Albumin Conjugates of the Anticancer Drug Chlorambucil: Synthesis, Characterization, and *In Vitro* Efficacy

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Summary

In our efforts to improve the selectivity and toxicity profile of antitumor agents, four maleimide derivatives of chlorambucil (**1**–**4**) were bound to thiolated human serum albumin which differ in the stability of the chemical link between drug and spacer. **1** is an aliphatic maleimide ester derivative of chlorambucil, whereas **2**–**4** are acetaldehyde, acetophenone, and benzaldehyde carboxylic hydrazone derivatives. HPLC stability studies at pH 5.0 with the related model compounds **5**, **7**, **8**, and **9**, in which chlorambucil was substituted by 4-phenylbutyric acid, demonstrated that the carboxylic hydrazone derivatives have acid-sensitive properties; the acid lability of **7** was particular prominent with a half-life of only a few hours. The alkylating activity of albumin-bound chlorambucil was determined with the aid of 4-(4-nitrobenzyl)-pyridine (NBP), demonstrating that on average three equivalents were protein-bound. Evaluation of the cytotoxicity of free chlorambucil and the respective albumin conjugates in the MCF7 mamma carcinoma and MOLT4 leukemia cell line employing a propidium iodide fluorescence assay demonstrated that the conjugate in which chlorambucil was bound to albumin through an ester bond was not as active as chlorambucil. In contrast, the conjugates in which chlorambucil was bound to albumin through carboxylic hydrazone bonds were as or more active than chlorambucil in both cell lines. In particular, the conjugate in which chlorambucil was bound to albumin through an acetaldehyde carboxylic hydrazone bond exhibited IC_{50} values which were approximately 4-fold (MCF7) to 13-fold (MOLT4) 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.

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].

A promising approach to circumvent the toxic side effects of anticancer drugs on normal cells and to improve their efficacy towards malignant cells is to couple anticancer drugs to suitable carrier proteins or macromolecules. Expected advantages of these protein conjugates are preferable tissue distribution [3], 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 addition, polymer or protein conjugates can accumulate in solid tumors due to the enhanced microvasculature of tumor tissue [4,5]. This phenomenon has been termed "enhanced permeability and retention" in relation to tumor targeting ("EPR-phenomenon") [6].

Due to our interest in the role which natural plasma proteins play in the *in vivo* distribution of anticancer drugs [7,8], we have developed chlorambucil conjugates of the serum protein albumin. Besides the fact that albumin has a molecular weight of 66 500, which is appropriate for the above concept of passive tumor targeting, this serum protein is suitable as a potential drug delivery system for the following reasons:

(a) Albumin exhibits a significant uptake in tumor tissue as demonstrated by Matsumura et al. [9] and Sinn et al. [10]; (b) it is a readily available protein in a pure and uniform form exhibiting good biological stability; (c) albumin is biodegradable, non-toxic and non-immunogenic.

Chlorambucil was one of the first anticancer agents which was used to prepare antibody conjugates [11–13]. However, in these reports [11,12] chlorambucil was either physically adsorbed or chemically linked to the carrier by mere incubation or by direct coupling using N-hydroxysuccinimide/N,N′-di-

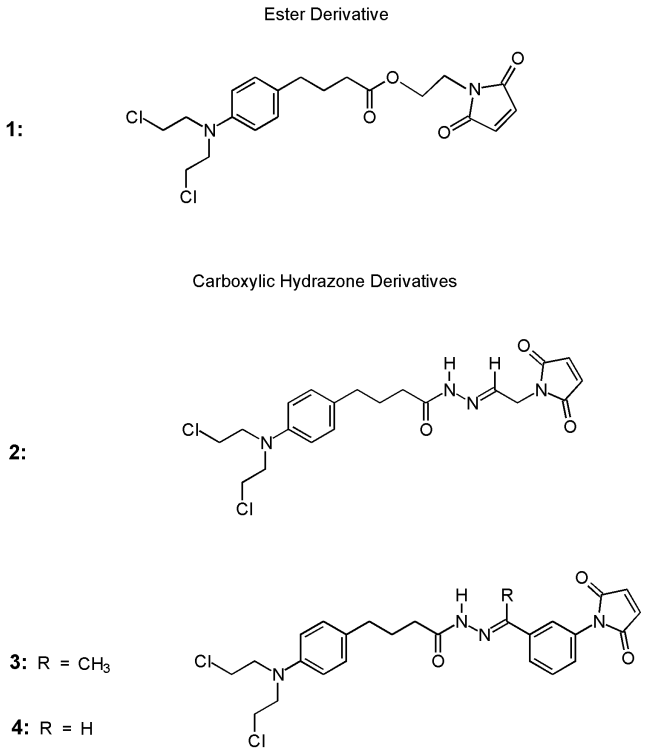

Figure 1 Structures of the maleimide derivatives of chlorambucil **1**–**4**.

