid chloride and 2 eq of triethylamine. Isolation of the compounds was performed by chromatography on a silica gel column as well as a preparative diol column. The benzoyl or phenylacetyl hydrazone derivatives **3** and **4** were obtained by reacting doxorubicin·HCl with an excess of 3-maleimidobenzoic acid hydrazide (trifluoroacetate salt) or 4-maleimidophenylacetic acid hydrazide (trifluoroacetate salt) in anhydrous methanol and precipitating the products by adding acetonitrile (Fig. 1). Analytical samples were obtained by recrystallization from methanol/acetonitrile.

The ^{13}C-NMR spectra revealed 36 distinct signals for **1** and **3** and 37 distinct signals for **2** and **4** (the sum of the number of NMR active carbon atoms of doxorubicin and the maleimide spacer group), and the characteristic peaks of the introduced maleimide group are observed at 134—135 and 168—170 ppm for the carbon atoms of the double bond and carbonyl group. Because we observed only one set of signals in the ^{13}C-NMR spectra of **3** and **4**, we tentatively concluded that the compounds are the (*Z*)-isomers. The ^{13}C-NMR signals for doxorubicin could be assigned by comparison with the literature data.[9] Compounds **1**—**4** showed characteristic peaks in the mass spectra as determined by fast atom bombardment (FAB-MS).

The stability of **1**—**4** was studied at pH values of 5.0 and 7.4 on a reverse-phase C^{18}-column with the aid of HPLC. Hydrolysis of the amide and acylhydrazone bond at room temperature was evaluated by following the appearance of free doxorubicin (retention time: 12.2 min) at $\lambda = 495$ nm and chromatograms were recorded every three hours for 24 h. The results are shown in Table 1. Whereas the amide derivatives **1** and **2** showed no release of doxorubicin at pH 5.0 or 7.4, the benzoyl or phenylacetyl hydrazone derivatives **3** and **4** showed good stability

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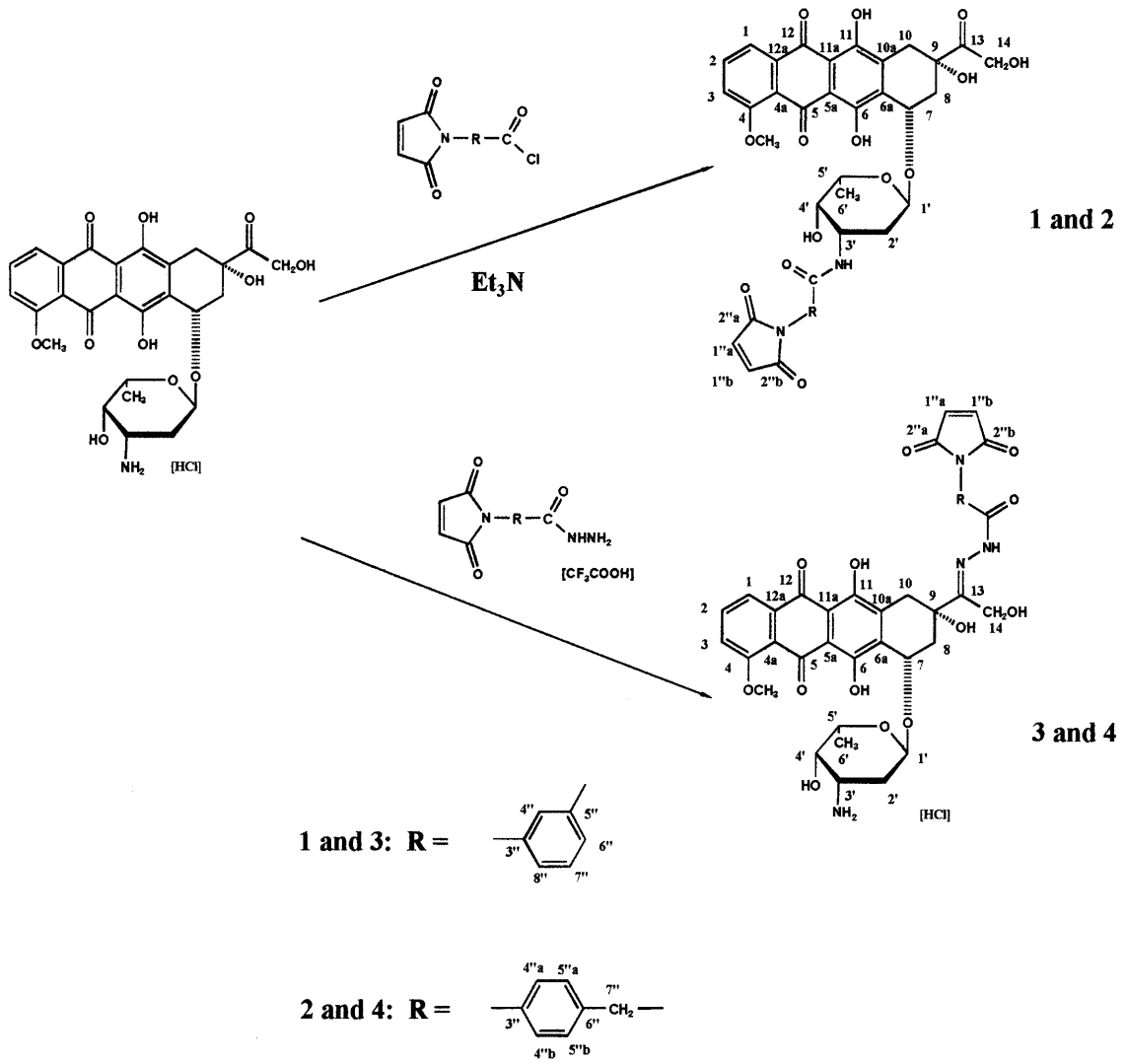

