European Journal of Medicinal Chemistry 46 (2011) 1604–1615

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Bivalent bendamustine and melphalan derivatives as anticancer agents

Ana Maria Scutaru [a], Maxi Wenzel [a], Ronald Gust [a,b,*]

[a] Institute of Pharmacy, Freie Universität Berlin, Königin Luise Str. 2+4, 14195 Berlin, Germany
[b] Institute of Pharmacy, Department of Pharmaceutical Chemistry, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria

ARTICLE INFO

Article history:
Received 10 August 2010
Received in revised form
1 February 2011
Accepted 4 February 2011
Available online 3 March 2011

Keywords:
Bendamustine
Melphalan
Dimer
Drug targeting
Breast cancer

ABSTRACT

The alkylating agents bendamustine and melphalan are currently used in the treatment of various tumoral diseases. In order to increase their antitumor potency and tumor selectivity both compounds were integrated in structure–activity relationship studies including new drug carrier systems. Here we describe the synthesis and the cytotoxicity of new bivalent bendamustine and melphalan derivatives. Two molecules each esterified with *N*-(2-hydroxyethyl)maleimide were connected by diamines with various chain lengths ($n = 6, 7, 8, 12$). It was supposed that these conjugates (**5a–d**, **10a–d**, **11a–d**) cause cytotoxic effects preferred as bivalent drug. Indeed the cytotoxicity of the new compounds increased compared to bendamustine and melphalan as determined in concentration-dependent *in vitro* assays using the human MCF-7 and MDA-MB-231 breast cancer cell lines.

1. Introduction

Bendamustine firstly synthesized by Ozegowski and Krebs [1] is a very promising drug with applicability in the treatment of various tumoral diseases such as non-Hodgkin's lymphoma and multiple myeloma [2–9].

Further clinical trials illustrated some remarkable results in the treatment of chronic lymphocytic leukemia (CLL) [10–12] and thus, in March 2008, bendamustine was also approved in the USA for this indication [13]. Furthermore, bendamustine has a promising future in the treatment of breast cancer as it has been shown by recent studies [14–16].

Its structure can be divided into three parts: a bis(2-chloroethyl)-amino (N-lost) group providing the alkylating properties, a benzimidazole core and a carboxylic acid side chain mediating water solubility. This structure assigned it to the class of nitrogen mustard agents comparable to melphalan, which is used in the treatment of cancer, including ovarian cancer, malignant melanoma, multiple myeloma (bone-marrow cancer), breast cancer and chronic myelogenous leukemia [17,18].

The design of bendamustine and melphalan is based on the knowledge that the use of alkylating agents in the treatment of cancer is restricted due to the diversity of the side effects (for example: nephrotoxicity, nausea and vomiting). Thus melphalan was obtained by combining the N-lost moiety with the amino acid phenylalanine whereas in bendamustine the N-lost group is bound to a purine-like heterocycle [19–22]. This drug design should guarantee a higher accumulation of the compounds in tumor cells compared to normal cells, because of a higher biomolecule trafficking in fast growing (tumor) cells.

Since the discovery of melphalan in the late 1950s, many efforts have been made to synthesize derivatives of melphalan with the purpose of diminishing the side effects of the drug [22–24]. A suitable concept might be the tumor targeting using natural and synthetic polymers as carrier as already demonstrated for chlorambucil by Kratz et al. [25,26]. They combined the anticancer agent in a first step with a maleimide spacer and bound it in a second step selectively to thiol groups present in the carrier protein, the Human Serum Albumin (HSA) [27,28]. These HSA conjugates accumulated in the tumors due to the EPR (enhanced permeability and retention) effect. The same conjugates were built in situ with endogenous HSA, if the drug-maleimide is administered iv. This pro-drug (drug-carrier) design resulted in an enhanced antitumor activity accompanied by a better compatibility due to lower unwanted toxic side effects.

Abbreviations: DADC, 1,12-diaminododecane; DAHEPT, 1,7-diaminoheptane; DAH, 1,6-diaminohexane; DAOCT, 1,8-diaminooctane; EPR (enhanced permeability and retention) effect, the property by which macromolecules build up in the tumor due to the special properties of the cells (e.g., poor lymphatic drainage); FCS, fetal calf serum; PBS, phosphate-buffered saline; DCC, *N*,*N*-dicyclohexylcarbodiimide; DMAP, (4-dimethyl-aminopyridine).