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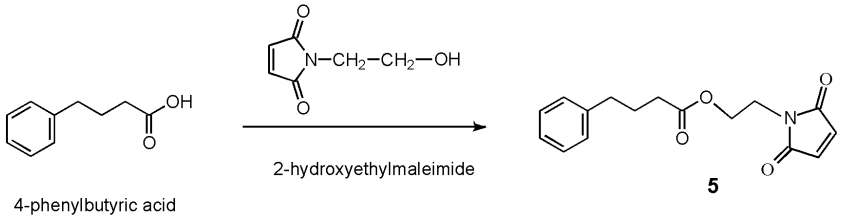
cyclohexylcarbodiimide (DCC) [13]. These direct methods have the disadvantage that during preparation polymeric products are likely to be formed and the resulting conjugates are chemically not well defined with respect to the chemical link between drug and carrier protein.

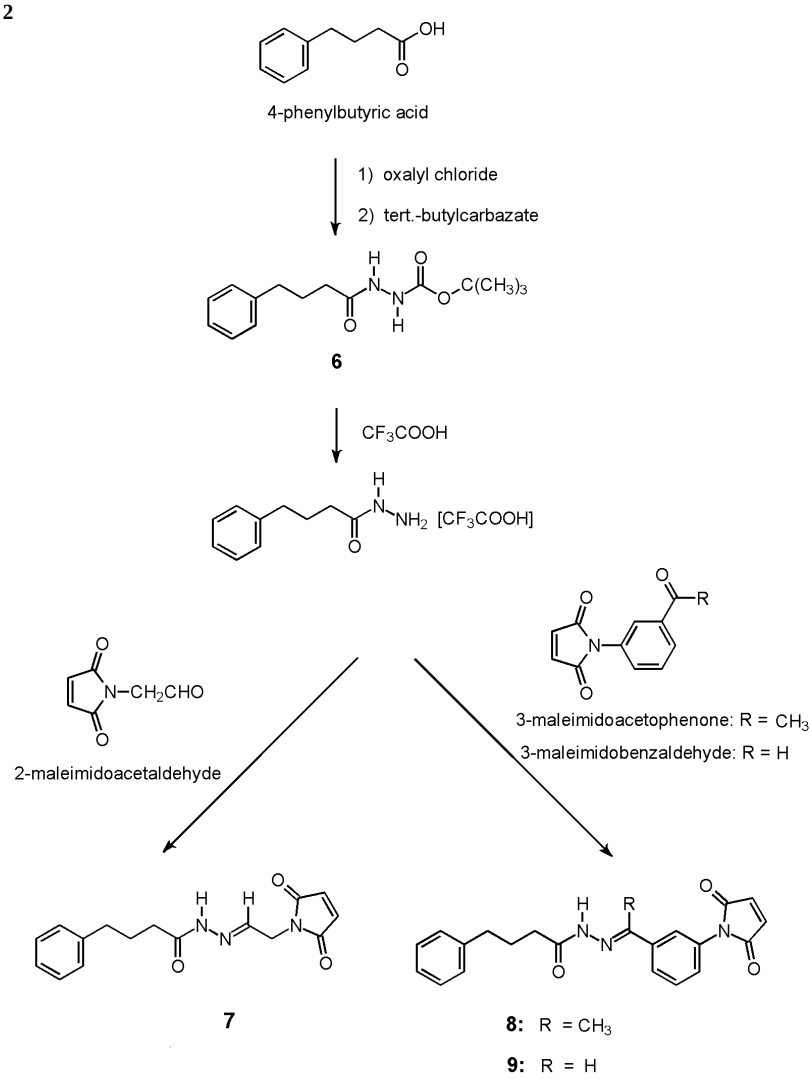
For these reasons we recently synthesized maleimide derivatives of chlorambucil which differed in the stability of the chemical link (ester and carboxylic hydrazone bond) between the drug and the spacer molecule (see Figure 1) [14]. The introduced maleimide group is able to bind selectively to sulfhydryl groups of carrier proteins. 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. Albumin is taken up by the cell through endocytosis [6,15]. During internalization the pH is reduced from 7.4 to 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.

As a first step in anticancer drug development, unbound chlorambucil as well as the albumin conjugates, which we obtained with the above mentioned derivatives, were subsequently evaluated for their inhibitory efficacy in two tumor cell lines. In this paper we report on the synthesis and characterization of the outlined albumin conjugates, their antiproliferative efficacy and the relationship between acid-sensitivity, serum stability, and cytotoxicity.