Fig. 1. Structures of Maleimide Derivatives of Doxorubicin **1**—**4**

Table 1. Time- and pH-Dependent Stability of **1**—**4** at pH 7.4 and 5.0 after 6 and 24 h[a)]

Compound	Release of doxorubicin in % after 6 h		Release of doxorubicin in % after 24 h	
	pH 7.4	pH 5.0	pH 7.4	pH 5.0
1	0	2	1	3
2	0	1	0	1
3	3	13	9	41
4	4	15	8	36

a) A 100 μl aliquot of an approximately 200 μM solution of **1**—**4** (dissolved in methanol) was added to 1.0 ml of 0.15 M NaCl, 0.01 M $NaHCO_3$, 0.004 M NaH_2PO_4 containing 10% acetonitrile (pH 7.4 or 4.0) at room temperature. Samples (50 μl) were taken every 3 h and subjected to HPLC. The release of doxorubicin was evaluated from the peak area of doxorubicin (retention time 12.2 min) at $\lambda = 495$ nm.

at pH 7.4 (less than 10% release of doxorubicin after 24 h) but exhibited a significant release at pH 5.0 (approximately 40% release of doxorubicin for **3** and approximately 35% release for **4** after 24 h). This was as expected from the character of the chemical links involved (amide and acylhydrazone bonds respectively).

Compounds **1**—**4** are therefore suitable candidates for binding to carrier proteins in order to evaluate the role of pH-dependent stability in relation to cellular uptake and *in vitro* efficacy. It should also be possible to investigate whether the activity of doxorubicin can be attributed to interactions with the cell membrane and not to intercalation with DNA considering that doxorubicin bound firmly to polymers retains its cytotoxic effect without entering cells.[10)] Compounds **1**—**4** have been bound to thiolated carrier proteins, and we will report on the *in vitro* activity of transferrin and albumin conjugates in selected tumor cell lines in the near future.

Experimental

Instruments used were as follows: melting points, Büchi 530; ^{13}C-NMR, Bruker 400 MHz AM 400 (internal standard: TMS); FAB-MS, Finnigan-MAT 312; elemental analysis, Perkin-Elmer Elemental Analyzer 240; preparative fast protein liquid chromatography (FPLC), LiChroPrepDiol-column (Merck AG, 310-25; 40—63 μm) with an LKB 2248 pump (flow: 3 ml/min), a fraction collector LKB 2211 and a Lambda 1000 UV/visible monitor from Bischoff (280 nm). Silica gel chromatography was carried out on silica gel 60 (0.063—0.100 mm) from Merck AG; TLC, silica coated plates $60F_{254}$ from Merck; and HPTLC on diol glass coated plates F_{254} (0.2 mm) from Merck AG. Organic solvents were of analytical grade and a gift from BASF; other organic or inorganic compounds were from Merck AG. Maleimide spacer groups were prepared previously.[5)] HPLC studies were performed on a C^{18}-Sephasil™ column (5 μm, 4 × 250 mm, Pharmacia; mobile phase, acetonitrile: 0.005 M NaH_2PO_4 (pH 5.0) = 70 : 30) with an LKB 2150 pump (flow: 1.5 ml/min), a Lambda 1000 UV/visible monitor from Bischoff (at $\lambda = 495$ nm), a Merck Hitachi AS400 auto sampler and a

Merck Hitachi D2500 integrator.

