Synthesis and in Vitro Efficacy of Transferrin Conjugates of the Anticancer Drug Chlorambucil

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One strategy for improving the selectivity and toxicity profile of antitumor agents is to design drug carrier systems employing soluble macromolecules or carrier proteins. Thus, five maleimide derivatives of chlorambucil were bound to thiolated human serum transferrin which differ in the stability of the chemical link between drug and spacer. The maleimide ester derivatives **1** and **2** were prepared by reacting 2-hydroxyethylmaleimide or 3-maleimidophenol with the carboxyl group of chlorambucil, and the carboxylic hydrazone derivatives **5**−**7** were obtained through reaction of 2-maleimidoacetaldehyde, 3-maleimidoacetophenone, or 3-maleimidobenzaldehyde with the carboxylic acid hydrazide derivative of chlorambucil. The alkylating activity of transferrin-bound chlorambucil was determined with the aid of 4-(4-nitrobenzyl)pyridine (NBP) demonstrating that on average 3 equivalents were protein-bound. Evaluation of the cytotoxicity of free chlorambucil and the respective transferrin conjugates in the MCF7 mammary carcinoma and MOLT4 leukemia cell line employing a propidium iodide fluorescence assay demonstrated that the conjugates in which chlorambucil was bound to transferrin through non-acid-sensitive linkers, i.e., an ester or benzaldehyde carboxylic hydrazone bond, were not, on the whole, as active as chlorambucil. In contrast, the two conjugates in which chlorambucil was bound to transferrin through acid-sensitive carboxylic hydrazone bonds were as active as or more active than chlorambucil in both cell lines. Especially, the conjugate in which chlorambucil was bound to transferrin through an acetaldehyde carboxylic hydrazone bond exhibited IC_{50} values which were approximately 3−18-fold lower than those of chlorambucil. Preliminary toxicity studies in mice showed that this conjugate can be administered at higher doses in comparison to unbound chlorambucil. The structure−activity relationships of the transferrin conjugates are discussed with respect to their pH-dependent acid sensitivity, their serum stability, and their cytotoxicity.

Introduction

Chlorambucil (Leukeran) is a nitrogen mustard which is used clinically against chronic lymphatic leukemia, lymphomas, and advanced ovarian and breast carcinomas.[1] The clinical application of this anticancer drug, which exhibits its cytotoxicity due to its alkylating properties, is, however, limited by its toxic side effects such as nausea, myelotoxicity, and neurotoxicity.[2]

One approach to overcome the toxicity of anticancer drugs to normal tissue is to attach cytotoxic drugs to suitable carrier proteins which accumulate in tumor tissue. Due to our interest in the role which human plasma proteins play in the in vivo distribution of anticancer drugs,[3,4] we have developed chlorambucil 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/cell) on the cell surface of tumor cells.[5,6] Antigenic heterogeneity or modulation is not present (in comparison to tumor-associated antigens) since the transferrin receptor is essential for cell growth.[7] Furthermore, transferrin has been used as a delivery system for toxins and DNA[8] and is a stable, commercially available protein, which has been intensively studied and characterized.[6]

Chlorambucil was one of the first anticancer agents which was used to prepare antibody conjugates.[9−11] However, in these reports[9,10] chlorambucil was either physically adsorbed or chemically linked to the carrier by mere incubation or by direct coupling using *N*-hydroxysuccinimide/*N*,*N*′-dicyclohexylcarbodiimide (DCC).[11] These direct methods have the disadvantage that during preparation polymeric products are likely to be formed and the resulting conjugates are chemically not well-defined with respect to the chemical link between drug and carrier protein.

We therefore wanted to improve the coupling technique in order to prepare better defined protein conjugates of chlorambucil in which the stability of the bond between carrier protein and chlorambucil could also be varied. An effective method of preparing protein conjugates 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

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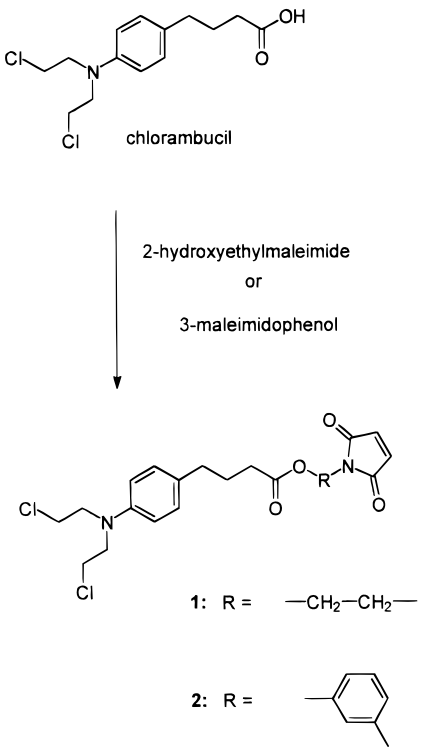
Scheme 1



bond.[12] Recently, we developed a number of maleimide compounds for this purpose.[13]

Chlorambucil is a suitable agent for chemical modification due to the presence of only one carboxyl group in the molecule, and thus we synthesized five maleimide derivatives of chlorambucil which differed in the stability of the chemical link (aliphatic and aromatic ester or carboxylic hydrazone bond) between the drug and the spacer group. The rationale for varying the stability of the chemical link was to assess the significance of the pH-dependent stability of the link between drug and carrier for in vitro and in vivo activity. Transferrin is taken up by the cell through receptor-mediated endocytosis. During internalization the pH is reduced from 7.4 to 5.5−5.0, and this pH change can be exploited through acid cleavage of a predetermined breaking point so that the drug can be released inside the tumor cell.[14]

In this paper we report on the synthesis and characterization of chlorambucil−transferrin conjugates, their antiproliferative efficacy, and the relationship between pH-dependent stability, serum stability, and cytotoxicity.

Chemistry

Synthesis of Maleimide Derivatives of Chlorambucil. The route of preparing the maleimide ester derivatives of chlorambucil (**1** and **2**) is depicted in Scheme 1. The aliphatic and aromatic esters of chlorambucil were synthesized by reacting chlorambucil with an excess of 2-hydroxyethylmaleimide or 3-maleimidophenol in CH_2Cl_2 and addition of DCC or *N*-cyclohexyl-*N*′-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and catalytic amounts of (dimethylamino)pyridine.