Scheme 1



Scheme 2



Results

*Synthesis of the model compounds **5**, **7**, **8**, and **9** and their stability at pH 5.0.* In order to assess the significance of the acid-sensitivity 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 in **1–4**. Unfortunately, due to the rapid hydrolysis of the chlorine atoms of chlorambucil (detection of a number of peaks within a few hours with the aid of HPLC [16]) aqueous stability studies of **1–4** with respect to the chemical link between the maleimide spacer and chlorambucil could not be carried out. We therefore synthesized the model compounds **5**, **7**, **8**, and **9** in which chlorambucil was substituted by 4-phenylbutyric acid.

The aliphatic ester of 4-phenylbutyric acid was synthesized by reacting 4-phenylbutyric acid with an excess of 2-hydroxyethylmaleimide in CH_2Cl_2 and addition of N-cyclohexyl-N′-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate and catalytic amounts of dimethylaminopyridine (Scheme 1). The carboxylic hydrazone derivatives **7**–**9** were obtained by reaction of 4-phenylbutyric acid hydrazide (trifluoroacetate) [prepared by reacting phenylbutyric acid chloride with tert.-butylcarbazate and subsequent cleavage with CF_3COOH] with 2-maleimidoacetaldehyde, 3-maleimidoacetophenone, or 3-maleimidobenzaldehyde (Scheme 2).

5, **7**, **8**, and **9** were characterized through ^{1}H NMR and ^{13}C NMR spectroscopy as well as elemental analysis. 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 ppm and 169–170 ppm for the carbon atoms of the double bonds and carbonyl groups in the ^{13}C NMR spectra (recorded in $CDCl_3$). In the ^{1}H NMR and ^{13}C NMR spectra of **7**–**9** only one set of signals is observed showing the presence of only one isomer. From a steric point of view the E-stereoisomer is favored, and we therefore tentatively suggest that this stereoisomer is present which is in accordance with studies on simple hydrazones [17].

The stability of **5**, **7**, **8**, and **9** was studied at pH 5.0 on a reverse-phase C-18-HPLC column. Stability studies were carried out by first reacting **5**, **7**, **8**, and **9** quantitatively in a Michael addition with one equivalent of 2-mercaptoethanol to produce the addition product as a single peak (**5**, **7**, **8**, and **9** were not used directly for HPLC-studies due to the slow hydrolysis of the maleimide group at pH 5.0–7.0). The decrease of this peak area for **5**, **7**, **8**, and **9** in the chromatograms was used to calculate the respective half-lives. Whereas the hydrolysis of **5** was slow (< 20% decomposition after 96 h), the half-lives of the carboxylic hydrazones at pH 5.0 were $t_{1/2} \approx 4$ h for **7**, $t_{1/2} \approx 20$ h for **8**, and $t_{1/2} \approx 65$ h for **9**. This hydrolytic classification can be expected from the character of the chemical link involved [18].

Preparation of albumin conjugates (A-1, A-2, A-3, A-4) [1]. The albumin conjugates were prepared by reacting **1–4** with thiolated albumin. The sulfhydryl 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, an average number of 2.8–3.0 HS-groups being introduced. For preparing the conjugates the respective maleimide derivative was added to the thiolated protein sample, and A-1, A-2, A-3, and A-4 were isolated by gel chromatography (Sephadex® G-25). In order to rule out the presence of unbound drug as well as polymeric by-products 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). We have previously shown that this column is suitable for separating and detecting serum proteins in the presence of low-molecular weight compounds [7]. As an example a typical chromatogram of A-2 recorded at λ = 280 nm is depicted in Fig. 2, showing a main peak at 7.2 min corresponding to that of monomeric albumin; 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 albumin also shows this peak with a peak area of approximately 3–5%).

The amount of chlorambucil bound to albumin was determined with the aid of the NBP-assay [19] which was modified accordingly (see Experimental Section) whereas the protein

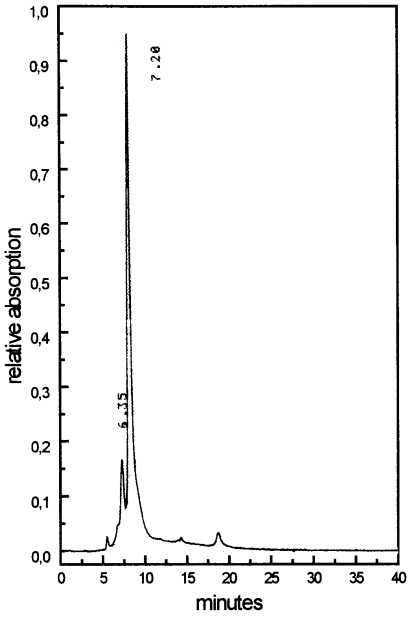


Figure 2. Chromatogram of A-2 at pH = 7.4. Chromatograms were performed on a size-exclusion column and recorded at 280 nm. Retention times: 6.35 min (dimeric albumin conjugate), 7.20 min (monomeric albumin conjugate).

concentration of the conjugates was determined using the BCA-protein-assay from Pierce (USA). In this way the number of equivalents of chlorambucil bound to albumin was calculated. The ratio of chlorambucil/albumin in the conjugates was 2.7–3.0 which corresponded well with the number of introduced HS-groups (see above).

