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SYNTHESIS OF NEW MALEIMIDE DERIVATIVES OF DAUNORUBICIN AND BIOLOGICAL ACTIVITY OF ACID LABILE TRANSFERRIN CONJUGATES

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Abstract: Maleimide groups were bound to the 3'-amino position of daunorubicin through a benzamide bond or to the 13-keto position through a benzoyl hydrazone or phenylacetyl hydrazone bond. The acid labile transferrin conjugates prepared with the latter two derivatives exhibited high activity in human melanoma cells (MEXF 989) using a clonogenic cell assay comparable to or exceeding that of daunorubicin.

Daunorubicin is a widely used antineoplastic agent in the treatment of acute leukemia[1]. The clinical application of this anthracycline drug is, however, limited by its toxic dose-related side effects, such as cumulative cardiotoxicity, myelosuppression, and nephrotoxicity[2].
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. Due to our interest in the role which natural plasma proteins play in the *in vivo* distribution of anticancer drugs[3], we developed daunorubicin conjugates of the serum protein transferrin, the iron(III) transport protein. Transferrin exhibits a significant uptake in tumor tissue due to high amounts of specific transferrin receptors (150 000 - 1 000 000 per cell) on the cell surface of tumor cells.[4] Furthermore, transferrin is a stable, commercially available protein and has been used as a drug delivery system for toxins and DNA.[5]

An effective method of preparing 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.[6] Recently, we developed a number of maleimide compounds for this purpose.[7]
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 appears to be 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.[8] During internalisation the pH is reduced from pH 7.4 to 5.5-5.0 within endosomes, and this pH change can be exploited through acid-labile crosslinking to the carrier so that the drug can be released inside the tumor cell. An antibody conjugate, in which the related anthracycline doxorubicin was bound through an acid-sensitive hexoylhydrazone link, has shown high *in vitro* antitumor activity[9], although it has been noted that this link with doxorubicin lacks stability under physiological conditions.[10]

Thus, we prepared three maleimide derivatives of daunorubicin which differed in the site (3'-amino or 13-keto position) and stability (benzamide or benzoyl and phenylacetyl hydrazone bond) of the chemical link between drug and spacer group. We introduced an aromatic moiety in the linker arm which would allow a subsequent variation of stability parameters by binding suitable substituents to the aromatic ring.
The method of preparing the maleimide amide derivative of daunorubicin (**1**) is depicted in scheme 1.

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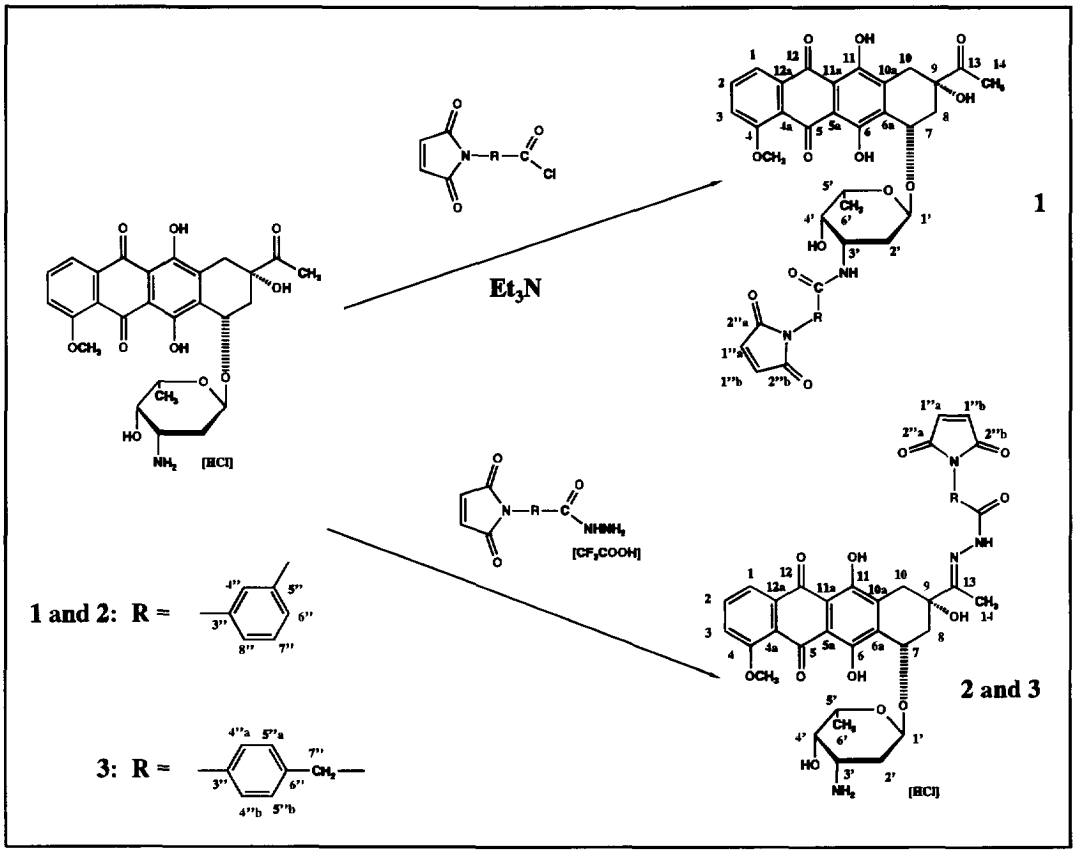
Scheme 1: Synthesis of the daunorubicin maleimide derivatives **1-3**