* Corresponding author. Institute of Pharmacy, Department of Pharmaceutical Chemistry, University of Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria. Tel.: +43 512 507 5245; fax: +43 512 507 2940.
E-mail address: ronald.gust@uibk.ac.at (R. Gust).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.02.008

In our approach bendamustine and melphalan were attached to small synthetic dendrimers. For this purpose, both drugs esterified with *N*-(2-hydroxyethyl)maleimide (analogously to the investigations of Kratz et al.) were bound via the maleimide moiety to 1,3,5-tris(3-aminopropyl)benzene (**G0**) and its **G1** analog 3,5-bis-(3-aminopropyl)-*N*-(3-{3,5-bis[3-{3,5-bis(3-aminopropyl)benzoylamino}propyl]phenyl}propyl)benzamide. Compounds **G0** and **G1** have seen to be suitable synthetic carriers in previous studies with platinum complexes [29,30].

The study of the drug-dendrimer conjugates includes also comparable investigations of melphalan and its maleimide ester with the free and Boc-protected amino groups (compounds **8** and **9**, see Scheme 2). In accordance to Bergel and Stock [23] the cytotoxic effects were unaffected by the esterification of the COOH group but strongly decreased by protection of the NH_2 group. It should be mentioned that these structural modifications did not reflect on the reactivity of the N-lost group of melphalan. The hydrolysis rates of **8** and **9** were comparable to melphalan.

In contrast, esterification of bendamustine with *N*-(2-hydroxyethyl)maleimide (**4a**, Scheme 1) strongly improved the hydrolytic stability of the N-lost moiety and consequently the cytotoxicity in cell culture experiments [30]. Although very active *in vivo*, bendamustine was inactive *in vitro* because of its fast hydrolysis under cell culture conditions. Therefore, it is assumed, that the DNA crosslinking achieved by the N-lost group plays a subordinate role in the antitumor activity of bendamustine.

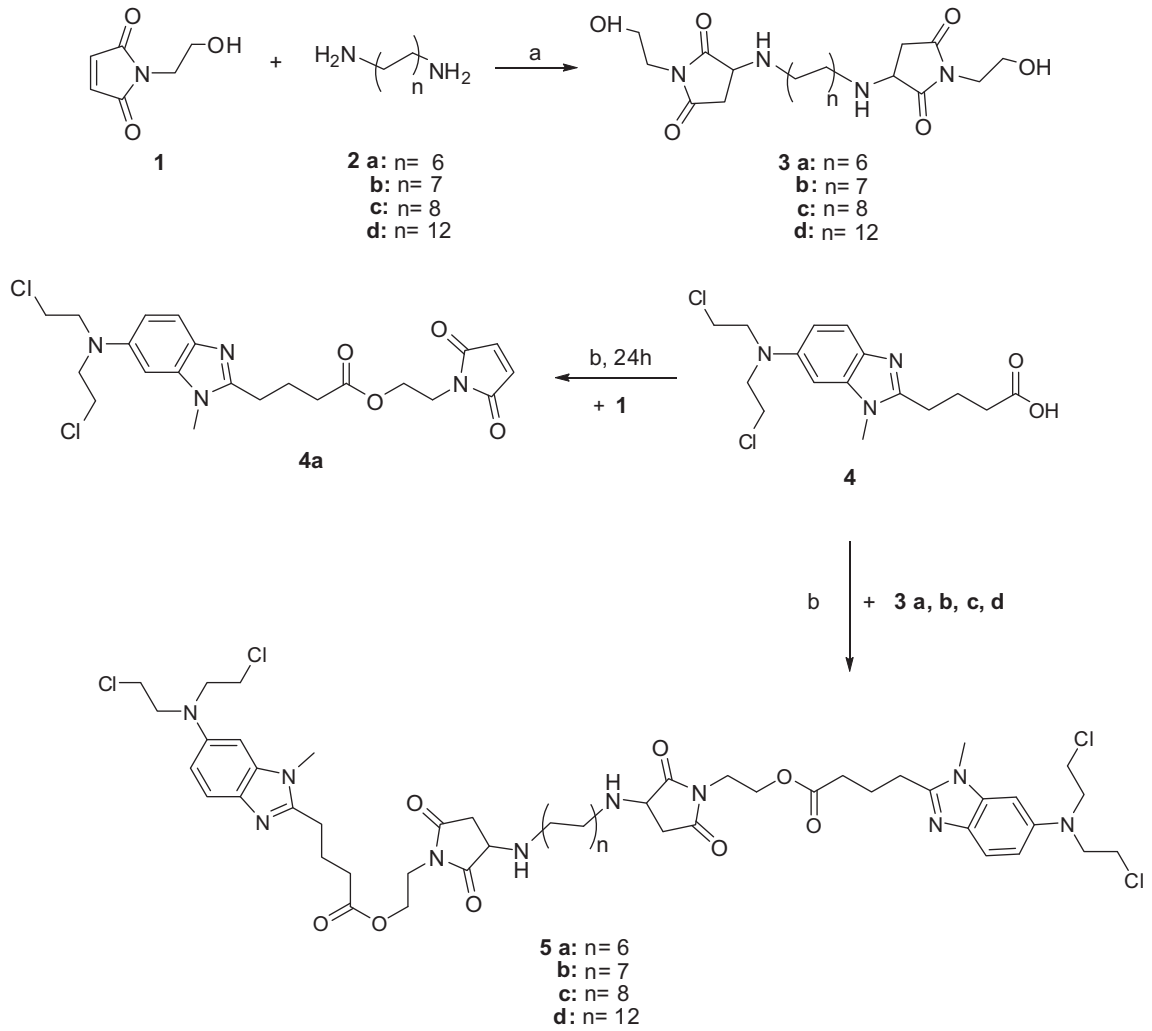
In order to further optimize the *in vitro* cytotoxicity of bendamustine and melphalan, we decided to use the concept of bivalent drugs. The positive results of amino-terminated dendrimers and the increased cellular accumulation of diaminoalkyl connected platinum drugs [31] induced us to link two *N*-(2-hydroxyethyl)-maleimide-drug conjugates to diamines with variable chain lengths (1,6-diaminohexane (DAH), 1,7-diaminoheptane (DAHEPT), 1,8-diaminooctane (DAOCT) and 1,12-diaminododecane (DADC)) (see Schemes 1 and 2). The synthesis and the activity against the MCF-7 and MDA-MB-231 mammary carcinoma cell lines are described in this paper.