The carboxylic hydrazone derivatives **5**−**7** were obtained by reaction of the acid hydrazide of chlorambucil (prepared by reacting the acid chloride of chlorambucil with *tert*-butyl carbazate and subsequent cleavage with CF_3COOH) with 2-maleimidoacetaldehyde, 3-maleimidoacetophenone, or 3-maleimidobenzaldehyde (see Scheme 2).

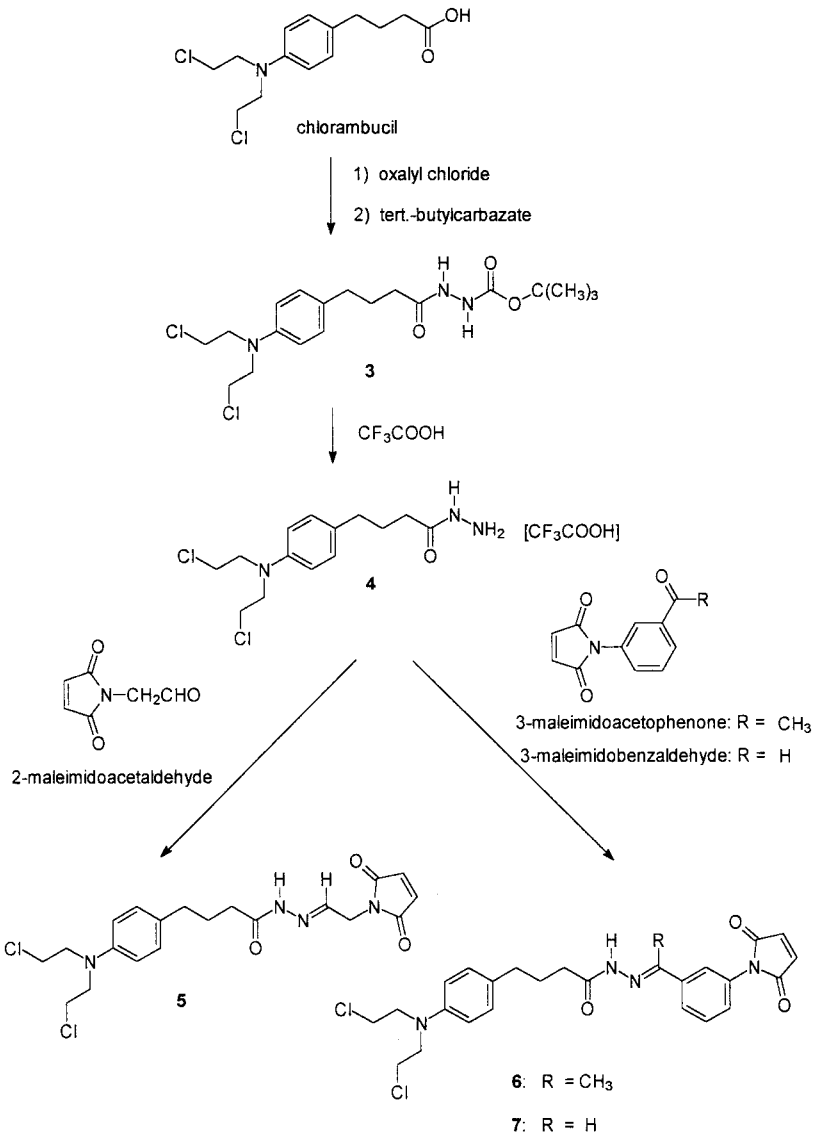
Characteristic peaks of the introduced maleimide groups are singlets in the range from 6.8 to 7.2 ppm for the proton signals of the double bond in the ^{1}H NMR spectra and at 134−135 and 169−170 ppm for the carbon atoms of the double bonds and carbonyl groups in the ^{13}C NMR spectra. Furthermore, the proton signal of the carboxyl group (12.1 ppm in chlorambucil) is not present in the spectra of **1**, **2**, and **5**−**7**. In the ^{1}H NMR and ^{13}C NMR spectra of **5**−**7** (recorded in $CDCl_3$) only one set of signals is observed showing the presence of only one steresoisomer. From a steric point of view the *E*-isomer is favored, and we therefore tentatively suggest that this stereoisomer is present which is in accordance with studies on simple hydrazones.[15]

The molecular ion peak of **1, 2**, and **5**−**7** was observed in the mass spectra (EI/CI, FAB) indicating that the chlorine atoms were not lost or substituted during synthesis which is also confirmed by elemental analysis (see the Experimental Section). In addition, we determined the alkylating activity of our derivatives with the aid of a nitrobenzylpyridine (NBP)-based assay according to Epstein et al.[16] This reagent produces a violet-colored complex ($\epsilon_{565} = 15\ 100\ M^{-1}\ cm^{-1}$) with alkylating agents under basic conditions. Compounds **1**, **2**, and **5**−**7** exhibited 97−100% alkylating activity when compared to pure chlorambucil, which was set as the standard.

Results and Discussion

pH-Dependent Stability of 8−12. To assess the significance of pH-dependent stability of the chemical link between drug and protein for subsequent in vitro studies, we wanted to determine the stability of the ester and hydrazone links realized in **1**, **2**, and **5**−**7**. Unfortunately, due to the rapid hydrolysis (detection of a number of peaks within a few hours with the aid of HPLC[17]) of the chlorine atoms of chlorambucil, aqueous stability studies of **1**, **2**, and **5**−**7** with respect to the chemical link between the maleimide spacer and chlorambucil could not be carried out. We therefore synthesized the model compounds **8**−**12** in which chlorambucil was substituted by 4-phenylbutyric acid and the maleimide spacer by ethanol, phenol, phenylacetaldehyde, acetophenone, and benzaldehyde, respectively[18] (see Chart 1). **8** and **9** were synthesized according to the literature[19] and **10**−**12** in analogy to that of **5**−**7** (synthesis and characterization data are available as Supporting Information). The pH-dependent stability of **8**−**12** was studied at pH values of 5.0 and 7.4 on a reverse-phase C18 HPLC column. The decrease of the peak areas of **8**−**12** in the chromatograms was used to calculate the respective half-lives. Whereas the half-lives of **8** and **9** were not reached after 4 days at either pH 5.0 or 7.4 (<10−20% decomposition after 96 h), the decomposition of the carboxylic hydrazones at pH 5.0 was fast for **10** and **11** [$t_{1/2} \approx 5$ h (**10**); $t_{1/2} \approx 11$ h (**11**)] but slow for **12** [$t_{1/2} \approx 90$ h]. This hydrolytic classification can be expected from the character of the chemical link involved.[20]

Scheme 2



All hydrazones showed good stability at pH 7.4 (<10% decomposition after 96 h). In a further set of experiments we analyzed the stability of **8**−**12** in cell-conditioned culture medium over a period of 6 days. Samples were incubated at 37 °C, ultrafiltered (cutoff MW 10 000), and analyzed through HPLC (see the Experimental Section) every 24 h. In all cases the decrease in the original peak area was less than 20% demonstrating that the model compounds are sufficiently stable under these experimental conditions.