Maleimide Amide Derivatives of Doxorubicin Synthesis of **1**: A doxorubicin hydrochloride/lactose mixture (3.0 g), which contained 500 mg (0.86 mmol) of pure doxorubicin hydrochloride, was suspended in 50 ml of anhydrous tetrahydrofuran and 264 mg (1.12 mmol) of *m*-maleimidobenzoic acid chloride and 276 μl (200 mg; 1.98 mmol) of triethylamine were added. This mixture was stirred at room temperature for 15 h. Lactose was removed by filtration and the clear red solution was evaporated *in vacuo*. The residue was dissolved in a minimal amount of tetrahydrofuran and purified using a silica gel column (tetrahydrofuran/hexane, 3/1). An analytical sample of the product was purified by chromatography on a diol-column (ethyl acetate/hexane, 3/1) to afford **1** (224 mg, 35%) as a red powder; *Rf* value, 0.30 (tetrahydrofuran/hexane, 3/1), mp 100—101 °C. ^{13}C-NMR (DMSO-d_6) δ: 16.99 (C-6'), 29.41 (C-2'), 32.03 (C-10), 36.57 (C-8), 46.15 (C-3'), 56.49 (–OCH_3), 63.67 (C-14), 66.66 (C-4'), 67.80 (C-5'), 69.86 (C-7), 74.93 (C-9), 100.37 (C-1'), 110.53 (C-5a), 110.67 (C-11a), 118.89 (C-4a), 119.40 (C-3), 119.62 (C-1), 125.96, 126.62, 128.67, 129.43, 131.49, 134.01 (C-3'' to C-8''), 134.53 (C-12a), 134.67 (C-1''a/1''b), 135.46 (C-10a), 135.48 (C-6a), 136.07 (C-2), 154.46 (C-11), 156.06 (C-6), 160.70 (C-4), 164.86 (amide-C), 169.64 (C-2''a/C-2''b), 186.25 (C-12), 186.36 (C-5), 213.70 (C-13). FAB-MS (rel intensity, 4-nitrobenzyl alcohol) Calcd for $C_{38}H_{34}N_2O_{14}$, M_r=742.68 g/mol, *m/z*: 743 (1.16%), 742 (0.98%), 215 (23.76%), 200 (13.87%). *Anal.* Calcd for $C_{38}H_{34}N_2O_{14}$: C, 61.43; H, 4.61; N, 3.77. Found: C, 60.84; H, 5.54; N, 3.95.

Synthesis of **2**: Doxorubicin hydrochloride (500 mg, 0.86 mmol) was dissolved in 50 ml of anhydrous *N,N*-dimethylformamide (DMF) and 1013 mg (4.30 mmol) of *p*-maleimidophenylacetic acid chloride and 719 μl (522 mg, 5.16 mmol) of triethylamine were added. The solution was stirred at room temperature for 15 h. DMF was removed under high vacuum and the residue was dissolved in a minimal amount of tetrahydrofuran. This solution was filtered and purified on a silica gel column (tetrahydrofuran/hexane, 3/1) to afford **2** (189 mg, 29%) as a red powder, *Rf* value, 0.26 (ethyl acetate/hexane, 3/1), mp 110 °C. ^{13}C-NMR (DMSO-d_6) δ: 16.79 (C-6'), 29.31 (C-2'), 38.67 (C-10), 38.95 (C-8), 46.63 (C-3'), 56.17 (C-7''), 56.40 (–OCH_3), 66.16 (C-14), 66.20 (C-4'), 71.85 (C-5'), 72.72 (C-7), 77.18 (C-9), 99.20 (C-1'), 110.33 (C-5a), 110.67 (C-11a), 118.97 (C-4a), 119.90 (C-3), 120.13 (C-1), 126.59, 129.43, 129.63, 129.95 (C-3'' to C-6''), 134.60 (C-1''a/1''b), 135.03 (C-12a), 135.48 (C-10a), 136.04 (C-6a), 136.20 (C-2), 153.57 (C-11), 153.65 (C-6), 160.60 (C-4), 169.75 (C-2''a/2''b), 171.73 (amide-C), 186.35 (C-12), 186.45 (C-5), 213.68 (C-13). FAB-MS (rel intensity, 4-nitrobenzyl alcohol) Calcd for $C_{39}H_{36}N_2O_{14}$, M_r=756.70 g/mol, *m/z*: 757 (1.52%), 214 (15.82%); *Anal.* Calcd for $C_{39}H_{36}N_2O_{14}$: C, 61.43; H, 4.61; N, 3.77. Found: C, 60.84; H, 5.54; N, 3.95.