2. Results and discussion

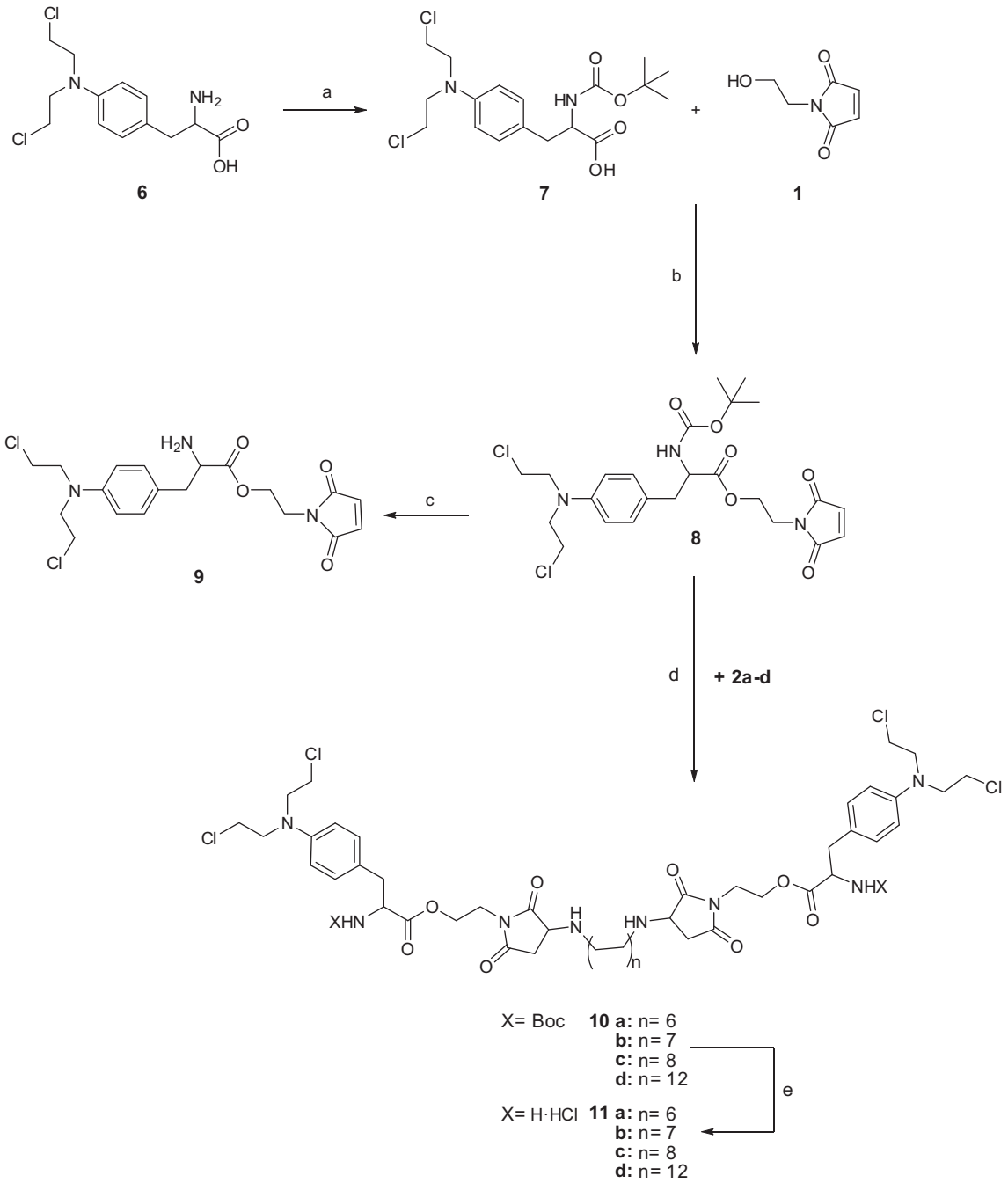
2.1. Chemistry

Two different strategies were evaluated for the synthesis of the dimers **5a**–**d** (bendamustine, Scheme 1), **10a**–**d** (melphalan Boc-protected, Scheme 2) and **11a**–**d** (melphalan, Scheme 2).

A first approach included a two steps route: 1) the reaction of *N*-(2-hydroxyethyl)maleimide with the diamine through a Michael-like addition; the maleimide being used as an electrophilic olefin and



Scheme 1. Synthesis of the bivalent bendamustine conjugates. Reagents and conditions: a) chloroform, reflux; b) DCC, DMAP, CH_2Cl_2, RT.



Scheme 2. Synthesis of the bivalent melphalan derivatives. Reagents and conditions: a) Boc_2O, TEA, methanol, RT, 1 h; b) DCC, DMAP, CH_2Cl_2, RT, 24 h; c) aqueous HCl, THF, RT, 24 h; d) chloroform, reflux; e) aqueous HCl, THF, RT.

the diamine as a nucleophile [32], 2) a Steglich reaction, involving the free acid group of the drug, the bismaleimide-adducts and typical reagents like DCC (*N*,*N*-dicyclohexylcarbodiimide) and catalytic amounts of DMAP (4-dimethylaminopyridine).

The second approach implicated at first the Steglich esterification of drug and spacer, followed by the addition of the diamines linker to the double bond at the maleimide moiety.

Taking the reaction conditions into consideration and the need for high yields, the first strategy was chosen for the formation of the bendamustine derivatives and the second one for the synthesis of the melphalan conjugates.