Preparation of Transferrin Conjugates (T-1, T-2, T-5, T-6, T-7). (Abbreviations: Tf, human serum transferrin; T-1, T-2, T-5, T-6, T-7, refer to the respective chlorambucil−transferrin conjugates prepared with the maleimide derivatives **1**, **2**, **5**, **6**, and **7**.) The transferrin conjugates were prepared by reacting **1**, **2**, and **5**−**7** with thiolated 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 using iminothiolane the formation of disulfide 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, with an average number of 3.0−3.2 HS groups being introduced. For preparing the conjugates the respective maleimide derivative was added to the thiolated protein sample and T-1, T-2, T-5, T-6, or T-7 purified by gel chromatography (Sephadex G-25). To rule out the presence of unbound drug as well as polymeric byproducts which may be formed in the coupling step, the purity of the conjugates was determined with an analytical HPLC size exclusion column (Bio-Sil SEC 250). Typical chromatograms recorded at $\lambda = 280$ nm show a main peak at 7.2 min corresponding to that of monomeric transferrin; a small peak at 6.35 min results from a dimeric product with a peak area of less than 10% in our conjugates (commercially available transferrin also shows this peak with a peak area of approximately 2−3%). In addition, the transferrin conjugates and native transferrin were analyzed by agarose electrophoresis as well as SDS−PAGE showing a dominant band at the position of monomeric transferrin (MW 80 000)—data not shown.

The amount of chlorambucil bound to transferrin was determined with the aid of the NBP assay which was modified accordingly (see the Experimental Section), whereas the protein concentration of the conjugates was determined using the BCA-protein assay from Pierce.

Chart 1. Structures of Model Compounds **8**−**12**

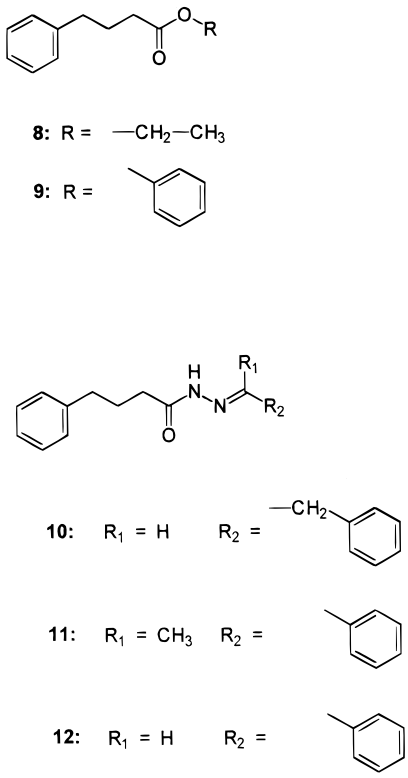


Table 1. Time-Dependent Stability of the Alkylating Activity of the Chlorambucil−Transferrin Conjugates in Comparison to Free Chlorambucil Incubated with 10% Human Blood Serum at 37 °C

		alkylating activity (% remaining after hours of incubation)[a]		
compound	$t_{1/2}$ (h)	24 h	48 h	96 h
chlorambucil	4.5	12.2	6.6	
T-1	3.1	20.9	13.8	11.0
T-2	4.3	22.3	15.2	12.1
T-5	4.0	25.0	19.5	15.2
T-6	4.5	22.9	13.7	10.9
T-7	5.9	24.1	14.5	13.4

[a] Starting concentrations in the incubated samples were $c \sim 330\ \mu$M in each case with respect to alkylating activity; this value at $t = 0$ h was set as 100%. Averages obtained in two independent experiments are presented (determinations were carried out in triplicate). Standard deviations were below ±5% and are not shown.

In this way the number of equivalents of chlorambucil bound to transferrin was calculated.[21] The ratio of chlorambucil/transferrin in the conjugates was 2.8−3.1 which corresponded well with the number of introduced HS groups (see above).

Stability in 10% Serum. To determine the loss of alkylating activity of chlorambucil and our transferrin conjugates in serum, we incubated chlorambucil and the conjugates with 10% human serum[22] at $T = 37$ °C and determined the loss of alkylating activity with the aid of the NBP assay over a period of 4 days. The results of these incubation studies are shown in Table 1. There is an initial rapid decrease of alkylating activity in all of the compounds (approximately 50% of activity remaining after 4−6 h). After this time a gradual loss of alkylating activity is observed which is larger for chlorambucil than for the conjugates, there being little difference between the individual transferrin conjugates (see Table 1).

Table 2. IC_{50} Values (μM)[a] for the Chlorambucil−Transferrin Conjugates in Comparison to Free Chlorambucil in the MCF7 Mammary Carcinoma and MOLT4 Leukemia Cell Lines

compound	MCF7	MOLT4
chlorambucil	24.6 ± 1.7	6.3 ± 0.8
T-1	−	7.8 ± 0.7
T-2	−	15.3 ± 1.3
T-5	6.8 ± 0.4	0.35 ± 0.08
T-6	23.2 ± 1.5	3.3 ± 0.3
T-7	35.5 ± 3.4	7.5 ± 0.9

[a] IC_{50} values (50% inhibitory concentration) represent the mean ± standard deviation ($n = 3$). Chlorambucil hydrazide (**4**) showed comparable activity to T-5 in the MOLT4 cell line and was as active as chlorambucil in the MCF7 cell line as demonstrated in subsequent cell culture experiments. Concentrations refer to the equialkylating activity of the respective compounds.