When free chlorambucil was reacted with thiolated albumin and the protein separated by gel filtration, the collected sample contained > 2.5 HS-groups/protein and less than 10% of chlorambucil with respect to the amount of protein present, demonstrating that chlorambucil does not couple to thiolated proteins under these experimental conditions.

Stability in 10% serum. In order to determine the loss of alkylating activity of chlorambucil and our albumin conjugates A-1–A-4 in 10% serum, we incubated chlorambucil and

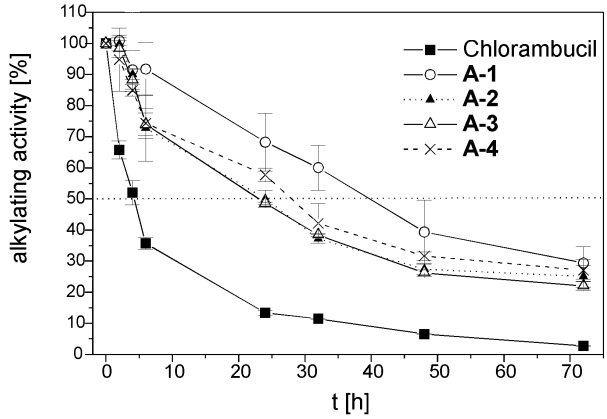


Figure 3. Time-dependent stability of the alkylating activity of the chlorambucil-albumin conjugates in comparison to free chlorambucil incubated with 10% human blood serum at 37 °C. Averages obtained in two independent experiments are presented (determinations were carried out in triplicate). Standard deviations were below ± 5% and are not shown; the value at t = 0 h was set as 100%.

[1] The abbreviations used are: HSA, human serum albumin; A-1, A-2, A-3, A-4 refer to the respective chlorambucil albumin conjugates prepared with the maleimide derivatives **1–4**.

the conjugates with 10% human serum at T = 37 °C and determined the loss of alkylating activity with the aid of the NBP-assay over a period of four days. The results of these incubation studies are shown in Fig. 3. An initial rapid decrease of alkylating activity for free chlorambucil is observed (approximately 50% of activity remaining after 5 h). This decrease in alkylating activity is significantly smaller for the albumin conjugates A-1–A-4 (approximately 50% of activity remaining after 25 to 35 h), there being little difference between the individual albumin conjugates. Stability studies carried out in 10% fetal calf serum (FCS) did not differ significantly to those performed in 10% human serum (data not shown).

Cell culture experiments. The newly synthesized albumin 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. Respective IC_{50} values are summarized in Table 1. Albumin 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). IC_{50} values were not reached for the albumin conjugate A-1 in both cell lines (concentration range 0.1–40 μM). In contrast, the conjugates A-3 and A-4 were as active as unbound chlorambucil (IC_{50} values in the range of 2.5–4 μM (MOLT4) and 14–17 μM (MCF7) respectively). The most active compound in both cell lines is A-2, in which chlorambucil was bound to albumin through an acetaldehyde carboxylic hydrazone bond (IC_{50} values ≈ 0.3 μM (MOLT4), and ≈ 4 μM (MCF7) respectively).

Table 1. IC_{50} values (μM)[a] for the chlorambucil-albumin conjugates in comparison to free chlorambucil in the MCF7 mamma carcinoma and MOLT4 leukemia cell line.

Compound	MCF7	MOLT4
Chlorambucil	14.3 ± 1.1	3.9 ± 0.3
A-1	–	–
A-2	3.7 ± 0.3	0.3 ± 0.02
A-3	17.7 ± 1.3	2.9 ± 0.2
A-4	15.8 ± 1.2	2.7 ± 0.3

[a] IC_{50} values (50% inhibitory concentration) represent the mean ± standard deviation from three independent experiments. Concentrations refer to the equialkylating activity of the respective compounds.

Preliminary toxicity studies. Subsequently, chlorambucil and A-2 were subjected to acute toxicity studies (dose: 20 mg/kg per day with respect to the amount of chlorambucil present; administration: ip (intraperitoneal), three consecutive days). Whereas two out of three mice in the group treated with free chlorambucil died within two or three days after the first injection, no mortality was observed in the conjugate group even after 16 days (see Table 2). An increase in body weight was observed in this group, which is an indication of very low systemic toxicity.