The route of preparing the dimers of bendamustine is depicted in Scheme 1. *N*-(2-hydroxyethyl)maleimide (**1**) was refluxed in chloroform with the respective diamine (**2a**–**d**) to give the products **3a**–**d**. The addition to the maleimides results in asymmetric centers and the building of isomers which were not separated. Bendamustine hydrochloride was transformed into the free base **4**, activated by addition of DCC followed by conversion into the desired compounds **5a**–**d** after addition of the correspondent bismaleimide-adducts **3a**–**d** and DMAP.

Melphalan–maleimide derivatives were synthesized from L-melphalan (**6**) after Boc-protection and reaction with the spacer

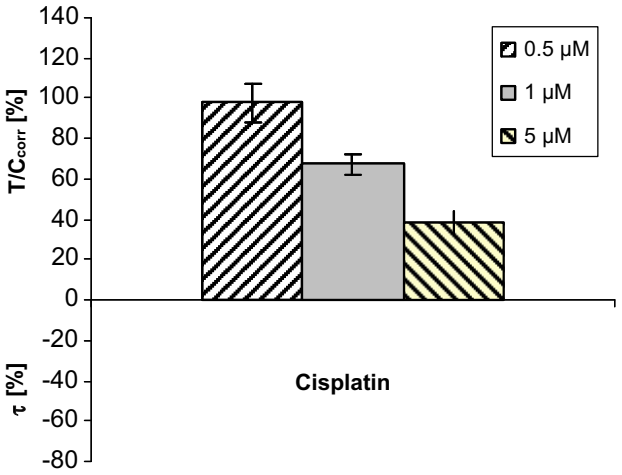


Fig. 1. *In vitro* cytotoxicity of cisplatin at 72 h in the MCF-7 cell line. For detailed information on the investigation of the T/C_{corr} [%] and τ [%] values see Experimental section.

as depicted in Scheme 2 and described in the literature [33,34]. The resulting ester **8** (Boc-protected) was purified by column chromatography and treatment with aqueous HCl to yield **9**.

Heating of **8** with the respective diamines **2a**–**d** in chloroform gave the Boc-protected dimers **10a**–**d** which were transformed into the final compounds **11a**–**d** by treatment with aqueous HCl (Scheme 2). No attempts were made to separate **10a**–**d** and **11a**–**d** into optically pure compounds.