1 was synthesized by reacting daunorubicin•HCl with 3-maleimidobenzoic acid chloride[6] and two equivalents of triethylamine in THF for 15 h at room temperature in the dark. Isolation of the compound was performed by chromatography on silica gel (THF/hexane 3/1) and subsequent chromatography on a LiChroPrepDiol-column (Merck AG, 310-25; 40-63 µm) (THF/hexane 3/1) to obtain an analytical pure sample (ethyl acetate/hexane 3/1); yield: 68 %, R_f-value: 0.35 (THF/hexane 3/1); m.p.: 110 °C; FAB-MS (4-nitrobenzylalcohol, 3 kV, Mr = 726.69 g/mole): *m/z* = 727 (7 %).

The ^{13}C-NMR-spectrum[11] revealed 36 distinct signals (the sum of the number of NMR active carbon atoms of daunorubicin and the maleimide benzoic ester group), and the characteristic peaks of the introduced maleimide group are observed at 134-135 ppm and 168-170 ppm for the carbon atoms of the double bond and carbonyl group. The ^{13}C-NMR-signals for daunorubicin could be assigned by a comparison with the literature data.[12]

The daunorubicin derivatives **2** and **3** were obtained by reacting daunorubicin•HCl with a five-fold excess of 3-maleimidobenzoic acid hydrazide (trifluoroacetate salt)[6] or 4-maleimidophenylacetic acid hydrazide (trifluoroacetate salt)[6] in anhydrous methanol, addition of catalytic amounts of CF_3COOH and stirring for 96 h at room temperature in the dark (scheme 1). The products were precipitated by repeated addition of acetonitrile. Analytical samples were obtained by crystallization from methanol/acetonitrile; yields: (**2**) 60 %, (**3**) 70 %; R_f-values (RP-C^{18}, Merck, acetonitrile/0.005 M NaH_2PO_4 (pH 5.0) = 70/30): **2**: 0.41 **3**: 0.38; m.p.: **2**: > 192 °C (decomp.), **3**: > 159 °C (decomp.); FAB-MS (4-nitrobenzylalcohol, 3 kV): **2**: (Mr = 777.82 g/mole) *m/z* = 741 [M^+-HCl] (3.0 %), **3**: Mr = (791.82 g/mole) *m/z* = 791 [M^+] (1.3 %); 755 [M^+-HCl] (2.9 %) ^{13}C-NMR-data (see Note 13).

A characteristic mole peak of **1-3** was observed in the mass spectra as determined by fast atom bombardment (FAB).

The pH-dependent stability of **1-3** was studied at pH-values of 5.0 and 7.4 on a reverse-phase C_{18}-column with the aid of HPLC.[14] Hydrolysis of the amide and hydrazone bond at room temperature was evaluated through the appearance of free daunorubicin (retention time: 13.5 min) at λ = 495 nm and chromatograms were recorded every three hours for 24 h. Whereas the amide derivative **1** showed no release of daunorubicin at pH 5.0 or 7.4, the hydrazone derivatives **2** and **3** showed good stability at pH 7.4 (less than 10% release of daunorubicin after 24 hours) and a significant release at pH 5.0 ($t_{1/2}$ (**2**) approximately 20 h, $t_{1/2}$ (**3**) approximately 30 h).
1-3 are therefore good candidates for binding to carrier proteins and evaluating the role of *pH-dependent stability* in relationship to cellular uptake and *in vitro* efficacy. Following this rationale it should also be possible to investigate whether activity of daunorubicin can be attributed to interactions with the cell membrane and not to intercalation with DNA considering that anthracyclins bound firmly to polymers retain their cytotoxic effect without entering cells.[15]