Table 2. Comparison of acute toxicity of chlorambucil and the chlorambucil-albumin conjugate A-2 in NMRI mice.

Compound	Dose, ip, day 1, 2, and 3	Number of mice	Mortality (days of death)	Average increase in body weight after 16 days
Chlorambucil	20 mg/kg	3	2 (2, 3)	9%
A-2	20 mg/kg	3	0	22%

Discussion

Among naturally occurring proteins, albumin is a potential carrier due to a suitable molecular size for passive tumor targeting and due to its biocompatibility. We have therefore coupled the anticancer drug chlorambucil to this serum protein. Preferably, these albumin conjugates should be preparable in sufficient purity, they should exhibit stability at physiological conditions (pH ≈ 7.4), and they should retain the alkylating activity of the drug.

When preparing our conjugates, the maleimide derivatives of chlorambucil **1**–**4** were coupled to thiolated albumin, and the resulting conjugates were isolated with a purity of > 90%.

Our cell culture experiments demonstrate that the conjugate in which chlorambucil was bound to albumin through an acetaldehyde carboxylic hydrazone bond (A-2) revealed IC_{50} values which were significantly lower than those of chlorambucil. The aldehyde carboxylic hydrazone bond in the model compound **7** shows marked acid-sensitivity with a half-life of approximately 4 h [2)]. The conjugates A-3 and A-4, in which acid-sensitivity is not that pronounced (half-lives of the model compounds **8** and **9** in the range of 20–70 h at pH 5.0) exhibit antiproliferative activity which is comparable to that of chlorambucil. In contrast, the albumin conjugates prepared with **1**, which contains an ester bond, is not as active as chlorambucil, IC_{50} values not being reached in the tested concentration range.

These results taken together indicate that antiproliferative *in vitro* activity is correlated with the chemical link between chlorambucil and albumin. The importance of the acid-sensitivity of the linker between anthracyclines and the carrier protein for *in vitro* and *in vivo* anticancer activity has been noted by Greenfield et al. [20] and our group [21,22]. Endocytosis is presumably responsible for cellular uptake with subsequent release of chlorambucil in acidic endosomes and/or

[2)] We confirmed the acid-sensitivity of the albumin conjugates through the following experiment: A-1 – A-4 were incubated at pH 7.4 and pH 5 and their time-dependent stability was determined by HPLC (Biosil SEC 250 column) over a period of six days. Chlorambucil or hydrolysis products such as chlorambucil hydrazide are seen on this column at around 10.5–11.0 min (detection at 260 nm). When the acid-sensitive conjugates A-2 and A-4 were incubated at pH 5.0, they showed a distinct peak at 10.5 min with time which steadily increased in size during the course of the experiment in the order A-2 > A-3 > A-4. At pH 7.4, however, only very small peaks were observed after 6 days of incubation. The appearance of the peak at 10.5 min at pH 5 was especially pronounced in A-2 which is the conjugate with the highest *in vitro* activity. The conjugate A-1 showed only a small peak at 10.5–11.0 at both pH-values.

lysosomes, which is well documented for macromolecules and serum albumin [6,15].

When comparing the serum stability of chlorambucil and the albumin conjugates with respect to their alkylating activity, we found an increased stability of the conjugates over a period of four days (Fig. 3). However, there was no significant difference between the conjugates themselves indicating that the superior antiproliferative activity of A-2, A-3, and A-4 over A-1 observed *in vitro* cannot, on the whole, be ascribed to the prolonged alkylating activity of chlorambucil albumin conjugates, but rather to their acid-sensitive properties.

Preliminary *in vivo* studies comparing the toxicity of "equialkylating" high doses of chlorambucil and A-2 in mice have shown that the albumin conjugate was better tolerated and no mortality was observed.

In conclusion, our results show that an acid-sensitive link between chlorambucil and albumin is an effective way of retaining or improving the alkylating activity of chlorambucil. In the light of these results and preliminary studies of acute toxicity, we will evaluate the antitumor efficacy of active albumin conjugates of chlorambucil in animal tumor models.