Biological Data. The newly synthesized transferrin conjugates and unbound chlorambucil were evaluated for inhibitory effects in a mammary carcinoma (MCF7) and an acute lymphoblastic leukemia (MOLT4) cell line employing a propidium iodide fluorescence assay. Both cell lines were transferrin receptor-positive as shown by flow cytometric analysis with an anti-human CD71 antibody directed against the transferrin receptor (FACS data are available as Supporting Information). Respective IC_{50} values are summarized in Table 2 (cell culture data is included as Supporting Information). Transferrin and the employed buffer (0.0025 M sodium borate, 0.15 M NaCl, pH 7.2) had no influence on cell growth in both cell lines (data not shown). All of the tested compounds showed dose-dependent activity in both cell lines (concentration range 0.15−40 μM). The transferrin conjugates T-1, T-2, and T-7, which do not contain an acid-sensitive bond according to our stability studies using model compounds, are the least active compounds in both cell lines, IC_{50} values being reached at high concentrations (>7 μM) or not at all in the tested concentration range. This result suggests that the low antiproliferative effect of these conjugates could be due either to a slow release of chlorambucil under cell culture conditions or to interactions with the plasma membrane, which has been suggested as an additional target for the activity of alkylating agents besides their binding to cellular DNA.[23]

In contrast, the acid-sensitive conjugates T-5 and T-6 exhibit antiproliferative activity which is comparable to or exceeds that of chlorambucil. Above all, T-5 exhibited IC_{50} values which were approximately 3-fold lower (MCF7) or 18-fold lower (MOLT4) than those of chlorambucil. Chlorambucil hydrazide (**4**), which is the hydrolysis product after cleavage of the carboxylic hydrazone bond, showed comparable activity to T-5 in the MOLT4 cell line and was as active as chlorambucil in the MCF7 cell line as demonstrated in subsequent cell culture experiments. Among the transferrin conjugates prepared with the carboxylic hydrazone derivatives **5**−**7**, cytotoxicity is enhanced with increasing acid sensitivity of the hydrazone link according to pH-dependent stability studies carried out with the related model compounds **10**−**12**.[24] Furthermore, stability studies of **8**−**12** in cell-conditioned medium show that the respective ester and carboxylic hydrazone bonds are not prone to rapid hydrolysis. These results taken together indicate

that antiproliferative in vitro activity is correlated with the chemical link between chlorambucil and transferrin. The importance of the acid sensitivity of the linker between drug (e.g., anthracyclines) and the carrier protein for in vitro and in vivo anticancer activity has been noted by Greenfield et al.[25] and our group.[26−28]

When comparing the serum stability of chlorambucil and the transferrin conjugates with respect to their alkylating activity, we found an increased stability of the conjugates over a period of 4 days. However, there was no significant difference between the conjugates themselves indicating that the superior antiproliferative activity of T-5 and T-6 over T-1, T-2, and T-7 observed in vitro cannot, on the whole, be ascribed to the prolonged alkylating activity of chlorambucil−transferrin conjugates, but rather to their acid-sensitive properties.

Admittedly, our results do not rule out the possibility that chlorambucil hydrazide might be released extracellularly by the acid-sensitive conjugates T-5 and T-6, although as we have mentioned above, only small amounts are liberated at pH 7.4, and the model spacers demonstrate a good stability in cell-conditioned medium. The significance of the transferrin receptor for uptake of the chlorambucil−transferrin conjugates is an important issue for the outlined approach of tumor cell targeting, and we are currently investigating the mechanistic aspects of conjugate cell transport (e.g., through inhibition experiments with antibodies directed against the transferrin receptor and influence of ionophores on the inhibitory effects of the conjugates, respectively).

Preliminary Toxicity Studies. Subsequently, chlorambucil and T-5 were subjected to acute toxicity studies (dose, 20 mg/kg per day with respect to the amount of chlorambucil present; administration: ip, 3 consecutive days). Whereas two out of three mice in the group treated with free chlorambucil died after the second or third injection, all mice treated with the conjugate survived. No body weight loss was observed in this group.

In summary, we have shown that maleimide derivatives of chlorambucil can be bound effectively to thiolated transferrin by which transferrin conjugates are obtained in high purity which retain the alkylating activity of the drug. Transferrin holds promise as a drug carrier due to the large number of transferrin receptors on the surface of tumor cells. Expected advantages of these protein conjugates are preferable tissue distribution, prolonged half-life of the drug in the plasma, and controlled drug release from the carrier protein by adjustment of the chemical properties of the bond between the drug and the carrier.

In our effort to prepare new active formulations of chlorambucil, we have demonstrated that acid-sensitive chlorambucil−transferrin conjugates (T-5 and T-6) demonstrate antiproliferative activity in two human tumor cell lines (MCF7 and MOLT4) which is comparable to or exceeds that of free chlorambucil. Especially, the conjugate in which chlorambucil was bound to transferrin through an acetaldehyde carboxylic hydrazone (T-5) revealed IC_{50} values which were significantly lower than those of chlorambucil. In light of these results and preliminary studies of acute toxicity, we will evaluate the antitumor efficacy of transferrin conjugates of chlorambucil in animal tumor models.

Experimental Section

Chemicals, Materials, and Spectroscopy. Melting points, Büchi 530; ^{1}H NMR and ^{13}C NMR, Bruker 400 MHz AM 400, Varian 300 (internal standard, TMS); EI-MS, FAB, Finnigan-MAT 312; elemental analysis, Perkin-Elmer elemental analyzer 240; preparative low-pressure chromatography, LiChroPrepDiol column (LOBAR, 310−25, 40−63 μm) from Merck AG with a LKB 2248 pump (flow, 3 mL/min), a LKB 2211 fraction collector, and a Lambda 1000 UV/visible spectrophotometer detector from Bischoff (254 or 280 nm); analytical HPLC, reverse-phase HPLC column (Spherisorb ODS 2−5 μm; MedChrom, Heidelberg, FRG); silica gel chromatography, silica gel 60 (0.063−0.100 mm) from Merck AG; TLC, silica-coated plates 60 F_{254} from Merck; HPTLC, diol glass-coated plates F_{254} (0.2 mm) from Merck AG. Chlorambucil (M_r 304.20) was a gift from Borroughs Wellcome, FRG. Organic solvents: HPLC grade (Merck) or analytical grade (gift from BASF AG). Other organic or inorganic compounds: Merck AG, FRG. Maleimide spacer molecules were prepared previously.[13] Materials for the preparation of conjugates: human serum transferrin (Tf) (98%, crystalline, iron-saturated, M_r 80 000) was a gift from Boehringer Mannheim, FRG. Iminothiolane·HCl, 5,5′-dithiobis(2-nitrobenzoic acid), and propidium iodide were purchased from Aldrich-Sigma-Chemie, FRG. The buffers used were vacuum-filtered through a 0.2-μm membrane (Sartorius, FRG) and thoroughly degassed with argon prior to use. Cell culture media, supplements (L-glutamine, antibiotics, trypsin versene/EDTA), and fetal calf serum (FCS) were purchased from Bio Whittaker (Serva, Heidelberg, FRG). All culture flasks were obtained from Greiner Labortechnik (Frickenhausen, FRG).