The transferrin conjugates were prepared by reacting the maleimide derivatives of daunorubicin **1-3** with thiolated human serum transferrin. The HS-group adds to the double bond of the maleimide group in a fast and selective reaction forming a stable thioether bond. During thiolation the formation of disulphide bonds was prevented by addition of 0.001 M EDTA and degassing all buffers with argon. Under these conditions the number of introduced HS-groups was highly reproducible, an average number of 3.2 - 3.5 HS-groups being introduced. For preparing the conjugates it proved advantageous to dilute the thiolated sample before adding the daunorubicin maleimide derivative dissolved in a minimal amount of dimethylformamide, then concentrating the mixture to a small volume, and finally isolating the conjugates by gel filtration. This procedure prevented the formation of polymeric products; the purity of the samples was > 90 % as determined at λ = 495 nm with an analytical HPLC-size exclusion column (Bio-Sil SEC 250 (300 mm x 7.8 mm) from Bio-RAD, mobile phase: 0.15 M NaCl, 0.01 M NaH_2PO_4, 5% CH_3CN - pH 7.0). The preparation of the conjugates is described in Note 16.

Biological data: The transferrin conjugates prepared with **1**, **2** and **3** (T-DAUNO-MBS, T-DAUNO-HYD and T-DAUNO-ARZID) were subsequently tested for biological activity in human melanoma cells (MEXF 989) using a clonogenic assay in which the inhibition of colony formation of tumor stem cells in soft agar is studied. The tumor cells used in the assay were prepared directly from the melanoma xenograft MEXF 989 growing as a solid tumor in athymic nude mice. The results of the colony forming assay are illustrated in Figure 1.

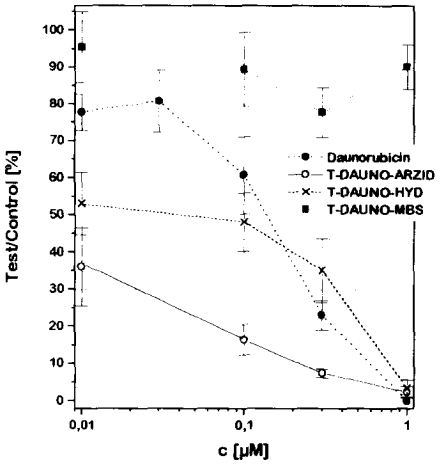


Fig. 1. Antitumor activity of daunorubicin and the transferrin conjugates T-DAUNO-MBS, T-DAUNO-HYD and T-DAUNO-ARZID in a human melanoma xenograft (MEXF 989) using a clonogenic assay.
The clonogenic assay was performed as a two-layer soft agar assay and is described in detail in Fiebig et al.[17] Drug effects were expressed in terms of the percentage of survival, obtained by comparison of the mean number of colonies in the treated plates with the mean colony count of the untreated controls (test-versus-control-group value, T/C = colony count$_{Treated\ Group}$ • 100 / colony count$_{Control\ Group}$).

The transferrin conjugate prepared with the amide derivative, T-DAUNO-MBS, showed no activity at the concentrations tested (0.01-1.0 μM) whereas daunorubicin, T-DAUNO-HYD and T-DAUNO-ARZID showed a dose-dependent reduction in the number of formed colonies (IC_{70}(daunorubicin) = 0.26 μM, IC_{70}(T-DAUNO-HYD) = 0.39 μM, IC_{70}(T-DAUNO-ARZID) = 0.022 μM). Especially, T-DAUNO-ARZID, the transferrin conjugate prepared with the benzoylhydrazone derivative **3**, was more active than free daunorubicin (IC_{70}-value approximately tenfold lower). Antitumor activity of the acid labile conjugates T-DAUNO-HYD and T-DAUNO-ARZID was confirmed in a further xenograft (LXFL 529/11, lung, large cell) -IC_{90}(daunorubicin) = 0.03 μM, IC_{90}(T-DAUNO-HYD) < 0.01 μM, IC_{90}(T-DAUNO-ARZID) < 0.01 μM.
It is worth mentioning, that a transferrin conjugate, in which doxorubicin is bound to transferrin through glutaraldehyde coupling has shown antitumor activity in tumor cell lines[18], although the authors do not present any data concerning the property of the chemical link involved.