Acknowledgements

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Experimental

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); elemental analysis: Perkin-Elmer Elemental Analyzer 240.– Analytical HPLC: reverse phase HPLC-column (Spherisorb-C18 ODS 2– 5 µm from MedChrom, Heidelberg, FRG); silica gel chromatography on silica gel 60 (0.063–0.100 mm) from Merck KG; TLC: silica coated plates 60 F_{254} from Merck AG; Chlorambucil (Mr 304.20) was a gift from Burroughs Wellcome, FRG. Organic solvents: HPLC grade (Merck KG) or analytical grade (gift from BASF AG).– Other organic or inorganic compounds: Merck KG, FRG. Maleimide spacer molecules were prepared previously [23]. Materials for the preparation of conjugates: human serum albumin (HSA) (98%, crystalline, Mr 66 500), iminothiolane HCl, 5,5′-dithio-bis-(2-nitrobenzoic acid), 4-(4-nitrobenzyl)pyridine, 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 albumin ε_{280} = 35 700 M^{-1} cm^{-1} [24] and the concentration of HS-groups with Ellmanns reagent ε_{412} = 13 600 M^{-1} cm^{-1} [25] 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 (USA). The amount of chlorambucil bound to albumin was determined using a modified 4-(4-nitrobenzyl)pyridine assay based on the assay of Epstein et al. [19] (see below). The purity of the albumin 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) and 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 **5**, **7**, **8**, and **9***

4-Phenylbutanoyl-2-hydroxyethylmaleimide **(5).** 4-Phenylbutyric acid (1.0 g, 6.08 mmol), 2-hydroxyethylmaleimide (1.72 g, 12.16 mmol), and a catalytic amount of 4-dimethylaminopyridine (DMAP; 20 mg, 0.16 mmol) were dissolved in 100 mL anhydrous CH_2Cl_2 at room temperature. To this solution N-cyclohexyl-N′-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate (2.97 g, 6.68 mmol), dissolved in 100 mL anhydrous CH_2Cl_2, was added dropwise within 1 h and the solution stirred for further 8 h at room temperature. The turbid solution was filtered and evaporated in vacuo. The residue was dissolved in 50 mL ethyl acetate, filtered, and chromatographed on a silica gel column (ethyl acetate/hexane 1:2) to yield 1.33 g (76%) pale yellow syrup; R_f 0.30 (ethyl acetate/hexane 1:2).– ^{1}H NMR ($CDCl_3$): λ = 1.92 (tt, 2H, *J* = 6.7/6.5 Hz, 6-H), 2.29 (t, 2H, *J* = 6.5 Hz, 7-H), 2.63 (t, 2H, *J* = 6.7 Hz, 5-H), 3.79 (t, 2H, *J* = 6.5 Hz, 3′-H), 4.24 (t, 2H, *J* = 6.5, 4′-H), 6.70 (s, 1H, 1′-H), 7.14–7.32 (m, 5H, aromatic H).– ^{13}C NMR ($CDCl_3$): δ = 26.21 (C-6), 33.38 (C-7), 35.08 (C-5), 36.99 (C-3′), 61.31 (C-4′), 125.99, 128.39, 128.49, 141.37 (aromatic C), 134.24 (C-1′), 170.40 (C-2′), 173.21 (C-8) ppm.– Anal. ($C_{16}H_{17}NO_4$).

4-Phenylbutanoyl tert.-butoxycarbonyl hydrazide **(6).** 4-Phenylbutyric acid (5.0 g, 30.4 mmol) was dissolved in 200 mL anhydrous CH_2Cl_2. Oxalyl chloride (3.97mL, 44.2 mmol) was added to this solution 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 was dissolved in 100 mL anhydrous CH_2Cl_2 and tert.-butylcarbazate (4.25 g, 32.2 mmol), dissolved in 100 mL anhydrous CH_2Cl_2, added dropwise while stirring at room temperature. The mixture was stirred for 36 h at room temperature, then filtered, and the filtrate evaporated in vacuo. After chromatography over a silica gel column (ethyl acetate/hexane 1:2) the product was crystallized from ethyl acetate/hexane in a yield of 6.95 g (82%) as a light brown solid; mp 94 °C.– R_f value: 0.15 (ethyl acetate/hexane 1:2).– ^{1}H NMR ($CDCl_3$): δ = 2.05 (tt, 2H, *J* = 6.6/6.4 Hz, 6-H), 2.23 (t, 2H, *J* = 6.4 Hz, 7-H), 2.68 (t, 2H, *J* = 6.6 Hz, 5-H), 6.63 (s, 1H, NH), 7.15–7.36 (m, 5H, aromatic H), 7.61 (s, 1H, NH).– Anal. ($C_{15}H_{22}N_2O_3$).

4-Phenylbutyric acid hydrazide (trifluoroacetate salt). 4-Phenylbutanoyl tert.-butoxycarbonyl hydrazide (0.70 g, 2.51 mmol) was dissolved in 10 mL anhydrous THF. To the stirred solution were added 10 mL trifluoroacetic acid, and the mixture was stirred for 1 h. Subsequently, the solvent was removed under high vacuum and the resulting hydrazide (trifluoroacetate salt) reacted with 2-maleimidoacetaldehyde, 3-maleimidobenzaldehyde or 3-maleimidoacetophenone to obtain the hydrazone derivatives **7–9** as described below.