Methods for the Preparation of Conjugates. FPLC for preparation of conjugates: P-500 pump, LCC 501 controller (Pharmacia), and LKB 2151 UV monitor (at λ = 280 nm); buffer, standard borate: 0.0025 M sodium borate, 0.15 M NaCl, pH 7.2. The protein concentration of the thiolated samples was determined using the ϵ values for transferrin (ϵ_{280} = 92 300 M^{-1} cm^{-1})[6] and the concentration of HS groups with Ellmann's reagent (ϵ_{412} = 13 600 M^{-1} cm^{-1})[29] with a double-beam UV/vis spectrophotometer U-2000 from Hitachi. The protein concentration of the conjugates was determined using the BCA-protein assay from Pierce. The amount of chlorambucil bound to transferrin was determined using a modified 4-nitrobenzylpyridine assay based on the assay of Epstein et al.[16] (see below). Purity of the transferrin conjugates was determined with the aid of HPLC on a Bio-Sil SEC 250 (300 mm × 7.8 mm) from Bio-RAD; mobile phase, 0.15 M NaCl, 0.01 M NaH_2PO_4, 5% CH_3CN, pH 7.0; with a LKB 2150 pump (flow, 1.5 mL/min), a Bischoff-Lambda 1000 UV/vis monitor at λ = 280 nm, an auto sampler (Merck Hitachi AS400), and an integrator (Merck Hitachi D2500). Conjugates were dissolved in 0.15 M NaCl, 0.01 M $NaHCO_3$, 0.004 M NaH_2PO_4, pH 7.4, and a 50-μL sample was injected.

Synthesis of Maleimide Derivatives of Chlorambucil: 1, 2, and 5−7. *O*-[4-[4-(Bis(2-chloroethyl)amino)-phenyl]butanoyl]-2-hydroxyethylmaleimide (1). 4-[4-(Bis(2-chloroethyl)amino)phenyl]butyric acid (chlorambucil; 1.0 g, 3.3 mmol), 2-hydroxyethylmaleimide (1.4 g, 13.2 mmol), and a catalytic amount of 4-(dimethylamino)pyridine (DMAP; 20 mg, 0.16 mmol) were dissolved in 100 mL of anhydrous CH_2Cl_2 at room temperature. DCC (0.747 g, 3.6 mmol), dissolved in 100 mL of anhydrous CH_2Cl_2, was added dropwise to this solution within 1 h, and the solution stirred for a further 8 h at room temperature. The solution was filtered and evaporated in vacuo. The residue was dissolved in 50 mL of ethyl acetate, filtered, and chromatographed on a silica gel column (ethyl acetate/hexane, 1:2) to yield 0.58 g (41%) of a light-yellow syrup: R_f 0.26 (ethyl acetate/hexane, 1:2); ^{1}H NMR ($CDCl_3$) δ 1.71 (tt, 2H, J = 6.7/6.5 Hz, 8-H), 2.21 (t, 2H, J = 6.5 Hz, 9-H), 2.42 (t, 2H, J = 6.7 Hz, 7-H), 3.63 (t, 2H, J = 5.5 Hz, 3′-H), 3.66 (t, 4H, J = 6.0 Hz, 1-H), 3.68 (t, 4H, J = 6.0

Hz, 2-H), 4.14 (t, 2H, J = 5.5 Hz, 4′-H), 6.65 (dd, 2H, J = 9.5/1.2 Hz, Ph), 7.03 (dd, 2H, J = 9.5/1.2 Hz, Ph), 7.05 (s, 2H, 1′-H); ^{13}C NMR ($CDCl_3$) δ 26.21 (C-8), 32.71 (C-9), 33.13 (C-7), 36.42 (C-3′), 41.08 (C-1), 52.17 (C-2), 61.81 (C-4′), 111.91, 129.19, 129.43, 144.44 (Ph), 134.53 (C-1′), 170.70 (C-2′), 172.52 (C-10); MS-EI m/z 426 ($M^+ - 1$), 377, 286, 181. Anal. ($C_{20}H_{24}N_2O_4Cl_2$) C, H, N, Cl.

***O*-[4-[4-(Bis(2-chloroethyl)amino)phenyl]butanoyl]-3-maleimidophenol (2).** *N*-Cyclohexyl-*N*′-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (1.6 g, 3.6 mmol) was dissolved in 100 mL of CH_2Cl_2 and added dropwise within 2 h to a solution of chlorambucil (1.0 g, 3.3 mmol), 3-maleimidophenol (1.9 g, 9.9 mmol), and a catalytic amount of DMAP (20 mg, 0.16 mmol) in 50 mL of anhydrous CH_2Cl_2. The solution was stirred for a further 6 h at room temperature and then filtered and the filtrate evaporated in vacuo. The residue was chromatographed on a silica gel column (ethyl acetate/hexane, 1:1) to yield 0.60 g (38%) of a yellow syrup: R_f 0.29 (ethyl acetate/hexane, 1:1); ^{1}H NMR (DMSO-d_6) δ 1.92 (tt, 2H, J = 6.7/6.5 Hz, 8-H), 2.58 (t, 2H, J = 6.5 Hz, 9-H), 2.63 (t, 2H, J = 6.7 Hz, 7-H), 3.66 (t, 4H, J = 6.2 Hz, 1-H), 3.68 (t, 4H, J = 6.2 Hz, 2-H), 6.68 (dd, 2H, J = 9.2/1.6 Hz, Ph), 7.09 (dd, 2H, J = 9.2/1.6 Hz, Ph), 7.21 (s, 2H, 1′-H), 7.14−7.60 (m, 4H, Ph); ^{13}C NMR (DMSO-d_6) δ 26.28 (C-8), 32.81 (C-9), 33.13 (C-7), 41.09 (C-1), 52.18 (C-2), 111.96, 120.05, 121.16, 123.89, 129.29, 129.46 144.52, 129.32, 132.40, 150.38 (Ph), 134.64 (C-1′), 169.54 (C-2′), 171.40 (C-10); MS-CI m/z 475 (M^+), 457, 377, 304, 190. Anal. ($C_{24}H_{24}N_2O_4Cl_2$) C, H, N, Cl.