From our preliminary data we conclude that the acid-labile properties of the link between transferrin and daunorubicin are important for retaining the antitumor activity of daunorubicin. Furthermore, the daunorubicin benzoylhydrazone derivative **3** appears to be an active model compound for preparing tailor-made acid-labile links between spacer and drug by introducing suitable substituents into the aromatic ring of the spacer group.
Relevant synthetic work in order to establish detailed structure-activity relationships of the acid-sensitivity of the chemical link with respect to *in vitro* activity and cellular uptake are under way in our research department.

Acknowledgments
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11. ^{13}C-NMR-data - 400 MHz (DMSO-d_6) for **1**: δ 16.99 (C-6'); 24.03 (C-14); 29.42 (C-2'); 31.50 (C-10); 6.06 (C-8); 46.17 (C-3'); 56.44 (-OCH_3); 66.66 (C-4'); 67.82 (C-5'); 70.08 (C-7); 75.15 (C-9); 100.34 (C-1'); 110.44 (C-5a); 110.56 (C-11a); 118.81 (C-4a); 119.51 (C-1); 119.80 (C-3); 125.95; 126.62; 128.66; 129.42; 131.49; 134.38 (C-3'' to C-8''); 134.44 (C-12a); 134.67 (C-1''a/1''b); 135.47 (C-6a); 135.52 (C-10a); 135.99 (C-2); 154.46 (C-11); 156.10 (C-6); 160.64 (C-4); 164.85 (amide-C); 169.74 (C-2''a/C-2''b); 186.11 (C-12); 186.24 (C-5); 211.75 (C-13)
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13. ^{13}C-NMR-data - 400 MHz (DMSO-d_6) for **2**: δ 16.73 (C-6'); 28.17 (C-2'); 33.19 (C-10); 38.28 (C-14); 38.67 (C-8); 46.63 (C-3'); 56.56 (-OCH_3); 66.04 (C-4'); 66.16 (C-5'); 71.38 (C-7); 72.30 (C-9); 99.20 (C-1'); 110.50 (C-5a); 110.54 (C-11a); 118.92 (C-4a); 119.64 (C-1); 119.84 (C-3); 125.72; 125.81; 126.23; 128.97;

129.72; 131.77 (C-3'' to C-8''); 133.62 (C-12a); 134.68 (C-1''a/1''b); 135.45 (C-6a); 135.87 (C-10a); 136.14 (C-2); 154.55 (C-11); 156.32 (C-6); 160.73 (C-4); 165.16 (C-13); 169.68 (C-2''a/2''b); 175.15 (acyl-C); 186.31 (C-12); 186.39 (C-5);
for **3**: δ 16.76 (C-6'); 27.99 (C-2'); 33.44 (C-10); 38.20 (C-14); 38.87 (C-8); 46.60 (C-3'); 50.28 (C-7''); 56.52 (-OCH_3); 65.98 (C-4'); 66.25 (C-5'); 71.67 (C-7); 71.70 (C-9); 98.94 (C-1'); 110.42 (C-5a); 110.62 (C-11a); 118.89 (C-4a); 119.60 (C-1); 119.93 (C-3); 126.06; 129.57; 129.86; 135.36; (C-3'' to C-6''); 134.56 (C-1''a/1''b); 135.76 (C-12a); 135.45 (C-6a); 135.89 (C-10a); 136.06 (C-2); 154.16 (C-11); 156.30 (C-6); 160.70 (C-4); 169.77 (C-13); 169.88 (C-2''a/2''b); 172.56 (acyl-C); 186.34 (C-12); 186.36 (C-5); because we observed only one set of signals in the ^{13}C-NMR-spectra of **2** and **3**, we tentatively propose the presence of (Z)-isomers.
14. HPLC studies were performed on a C18-Sephasil™ (5 µm, 4 x 250 mm, Pharmacia), mobile phase: acetonitrile/0.005 M NaH_2PO_4 (pH 5.0) = 70/30; LKB 2150 pump (flow: 1.5 ml/min), Lambda 1000 UV/visible monitor from Bischoff (at λ = 495 nm), Autosampler Merck Hitachi AS400, Integrator Merck Hitachi D2500.
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16. Preparation of transferrin conjugates: Data for one representative experiment is given.