*Carboxylic hydrazone derivative **7** of 4-phenylbutyric acid hydrazide and 2-maleimidoacetaldehyde.* 4-Phenylbutyric acid hydrazide (trifluoroacetate salt; 0.73 g, 2.51 mmol) was dissolved in 30 mL anhydrous THF and 2-maleimidoacetaldehyde (0.39 g, 2.75 mmol) added at room temperature. The reaction mixture was stirred for 36 h. The solution was then evaporated in vacuo and the residue crystallized from ethyl acetate/hexane to yield 0.34 g (45%) of a white solid.– Mp 113 °C.– R_f 0.27 (ethyl acetate/hexane 1:1).– ^{1}H NMR ($CDCl_3$): δ = 1.90 (tt, 2H, *J* = 6.5/6.4 Hz, 6-H), 2.51 (t, 2H, *J* = 6.4 Hz, 7-H), 2.65 (t, 2H, *J* = 6.5 Hz, 5-H), 4.33 (t, 2H, *J* = 2.5 Hz, 3′-H), 6.70 (s, 2H, 1′-H), 7.12 (t, 1H, 4′-H), 7.18–7.39 (m, 5H, Ph), 9.94 (s, 1H, NH).– ^{13}C NMR ($CDCl_3$): δ = 25.99 (C-6), 32.02 (C-7), 35.08 (C-5), 38.44 (C-3′), 125.88, 128.35, 128.45, 141.66 (aromatic C), 134.38 (C-1′), 138.72 (C-4′), 170.04 (C-2′), 176.08 (C-8) ppm.– Anal. ($C_{16}H_{17}N_3O_3$).

*Carboxylic hydrazone derivative **8** of 4-phenylbutyric acid hydrazide and 3-maleimidoacetophenone.* 4-Phenylbutyric acid hydrazide (trifluoroacetate salt; 0.73 g, 2.51 mmol) was dissolved in 30 mL anhydrous THF and 3-maleimidoacetophenone (0.59 g, 2.75 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: 0.77 g (82%) yellow powder.– Mp 148 °C.– R_f 0.41 (ethyl acetate/hexane 2:1).– ^{1}H NMR ($CDCl_3$): δ = 2.08 (tt, 2H, *J* = 6.7/6.5 Hz, 6-H), 2.27 (s, 3H,

CH_3), 2.73 (t, 2H, *J* = 6.5 Hz, 7-H), 2.84 (t, 2H, *J* = 6.7 Hz, 5-H), 6.84 (s, 2H, 1′-H), 7.15–7.72 (m, 9H, aromatic H), 9.75 (s, 1H, NH).– ^{13}C NMR-data ($CDCl_3$): δ = 12.68 (CH_3), 26.33 (C-6), 32.33 (C-7), 35.48 (C-5), 123.77, 125.52, 125.82, 126.64, 128.31, 128.56, 129.22, 131.56, 141.88, 145.53 (aromatic C), 134.30 (C-1′), 139.23 (C-9′), 169.38 (C-2′), 176.05 (C-8) ppm.– Anal. ($C_{22}H_{21}N_3O_3$).

*Carboxylic hydrazone derivative **9** of 4-phenylbutyric acid hydrazide and 3-maleimidobenzaldehyde.* 4-Phenylbutyric acid hydrazide (trifluoroacetate salt; 0.50 g, 1.72 mmol) was dissolved in 50 mL anhydrous THF and 3-maleimidobenzaldehyde (0.42 g, 2.06 mmol) added at room temperature. The reaction mixture was stirred for 24 h. The solution was evaporated in vacuo and the product purified by repeated crystallization from ethyl acetate/hexane.– Yield: 0.45 g (72%) yellow powder.– Mp 108 °C.– R_f 0.24 (ethyl acetate/hexane 2:1).– ^{1}H NMR ($CDCl_3$): δ = 2.10 (tt, 2H, *J* = 6.8/6.7 Hz, 6-H), 2.74 (t, 2H, *J* = 6.8 Hz, 7-H), 2.80 (t, 2H, *J* = 6.7 Hz, 5-H), 6.89 (s, 2H, 1′-H), 7.12–7.64 (m, 9H, aromatic H), 7.81 (s, 1H, 9′-H), 9.84 (s, 1H, NH).– ^{13}C NMR-data ($CDCl_3$): δ = 26.36 (C-6), 32.16 (C-7), 35.44 (C-5), 124.51, 125.81, 126.35, 127.28, 128.30, 128.536, 129.49, 131.82, 135.12, 141.76 (aromatic C), 134.29 (C-1′), 142.16 (C-9′), 169.24 (C-2′), 176.18 (C-8) ppm.– Anal. ($C_{21}H_{19}N_3O_3$).