All final compounds and those used in the biological tests were characterized by their fully assigned ^{1}H and ^{13}C NMR spectra and molecular ion peaks in the mass spectra. The conjugates were soluble in water and in buffered cell culture media as judged by visual examination.

2.2. Antitumor activity

The antiproliferative activity of the synthesized compounds was determined utilizing human MCF-7 and MDA-MB-231 breast cancer cell lines. From the graphs antiproliferative and cytostatic effects ($80\% > T/C_{corr} > 0\%$) as well as cytocidal effects ($\tau = T/C_{corr} < 0\%$) can be deduced.

Cisplatin, bendamustine (**4**) and melphalan (**6**) were used as references. The new synthesized compounds (as well as the reference substances) possessed comparable activity in both cell lines. The time response curves of MCF-7 and MDA-MB-231 cells are presented in supporting information. For discussion the (maximal) effect against MCF-7 cells after an incubation time of 72 h are presented in Figs. 1–4.

Cisplatin influenced the cell growth in a concentration-dependent manner and caused 40% growth inhibition at the highest applied concentration (5 µM) (Fig. 1).

Bendamustine (4) was nearly inactive at all tested concentrations (1, 5 and 10 µM, see Fig. 2), while melphalan (**6**) caused cytostatic effects ($T/C_{corr} = 20\%$, Fig. 3) at 10 µM.

The esterification of bendamustine (**4**) with the *N*-(2-hydroxyethyl)maleimide giving compound **4a** increased the activity highly (Fig. 2). Already 5 µM of **4a** caused cytostatic effects. All bivalent bendamustine derivatives (**5a**–**d**) were more active than the parent compound **4** but not as active as **4a**. The dimers **5b** and **5c** caused similar antiproliferative effects at 10 µM and were slightly more active than **5a** and **5d**. A clear preference of a chain length could not be detected from these results.

The antiproliferative effects of L-melphalan (**6**) depended on the derivatization of the amino group as well as the carboxyl group. The Boc-protected compound **7** proved to be inactive in both cell lines [30]. The esterification with *N*-(2-hydroxyethyl)maleimide not only compensated this inactivation but led to compound **8** with increased cytotoxicity (Fig. 3). Interestingly, the connection of two molecules by a diaminoalkyl spacer (**10a**–**d**) drastically reduced the antiproliferative activity (T/C_{corr} values of about 50% were achieved at 10 µM). The effect of **10b** and **10c** were somewhat more active than **10a** and **10d** (see Fig. 3).

The cleavage of the Boc groups in **10a**–**d** highly increased the cytotoxicity of the bivalent drugs (Fig. 3). Compound **11a** was cytostatic at 10 µM, while **11bc** were even cytocidal at 5 and 10 µM. The most active compound **11d** was tested in lower concentrations (Fig. 4) and caused cytotoxic effects even at 1 µM.

From the results depicted in Figs. 2–4 (and also from the data included in the supporting information) it is obvious that the concept of bivalent drugs was successful. The activity of melphalan was strongly increased and in the case of bendamustine *in vitro* active bendamustine compounds were obtained.

2.3. Discussion

Melphalan and bendamustine are alkylating agents and currently used in the treatment of various tumoral diseases. The

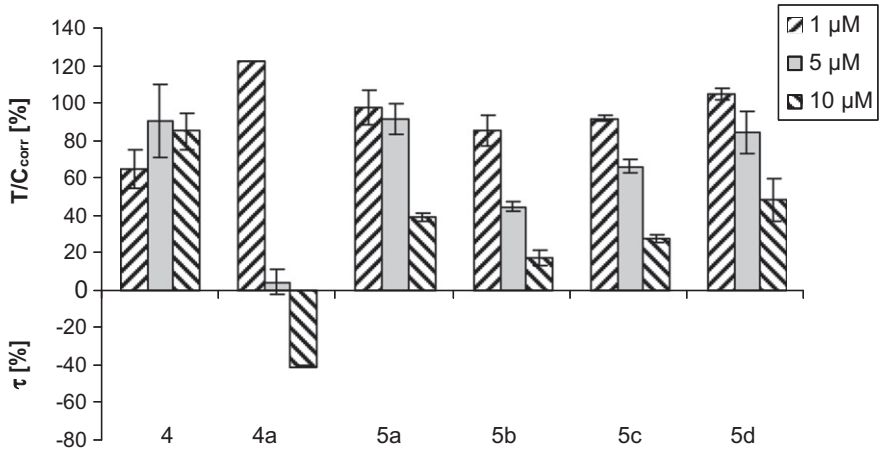


Fig. 2. *In vitro* cytotoxicity of bendamustine (**4**), its maleimide (**4a**) and bivalent derivatives (**5a**–**d**) at 72 h in the MCF-7 cell line. For detailed information on the investigation of the T/C_{corr} [%] and τ [%] values see Experimental section.