Maleimide Hydrazone Derivatives of Doxorubicin Synthesis of **3** and **4**: A solution of 116 mg (0.2 mmol) of doxorubicin hydrochloride and 343 mg (1.0 mmol) of *m*-maleimidobenzoic acid hydrazide (trifluoroacetate salt) or 357 mg (1.0 mmol) of *p*-maleimidophenylacetic acid hydrazide (trifluoroacetate salt) in 100 ml of anhydrous methanol was prepared. To this solution 100 μl of CF_3COOH were added, and the mixture was stirred for 96 h at room temperature and then concentrated to a volume of approximately 50 ml. Acetonitrile was added to the solution until a slight turbidity appeared. The resulting suspension was allowed to stand at −20 °C for 24 h for crystallization of the product. The solid red hydrazone was collected by centrifugation. The supernatant was evaporated to a small volume and treated with acetonitrile as above. The hydrazone fractions were combined and recrystallized from methanol/acetonitrile to give a red microcrystalline powder. (**3**: 89 mg, 55%, **4**: 95 mg, 60%), *Rf* values (reverse phase, acetonitrile: 0.005 M NaH_2PO_4 (pH 5.0)=70/30), **3**: 0.33, **4**: 0.35. mp **3**: >185 °C (dec.), **4**: 156 °C. ^{13}C-NMR (CD_3OD) **3** δ: 16.71 (C-6'), 28.22 (C-2'), 33.17 (C-10), 38.71 (C-8), 46.64 (C-3'), 56.59 (–OCH_3), 66.11 (C-14), 66.18 (C-4'), 71.26 (C-5'), 71.81 (C-7), 71.98 (C-9), 99.27 (C-1'), 110.03 (C-5a), 110.54 (C-11a), 118.69 (C-4a), 118.96 (C-3), 121.30 (C-1), 124.18, 126.73, 130.95, 131.47, 131.91, 132.34 (C-3'' to C-8''), 134.15 (C-12a), 134.72 (C-1''a/1''b), 134.76 (C-10a), 135.21 (C-6a), 136.19 (C-2), 153.65 (C-11), 154.72 (C-6), 156.27 (C-13), 160.77 (C-4), 169.63 (C-2''a/2''b), 184.39 (acyl-C), 184.40 (C-12), 186.48 (C-5); **4**: 16.77 (C-6'), 28.05 (C-2'), 37.19 (C-10), 38.94 (C-8), 46.58 (C-3'), 56.19 (C-7''), 56.57 (–OCH_3), 66.02 (C-14), 66.27 (C-4'), 71.73 (C-5'), 72.28 (C-7), 77.14 (C-9), 98.96 (C-1'), 110.57 (C-5a), 110.66 (C-11a), 118.95 (C-4a), 119.67 (C-3), 120.07 (C-1), 126.58, 129.35, 129.54, 129.84 (C-3'' to C-6''), 134.57 (C-1''a/1''b), 135.58 (C-12a), 135.61 (C-10a), 136.16 (C-6a), 136.19 (C-2), 153.55 (C-11), 153.65 (C-6), 156.32 (C-13), 160.64 (C-4), 169.75 (C-2''a/2''b), 171.76 (acyl-C), 186.40 (C-12), 186.44 (C-5). FAB-MS (rel intensity; 4-nitrobenzyl alcohol) **3**: Calcd for $C_{38}H_{38}N_4O_{12}Cl$, M_r=793.81 g/mol, *m/z*: [M^+−HCl] 758 (2.58%); **4**: Calcd for $C_{39}H_{40}N_4O_{12}Cl$, M_r=807.83 g/mol, *m/z*: [M^+−HCl] 772 (0.16%), 771 (0.26%), 544 (3.34%), 397 (3.84%), 369 (3.27%). *Anal.* **3**: Calcd for $C_{38}H_{38}N_4O_{12}Cl$: C, 57.50; H, 4.82; Cl, 4.46; N, 7.06. Found: C, 57.84; H, 5.24; Cl, 4.12; N, 7.35. **4**: Calcd for $C_{39}H_{40}N_4O_{12}Cl$: C, 57.99; H, 4.96; Cl, 4.39; N, 6.94. Found: C, 60.33; H, 5.19; Cl, 4.01; N, 6.55.

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