4-[4-(Bis(2-chloroethyl)amino)phenyl]butanoyl *tert*-Butoxycarbonyl Hydrazide (3). Chlorambucil (1.0 g, 3.3 mmol) was dissolved in 50 mL of anhydrous CH_2Cl_2. To this solution was added coxalyl chloride (431 μL, 4.8 mmol) and the solution stirred for 15 h at T = 35 °C. The yellow solution was evaporated in vacuo. Remaining amounts of oxalyl chloride were removed under high vacuum. The thus prepared acid chloride of chlorambucil was dissolved in 20 mL of anhydrous CH_2Cl_2, and *tert*-butyl carbazate (0.46 g, 3.5 mmol), dissolved in 20 mL of anhydrous CH_2Cl_2, was added dropwise while stirring at room temperature. The mixture was stirred for 36 h at room temperature and then filtered and the filtrate evaporated in vacuo. After chromatography over a silica gel column (ethyl acetate/hexane 2:1) the product was obtained in a yield of 0.70 g (51%) as a yellow oil: R_f value 0.52 (ethyl acetate/hexane, 2:1); ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 9H, $C(CH_3)_3$), 1.72 (tt, 2H, J = 6.6/6.4 Hz, 8-H), 2.04 (t, 2H, J = 6.4 Hz, 9-H), 2.46 (t, 2H, J = 6.6 Hz, 7-H), 3.62 (s, 4H, 1-H), 3.69 (s, 4H, 2-H), 6.64 (dd, 2H, J = 9.5/1.5 Hz, Ph), 7.03 (dd, 2H, J = 9.5/1.5 Hz, Ph), 8.59 (s, 1H, NH), 9.44 (s, 1H, NH). Anal. ($C_{19}H_{28}N_3O_3Cl_2$) C, H, N, Cl.

4-[4-(Bis(2-chloroethyl)amino)phenyl]butyric Acid Hydrazide (4). 4-[4-(Bis(2-chloroethyl)amino)phenyl]butanoyl *tert*-butoxycarbonyl hydrazide (0.70 g, 1.7 mmol) was dissolved in 10 mL of anhydrous THF. To the stirred solution was added 10 mL of trifluoroacetic acid, and the mixture stirred for 1 h. Subsequently, the solvent was removed under high vacuum and the resulting hydrazide of chlorambucil (trifluoroacetate salt) reacted with 2-maleimidoacetaldehyde, 3-maleimidoacetophenone, and 3-maleimidobenzaldehye to obtain the hydrazone derivatives **5**−**7** as described below.

Carboxylic Hydrazone Derivative 5 of 4-[4-(Bis(2-chloroethyl)amino)phenyl]butyric Acid Hydrazide (4) and 2-Maleimidoacetaldehyde. 4-[4-(Bis(2-chloroethyl)-amino)phenyl]butyric acid hydrazide (trifluoroacetate salt, **4**; 0.72 g, 1.7 mmol) was dissolved in 30 mL of anhydrous THF and 2-maleimidoacetaldehyde (0.35 g, 2.5 mmol) added at room temperature. The reaction mixture was stirred for 36 h. The solution was then evaporated in vacuo and the product purified by chromatography over a LOBAR column (ethyl acetate/hexane, 2:1) to yield 0.30 g (45%) of a pale-yellow solid: mp 68 °C; R_f 0.31 (ethyl acetate/hexane, 2:1); ^{1}H NMR ($CDCl_3$) δ 1.90 (tt, 2H, J = 6.5/6.4 Hz, 8-H), 2.54 (t, 2H, J = 6.4 Hz, 9-H), 2.59 (t, 2H, J = 6.5 Hz, 7-H), 3.63 (t, 4H, J = 6.0 Hz, 1-H), 3.69 (t, 4H, J = 6.0 Hz, 2-H), 4.37 (t, 2H, J = 2.5 Hz, 3′-H), 6.63 (dd, 2H, J = 8.5/1.3 Hz, Ph), 6.80 (s, 2H, 1′-H), 7.10 (dd, 2H, J = 8.5/1.3 Hz, Ph), 7.12 (t, 1H, J = 10.2 Hz, 4′-H), 9.94 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 26.31 (C-8), 32.03 (C-9), 34.27 (C-7), 38.52 (C-3′), 40.65 (C-1), 53.72 (C-2), 112.37, 129.72, 131.01, 144.38 (Ph), 134.46 (C-1′), 138.83 (C-4′), 170.07 (C-2′), 176.28 (C-10); MS-FAB m/z 439 (M^+), 286, 136. Anal. ($C_{20}H_{24}N_4O_3Cl_2$) C, H, N, Cl.

Carboxylic Hydrazone Derivative 6 of 4-[4-(Bis(2-chloroethyl)amino)phenyl]butyric Acid Hydrazide (4) and 3-Maleimidoacetophenone. 4-[4-(Bis(2-chloroethyl)-amino)phenyl]butyric acid hydrazide (trifluoroacetate salt, **4**; 0.71 g, 1.7 mmol) was dissolved in 30 mL of anhydrous THF and 3-maleimidoacetophenone (0.54 g, 2.5 mmol) added at room temperature. The reaction mixture was stirred for 36 h. The solution was evaporated in vacuo and the product purified by crystallization from ethyl acetate: yield 0.54 g (63%) of a yellow powder; mp 164 °C; R_f 0.27 (ethyl acetate/hexane, 3:2); ^{1}H NMR ($CDCl_3$) δ 2.02 (tt, 2H, J = 7.6/7.3 Hz, 8-H), 2.21 (2s, 3H, CH_3), 2.64 (t, 2H, J = 7.6 Hz, 9-H), 2.81 (t, 2H, J = 7.3 Hz, 7-H), 3.62 (t, 4H, J = 5.8 Hz, 1-H), 3.70 (s, 4H, J = 6.1 Hz, 2-H), 6.67 (d, 2H, J = 8.8 Hz, Ph), 6.89 (s, 2H, 1′-H), 7.04 (d, 2H, J = 8.8 Hz, Ph), 7.36−7.77 (m, 4H, Ph), 8.58 (2s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 12.50 (CH_3), 26.51 (C-8), 32.36 (C-9), 34.30 (C-7), 40.56 (C-1), 53.66 (C-2), 112.22, 123.72, 125.47, 126.65, 129.21, 129.73, 131.16, 131.62, 144.27, 145.22 (Ph), 134.31 (C-1′), 139.15 (C-9′), 169.34 (C-2′), 175.83 (C-10); MS-FAB m/z 515 (M^+), 451, 216. Anal. ($C_{26}H_{28}N_4O_3Cl_2$) C, H, N, Cl.