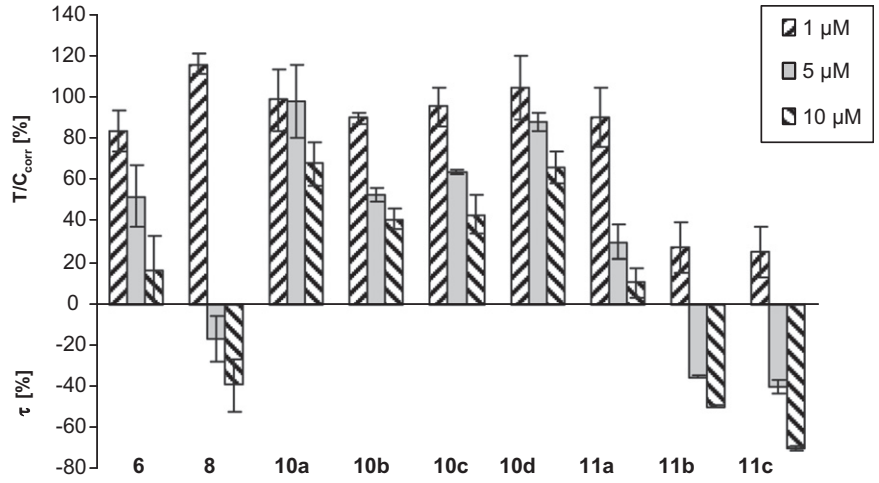


Fig. 3. *In vitro* cytotoxicity of melphalan (**6**), its Boc-protected maleimide derivative (**8**) as well as its Boc-protected bivalent derivatives (**10a**–**d**) and of the compounds **11a**–**c** at 72 h in the MCF-7 cell line. For detailed information on the investigation of the T/C$_{corr}$ [%] and τ [%] values see Experimental section.

mode of action of alkylating drugs included a selective DNA-interaction and the formation of interstrand cross links [35–37]. The N-lost moiety forms an aziridinium ion which attacks the nucleobases. Therefore, it is necessary to transfer the intact alkylator into the cells and prevent hydrolysis in the cell medium.

Bendamustine and melphalan are non-stable in aqueous solutions including the media used for cell culture experiments [38–40]. After hydrolysis into the dihydroxyl species the cytotoxicity is lost. Nevertheless, melphalan was distinctly more active than bendamustine against MCF-7 and MDA-MB-231 cells (see Figs. 2 and 3 and supporting information) which point to different accumulation kinetics into the cells. Melphalan might be transferred through the cell membrane e.g. by amino acid carrier systems prior to hydrolysis. This would explain the loss of activity after Boc-protection, which prevent the recognition by the transporter. Active transport of melphalan into cells was confirmed e. g. for the ASC-like amino acid system at low concentrations and the amino acid transport system L at higher concentrations [41].

With the objective to increase uptake and accumulation of melphalan in tumor cells a series of dipeptide derivatives were synthesized [23,24]. Other approaches focused on the combinations with carriers for instance to increase the uptake in the central nervous system for the therapy of intracerebral tumors (for further information about pharmaceutics, combinations, Co-drugs and prodrugs of melphalan see Wickström et al. [22]).

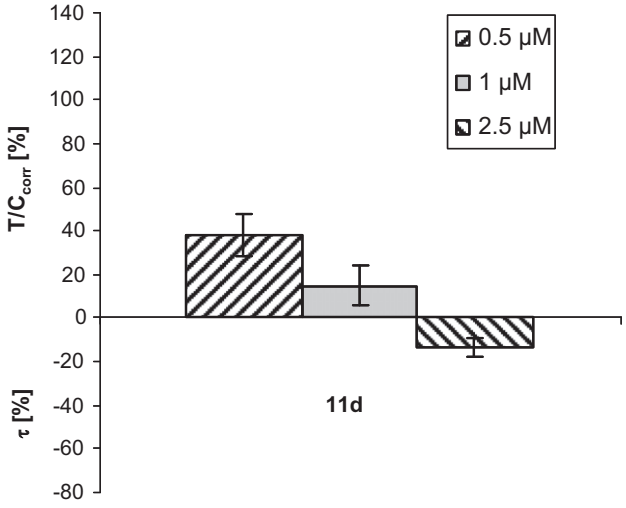


Fig. 4. *In vitro* cytotoxicity of the bivalent melphalan derivative 11d at 72 h in the MCF-7 cell line. For detailed information on the investigation of the T/C$_{corr}$ [%] and τ [%] values see Experimental Section.