(1) Thiolation of Transferrin (Tf) using Iminothiolane - 64 mg Tf (a gift from Boehringer Mannheim, 98 %, crystalline) were dissolved in 2.0 ml degassed buffer (0.1 M sodium borate, 0.001M EDTA, 0.15 M NaCl - pH = 8.0, c(Tf) ≈ 4,0 x 10^{-4} M) and 140 µl of a 4 x 10^{-2} M iminothiolane•HCl solution (5.5 mg iminothiolane•HCl dissolved in 1.0 ml of the same buffer) were added. After 60 min thiolated transferrin is isolated through size exclusion chromatography (Sephadex G-25F, Pharmacia, column: d = 1.0 cm, l = 10 cm, buffer: standard borate). The protein concentration of the thiolated samples was determined using the ε-values for transferrin ε_{280} = 92 300 M^{-1} cm^{-1} [3] and the concentration of HS-groups with Ellmann's reagent ε_{412} = 13 600 M^{-1} cm^{-1} [19]. The average number of introduced HS-groups was 3.2. To ensure that Fe(III) had not been released during thiolation, the Fe(III)-concentration was determined using ε $[Tf]_{465}$ = 4650 M^{-1} cm^{-1} [3] and showed that samples of thiolated transferrin contained at least 90% iron. The sample of thiolated transferrin (3.5 ml) was used directly for the synthesis of the conjugate.

*(2) Reaction of Maleimide Derivative **1** with Thiolated Transferrin* - 3.5 ml thiolated sample were diluted to a volume of 30 ml with standard borate (0.0025 M sodium borate, 0.15 M NaCl - pH 7.5) and 1.0 ml of a solution of **1** (Mr 726.69) in dimethylformamide (1.8 mg dissolved in 1.0 ml dimethylformamide) were added, homogenised and the slightly turbid mixture was kept at room temperature for 10 min. Concentration of this mixture to a volume of approximately 2.0 ml was carried out with CENTRIPREP®-10-concentrators from Amicon, FRG (60 min at 4 °C and 4500 rpm). The concentrated sample was centrifuged for 5 min with a Sigma 112 centrifuge and the supernatant loaded on a Sephadex G-25F column and the conjugate isolated (retention volume: 3.5 - 7.0 ml, buffer: standard borate). The protein concentration of the conjugates was determined using a BCA-protein-assay from Pierce (USA). The amount of daunorubicin bound to transferrin was determined using the ε-value for daunorubicin in physiological buffer ε_{477} = 9280 M^{-1} cm^{-1} [20] and subtracting the contribution from transferrin at this wavelength (ε$[Tf]_{477}$ = 4300 M^{-1} cm^{-1}) with respect to the number of introduced HS-groups. The concentration of bound daunorubicin was 310 µM and that of transferrin 101 µM which corresponds to approximately 3.1 equivalents of daunorubicin bound to transferrin.
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The melanoma xenograft MEXF 989 growing subcutaneously in thymus aplastic nude mice (nu/nu strain) was removed under sterile conditions. After mechanical disaggregation a single cell suspension was prepared which was used for the clonogenic assay. The bottom layer in the soft agar assay consisted of 0.2 ml Iscoves's Modified Dulbecco's Medium with 20% fetal calf serum and 0.75% agar. 6x10^4 cells were added to 0.2 ml of the same culture medium and 0.4% agar and plated in 24-multiwell dishes on to the base layer. Drugs were applied by continuous exposure (drug overlay) in 0.2 ml medium. Every dish included six control wells containing the vehicle and drug treated groups in triplicate at 6 concentrations. Cultures were incubated at 37 °C and 7% CO_2 in a humidified atmosphere for 12 days and monitored closely for colony growth using an inverted microscope. Within this period, *in vitro* tumor growth led to the formation of colonies with a diameter of 50 µm. At the time

of maximum colony formation, counts were performed with an automatic image analysis system (OMNICON FAS IV, Biosys GmbH). 24 hours prior to evaluation, vital colonies were stained with a sterile aqueous solution of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (1 mg/ml, 100 µl/well). An assay was considered fully evaluable, if the following quality control criteria were fulfilled:

- Mean number of colonies in the control group dishes for 24-multiwells of 20 colonies with a colony diameter of > 50 µm
- The positive reference compound 5-FU (at the toxic dose of 1000 µg/ml) must produce a colony survival of < 30% of the controls
- Coefficient of variation in the control group of 50%

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