Carboxylic Hydrazone Derivative 7 of 4-[4-(Bis(2-chloroethyl)amino)phenyl]butyric Acid Hydrazide (4) and 3-Maleimidobenzaldehyde. 4-[4-(Bis(2-chloroethyl)-amino)phenyl]butyric acid hydrazide (trifluoroacetate salt, **4**; 0.5 g, 1.2 mmol) was dissolved in 30 mL of anhydrous THF and 3-maleimidobenzaldehyde (0.29 g, 1.4 mmol) added at room temperature. The reaction mixture was stirred for 36 h. The solution was then evaporated in vacuo and the product purified by chromatography over a LOBAR column (ethyl acetate/hexane, 3:2) to yield 0.15 g (25%) of a pale-yellow solid: mp 62 °C; R_f 0.43 (ethyl acetate/hexane, 2:1); ^{1}H NMR ($CDCl_3$) δ 2.04 (tt, 2H, J = 7.3/7.0 Hz, 8-H), 2.63 (t, 2H, J = 7.3 Hz, 9-H), 2.81 (t, 2H, J = 7.0 Hz, 7-H), 3.62 (t, 4H, J = 5.6 Hz, 1-H), 3.70 (s, 4H, J = 5.6 Hz, 2-H), 6.65 (d, 2H, J = 8.4 Hz, Ph), 6.92 (s, 2H, 1′-H), 7.11 (d, 2H, J = 8.4 Hz, Ph), 7.34−7.68 (m, 4H, Ph), 7.78 (s, 1H, 9′-H), 9.52 (2s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 26.49 (C-8), 32.16 (C-9), 34.29 (C-7), 40.54 (C-1), 53.70 (C-2), 112.31, 124.50, 126.35, 127.39, 129.56, 129.76, 131.12, 131.94, 144.26, 145.28 (Ph), 134.35 (C-1′), 134.97 (C-9′), 169.26 (C-2′), 176.20 (C-10); MS-FAB m/z 501 (M^+). Anal. ($C_{25}H_{26}N_4O_3Cl_2$) Calcd: C, 59.9; H, 5.19; N, 11.2; Cl, 14.2. Found: C, 60.2; H, 5.31; N, 10.9; Cl, 13.6.

Synthesis of the Transferrin Conjugates T-1, T-2, T-5, T-6, T-7. All reactions were performed at room temperature unless otherwise stated. Data for one representative experiment is given.

(1) Thiolation of Transferrin Using Iminothiolane: 64 mg of Tf was dissolved in 4.0 mL of degassed buffer (0.1 M sodium borate, 0.001 M EDTA, 0.15 M NaCl, pH 8.0, c(Tf) ≈ 4.0×10^{-4} M), and 66 μL of a 8×10^{-2} M iminothiolane·HCl solution (2.75 mg of iminothiolane·HCl dissolved in 250 μL of the same buffer) was added. After 60 min thiolated transferrin was isolated through size exclusion chromatography (Sephadex G-25F, Pharmacia; column, d = 2.0 cm, l = 10 cm; buffer, standard borate). The average number of introduced HS groups was 3.1. (A smaller number of thiol groups can be introduced by reducing the amount of added iminothiolane, e.g., 40 μL of the iminothiolane solution are added for introducing two HS groups.) To ensure that Fe(III) had not been released during thiolation, the Fe(III) concentration was determined using $\epsilon[Tf]_{465} = 4650\ M^{-1}\ cm^{-1}$ [10] and showed that samples of thiolated transferrin contained at least 95% iron. The sample of thiolated transferrin (7.0 mL) was used directly for the synthesis of the conjugate.

(2) Reaction of Maleimide Derivative 5 with Thiolated Transferrin: 150 μL of a solution of **5** (M_r 439.1) in dimethylformamide (2.0 mg dissolved in 150 μL of dimethylforma-

mide) was added to 7.0 mL of thiolated sample and homogenized, 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 (10 min at 4 °C and 4500 rpm). The concentrated sample was centrifuged for 5 min with a Sigma 112 centrifuge, the supernatant loaded on a Sephadex G-25F column ($d = 1.0$ cm, $l = 10$ cm; buffer, standard borate), and the conjugate isolated. The concentration of bound chlorambucil was 390 ± 20 μM and that of transferrin 135 ± 6 μM which corresponds to approximately 2.9 equivalents of chlorambucil bound to transferrin.

Determination of the Alkylating Activity of Derivatives 1, 2, and 5−7 and Respective Transferrin Conjugates with 4-(4-Nitrobenzyl)pyridine (NBP)−Modified According to Epstein et al.[16] All determinations were carried out three times and mean values calculated.

(a) Chlorambucil and Chlorambucil Derivatives 1, 2, and 5−7: 50-μL samples ($c \approx 300$ μM) of chlorambucil or the respective chlorambucil derivative dissolved in anhydrous THF were filled into 10-mL tubes. To this solution were added 50 μL of acetic acid (5%), 100 μL of buffer (pH 7.4, 0.0025 M sodium borate, 0.15 M NaCl) 195 μL of distilled water, 50 μL of ethanol, and 55 μL of a NBP solution (500 mg of NBP dissolved in 10 μL of ethanol). The sealed tubes were heated for 90 min at 80 °C in a water bath and then cooled to room temperature. To the samples was added 500 μL of a triethylamine/acetone solution (1/1, v/v), and the resulting violet color was determined at 565 nm by spectrophotometry against a blank ($\epsilon_{565} = 15\ 100\ M^{-1}\ cm^{-1}$).

(b) Transferrin Conjugates of Chlorambucil: 50-μL samples of T-1, T-2, T-5, T-6, or T-7 were filled into tubes and diluted with buffer to 100 μL. To this solution were added 50 μL of acetic acid (5%), 245 μL of distilled water, 50 μL of ethanol, and 55 μL of the NBP solution. The samples were heated for 90 min at 80 °C and analyzed as above.

For the cell culture experiments and serum stability studies the alkylating activity of T-1, T-2, T-5, T-6 and T-7 was adjusted to 720 or 1000 μM by concentrating the samples using CENTRIPREP-10-concentrators.

Modified Procedure for Determination of the Alkylating Activity of the Transferrin−Chlorambucil Conjugates Incubated with 10% Serum. A 100-μL portion of the respective conjugate serum sample (see below) was filled into 10-mL tubes. To this solution were added 50 μL of acetic acid (5%), 245 μL of water, 50 μL of ethanol, and 55 μL of the NBP solution. The sealed tubes were heated for 90 min at 80 °C in a water bath and then cooled to room temperature; 400 μL of the samples was filled into Eppendorf tubes and centrifuged for 5 min; 250 μL of the supernatant was mixed with 250 μL of an Et_3N/acetone solution (1/1, v/v), and the resulting violet-colored solution was determined at 565 nm by spectrophotometry against a blank (sample without conjugate).