A new melphalan derivative represents the L-melphalanyl-p-L-fluorophenylalanine ethyl ester (J1) which was designed for the treatment of neuroblastoma, the most common and deadly tumor of childhood [42]. J1 is rapidly incorporated into the cytoplasm followed by intracellular ester hydrolysis resulting in a release of melphalan. The enzymes responsible for the activation have to be identified to be aminopeptidases. This concept could also be realized using ester derivatives which can be cleaved due to the low lysosomal pH or intracellular esterases.

Because up to now a carrier system for the uptake of bendamustine into tumor cells could not be confirmed we bound bendamustine to low molecular weight dendrimers of the **G0** and **G1** type [43] (Fig. 5) to achieve endocytotic or macropinocytotic accumulation in tumor cells as already demonstrated for platinum complexes [29]. The same modification was done with melphalan.

In the first part of our structure-activity relationship (SAR) study [30] we investigated the stability of the dendrimer bound drugs in aqueous solution as well as the cytotoxicity against MCF-7 and MDA-MB-231 cells in relation to the free drugs and the drug-maleimide conjugates.

Data obtained from the stability studies clearly documented that the esterification of bendamustine decreased the degradation in aqueous solution. The same experiments done with melphalan did not indicate higher stability. Under physiological conditions (PBS, pH 7.4) the bendamustine-maleimide ester showed a half-life of 10h. Interestingly, the maleimide derivative of melphalan underwent in this experiment a complete hydrolysis. Binding of the maleimide esters to diaminoalkanes did not change the hydrolysis profile. For instance bendamustine release by ester cleavage and hydrolysis processes were detected in comparable amounts also for the dimeric compounds.

The dimeric bendamustine derivatives were more active than the dendrimer derivatives (under consideration of the number of bound drug molecules) but not as active as the monomeric maleimide ester **4a**. This is an indication for the participation of a secondary effect of the maleimide moiety in the case of **4a**. As demonstrated by Kratz et al., maleimide derivatives can effectively bind to HSA which act as a carrier molecule but also prevent

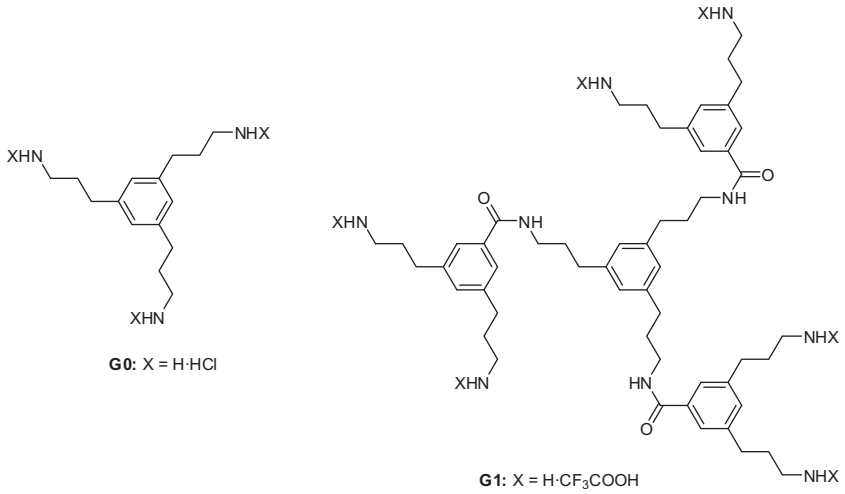


Fig. 5. Structure of **G0** and **G1** basic dendrimers.

hydrolysis reactions. Hopwood and Stock showed that, by coupling nitrogen mustard to proteins or semi-synthetic polymers, the rate of hydrolysis can be slowed down and, in some cases, this procedure led to a better biological activity [44]. In the first SAR study [30] fast interactions of the compounds **4a** and **9** with HSA were observed.

The derivatization of melphalan to bivalent drugs was more efficient than in the case of bendamustine. Compound **5b** as dimeric bendamustine derivative was cytostatic ($T/C_{corr} < 20\%$), whereas **5a**, **5c** and **5d** exhibited antiproliferative effects ($20\% < T/C_{corr} < 80\%$). In contrast all dimeric melphalan derivatives were cytocidally active ($T/C_{corr} < 0\%$).

It should be emphasized that all bivalent derivatives of melphalan and bendamustine exist as a mixture of isomers, therefore it can be argued that higher cytostatic effects may be expected for some of the optically pure compounds. A separation of the optically pure compounds is planned in a subsequent study.