Synthesis of the albumin conjugates A-1–A-4. All reactions were performed at room temperature unless otherwise stated. Data for one representative experiment is given:

(1) Thiolation of albumin using iminothiolane – 54 mg HSA were dissolved in 4.0 mL degassed buffer (0.1 M sodium borate, 0.001 M EDTA, 0.15 M NaCl – pH = 8.0, *c* (HSA) ≈ 2.0×10^{-4} M) and 130 µL of a 4×10^{-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 albumin is 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 3.0 [3)]. The sample of thiolated albumin (7.0–7.5 mL) was used directly for the synthesis of the conjugate.

*(2) Reaction of maleimide derivative **2** with thiolated albumin* – 150 µL of a solution of **2** (*M*r 439.1) in dimethylformamide (2.0 mg dissolved in 150 µL dimethylformamide) were added to 7.0 mL thiolated sample, 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) and the conjugate isolated (retention volume: 3.6 – 7.5 mL, buffer: standard borate). The concentration of bound chlorambucil was 334 ± 15 µM and that of albumin 118 ± 8 µM which corresponds to approximately 2.8 equivalents of chlorambucil bound to albumin.

Determination of the alkylating activity of the albumin conjugates A-1–A-4 with 4-(4-nitrobenzyl)pyridine (NBP) – modified according to Epstein et al.[19] All determinations were carried out three times and mean values calculated. 50 µL samples of A-1, A-2, A-3, or A-4 were filled into tubes and diluted with standard borate to 100 µL. To this solution were added 50 µL acetic acid (5%), 245 µL distilled water, 50 µL ethanol, and 55 µL of a NBP solution (500 mg NBP dissolved in 10 mL 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 were 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 (ε_{565} = 15 100 M^{-1} cm^{-1}).

For the cell culture experiments and serum stability studies the alkylating activity of the A-1, A-2, A-3, and A-4 was adjusted to 720 or 1000 µM by concentrating the samples using CENTRIPREP®-10-concentrators.

Modified procedure for the determination of the alkylating activity of the albumin-chlorambucil conjugates incubated with 10% serum. 100 µL of the respective conjugate serum sample (see below) were filled into 10 mL tubes. To this solution were added 50 µL acetic acid (5%), 245 µL water, 50 µL ethanol, and 55 µL of the NBP solution. The sealed tubes were heated for 90 minutes at 80 °C in a water bath and then cooled to room temperature. 400 µL of the samples were filled into Eppendorf tubes and centrifuged for 5 minutes. 250 µL of the supernatant were mixed with 250 µL of a 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).

Serum stability studies (10% serum). Preparation of the stock solution containing 10% serum: 330 µL human blood serum were filled into a 10 mL glass tube. To the serum were added 1970 µL buffer (pH = 7.4; 0.0025 M sodium borate, 0.15 M NaCl) and 1000 µL of the respective albumin-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, 24, 32, 48, and 72 h.

*Hydrolysis studies with **5**, **7**, **8**, and **9** at pH 5.0.* Stock solutions of **5**, 7**,** 8**,** and **9** (*c* = 10^{-2} M) in acetonitrile were prepared. To 50 µL of the respective stock solution were added 50 µL of a mercaptoethanol solution (10^{-2} M in acetonitrile) and 900 µL of buffer (0.01 M NaOAc, 0.15 M NaCl; pH 5.0). The solutions were incubated at room temperature for 30 minutes. Then 20 µL samples were analyzed at *t* = 0, 2, 4, 8, 24, 32, 48, 72, and 96 h on a reverse phase HPLC-column (acetonitrile/water 40/60 for **7**–**9**; acetonitrile/water 50/50 for **5**); retention times for **5**: 4.4 min, **7**: 7.1 min, **8**: 3.62 min, **9**: 8.1 min. The UV-absorption was detected at a wavelength of 212 nm (**5**, **7**) or 280 nm (**8**, **9**).

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 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.[26]. Briefly, cells were harvested from exponential phase cultures growing in RPMI culture medium by trypsinization, counted, and plated in 96 well flat-bottomed microtitre plates (50 µL 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 culture medium (6 control wells per 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).

Toxicity studies. Toxicity studies were carried out at the Chemistry Department of the University Heidelberg, Experimental Division. Using NMRI mice (three female mice per group) as animal models, 20 mg/kg of chlorambucil (5.0 mg chlorambucil dissolved in 10 mL 0.3 M $NaHCO_3$, 0.15 M NaCl) and A-2 (1650 µM with respect to its alkylating activity) were administered by ip injection on three 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). Relevant data are summarized in Table 2.

[3)] A smaller number of thiol groups can be introduced by reducing the amount of added iminothiolane, e.g. for introducing two HS groups 90 µL of the iminothiolane solution are added.

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