10% Serum Stability Studies with T-1, T-2, T-5, T-6, T-7. Preparation of the stock solution containing 10% serum: 330 μL of human blood serum was filled into a 10-mL glass tube. To the serum were added 1970 μL of buffer (pH 7.4, 0.0025 M sodium borate, 0.15 M NaCl) and 1000 μL of the respective transferrin−chlorambucil conjugate ($c = 1000$ μM). The stock solution was incubated at 37 °C for the hydrolysis studies. The alkylating activity of the samples (3 × 100 μL) was determined at $t = 0$, 2, 4, 6, 8, 24, 32, 48, 72, and 96 h.

Hydrolysis Studies of 8−12. Stock solutions of the 4-phenylbutyric acid derivatives ($c = 5 \times 10^{-3}$ M for **8**, 2×10^{-3} M for **9-12**) in acetonitrile were prepared; 150 μL of the respective stock solution was added to 1350 μL of a buffer at pH 5.0 (0.01 M NaOAc, 0.15 M NaCl) or of a buffer at pH 7.4 (0.15 M NaCl, 0.004 M NaH_2PO_4, 0.025 M $NaHCO_3$). The solutions were incubated at room temperature; 20-μL samples of the solutions (pH 5.0 and 7.4) were analyzed at different times ($t = 0$, 2, 4, 8, 24, 32, 48, 72, and 96 h) on a reverse-phase HPLC column (acetonitrile/water, 1/1): retention times for **8**, 16.9 min; **9**, 28.9 min; **10**, 10.3 min; **11**, 16.0 min; **12**, 11.5 min. The UV absorption was detected at a wavelength of 280 nm (**11**, **12**) or 254 nm (**8−10**).

For stability studies in cell-conditioned culture medium 0.5 mL of the respective stock solution and 0.5 mL of acetonitrile were added to 9.0 mL of cell-conditioned culture medium (from MCF7 cells) and the samples incubated at $T = 37$ °C. For **8−10** 8.0 mL of 2-propanol had to be added to keep the compounds in solution during the course of the experiment. In each case three 400-μL samples were ultrafiltered with Centrisart C-4 Eppendorfs (from Sartorius AG, FRG; 10 000 rpm, 45 min) at $t = 0$, 24, 48, 72, 96, and 120 h, and 20 μL of the filtrate was analyzed on a reverse-phase HPLC column (acetonitrile/water, 1/1) and detected as described above.

Biology. Human tumor cells were grown at 37 °C in a humidified atmosphere (95% air, 5% CO_2) in monolayer (MCF7 cells) or suspension (MOLT4 cells) RPMI 1640 culture medium with phenol red supplemented with 10% heat-inactivated FCS, 300 mg/L glutamine, and 1% antibiotic solution (5.000 μg of gentamycin/mL). Cells were trypsinized and maintained twice a week. The concentration of chlorambucil in the stock solution of the conjugates was 720 μM; chlorambucil was freshly dissolved in standard borate containing 5% ethanol at the same concentration.

Propidium Iodide Fluorescence Assay. The fluorescence assay was performed according to the method of Dengler et al.[30] Briefly, cells were harvested from exponential phase cultures growing in RPMI culture medium by trypsinization, counted, and plated in 96-well flat-bottomed microtiter plates (50 μL of cell suspension/well, 1.0×10^5 cells/mL). After a 24-h recovery in order to allow cells to resume exponential growth, 100 μL of culture medium (6 control wells/plate) or culture medium containing drug was added to the wells. Each drug concentration was plated in triplicate. After 6 days of continuous drug exposure nonviable cells were stained by addition of 25 μL of a propidium iodide solution (50 μg/mL). Fluorescence (FU_1) was measured using a Millipore Cytofluor 2350 microplate reader (excitation 530 nm, emission 620 nm). Microplates were then kept at −18 °C for 24 h, which resulted in a total cell kill. After thawing of the plates and a second fluorescence measurement (FU_2), the amount of viable cells was calculated by $FU_2 - FU_1$. Growth inhibition was expressed as treated/control × 100 (%T/C).

Flow Cytometric Analysis of Transferrin Receptor Expression. Adherent growing cells (MCF7) were detached from culture dishes by trypsinization or employed as cell suspension (MOLT4). The following steps were performed at 5 °C. Cells were washed twice with PBS and resuspended in PBS at a density of 1×10^7 cells/mL; 5 μL of anti-human CD71 antibody (biotinylated, monoclonal antibody from mouse; Dianova, Hamburg) was added to 100 μL of cell suspension (10^6 cells), and cells were incubated for 45 min. Cells were then washed twice with PBS and resuspended in 500 μL of PBS, 2 μL of streptavidin-DTAF [(dichlorortrianzinylamino)fluoroscein; Coulter Immunotech, Hamburg] was added, and cells were incubated for 30 min. Cells were washed twice with PBS and resuspended in 500 μL of PBS, and FACS analysis was performed using a FACSort (Becton Dickinson, Heidelberg) with the Lysis II software. Controls were prepared as described above, except that isotype-specific antibody (anti-goat IgG, biotinylated, monoclonal from mouse; Sigma) was used instead of anti-CD71 antibody.

Toxicity Studies. Toxicity studies were carried out at the Chemistry Department of the University Heidelberg, Experimental Division. Using NMRI mice (3 female mice/group) as animal models, 20 mg/kg chlorambucil (5.0 mg of chlorambucil dissolved in 10 mL of 0.3 M $NaHCO_3$, 0.15 M NaCl) and T-5 (1650 μM with respect to its alkylating activity) were administered by ip injection on 3 consecutive days (due to solubility problems of free chlorambucil, acute toxicity studies could not be performed with one ip injection alone at higher doses so that the above-mentioned administration schedule was chosen). Whereas two out of three mice in the group treated with free chlorambucil died within 1 or 2 days after the second and third injections, no deaths were recorded in the conjugate

group even after 14 days of administration. No body weight loss was observed in this group.

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Supporting Information Available: Syntheses and characterization of **10**−**12**; cell culture data of T-1, T-2, T-5, T-6, T-7, and chlorambucil in MCF7 and MOLT4 cell lines; and flow cytometric analysis of transferrin receptor expression in MCF7 and MOLT4 cell lines (4 pages). Ordering information is given on any current masthead page.

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