This finding confirms the positive results of other attempts to optimize the pharmacological effects of antitumor drugs, e.g. platinum complexes. Farrell and co-workers have successfully synthesized multinuclear platinum complexes with bridging linkers leading to circumvention of cisplatin resistance. These compounds were found to possess a different mode of action [45]. In our group we could demonstrate a higher accumulation of platinum complexes in MCF-7 cells due to an active transport into the tumor cells [46].

Unfortunately, the bivalent bendamustine and melphalan derivatives are not suitable to study the uptake into tumor cells, because of a missing tracer. Therefore, we will label in a forthcoming study the derivatives in such a way that either high resolution continuum source atomic absorption or fluorescence analysis can be used for pharmacological studies.

3. Conclusions

This work focused on the development of bivalent bendamustine and melphalan derivatives with increased cytotoxicity. The *in vitro* assay in MCF-7 and MDA-MB-231 breast cancer cell lines showed an important increase of the antitumor activity of bendamustine conjugates compared to the free drug. With exception of the diaminododecane-derivative, all bivalent compounds exhibited cytostatic properties in both cell lines. The analogous melphalan derivatives were much more active and reached cytocidal effects at the highest used concentrations (5 and 10 μM). Dimers with C7, C8 and C12 spacer were about 10 fold higher cytotoxic than melphalan. In continuation of this study, we will quantify the cellular uptake and the mode of action of the anticancer agents, e.g. the DNA-binding.

4. Material and methods

4.1. General

^1H and ^{13}C NMR spectra were taken on a Bruker Avance/DPX 400 MHz with TMS as internal standard. Melting points (uncorrected) were measured with a Büchi Melting Point B-545. All column chromatography purifications were done using silica gel 60H (230 mesh ASTM, Merck). TLC was performed on silica gel 60 GF_{254} plates (Merck). EI spectra were recorded on a Thermo-Fisions VG Auto Spec (70 eV). The ESI-TOF spectra were measured on an Agilent 6210 ESI-TOF, Agilent Technologies, Santa Clara, CA, USA. Solvent flow rate was adjusted to 4 μL/min, Spray voltage set to 4 kV. Drying gas flow rate was set to 15 psi (1 bar). All other parameters were adjusted for a maximum abundance of the relative $[M + H]^+$.

4.2. Synthesis

N-tert-Butoxycarbonyl-4-[bis(2-chloroethyl)amino]-L-phenylalanine (**7**) was prepared according to literature procedures [33]. Bendamustine hydrochloride was a gift from Bendalis. L-Melphalan hydrochloride was synthesized as described by Bergel and Stock [47] starting from L-phenylalanine. All other starting materials were purchased from commercial sources and were used without purification. Solvents were dried under standard conditions.

4.2.1. General procedure for the synthesis of the bismaleimide-adducts ***3a–d*** *(mixture of optical isomers)*

N-(2-Hydroxyethyl)maleimide (**1**) and the diamine (**2a–d**) were dissolved in 50 mL of chloroform and refluxed for 24 h. The solvent was removed *in vacuo*. The residue was chromatographed on a silica gel column to afford the desired product.

*4.2.1.1. N,N′-Bis[1-(2-hydroxyethyl)pyrrolidine-2,5-dione-3-yl]-1,6-diaminohexane (**3a**).* *N*-(2-Hydroxyethyl)maleimide (2.12 g, 15 mmol), 1,6-diaminohexane (581 mg, 5 mmol), column chromatography with chloroform/methanol = 5:1. Yield: 1.6 g (80%) as an yellow gum: $R_f = 0.22$ (chloroform/methanol 5:1). ^1H NMR ($CDCl_3$): $\delta = 1.37$ (s, 4H, CH_{2DAH}), 1.50 (quin, $^3J(H,H) = 6.49$ Hz, 4H, CH_{2DAH}), 2.54–2.63 (m, 3H, **1** CH_2), 2.67–2.73 (m, 3H, **1** CH_2), 2.93–2.97 (m,

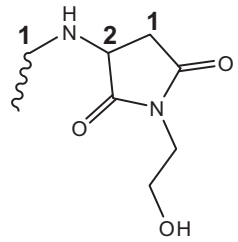


Chart 1. (Supporting scheme for NMR data of compound **3a**).

2H, **2** CH), 3.66–3.74 (m, 4H, $CH_2N_{succinimide}$), 3.77–3.81 (m, 4H + 2H, CH_2OH + CH) (Chart 1).

*4.2.1.2. N,N′-Bis[1-(2-hydroxyethyl)pyrrolidine-2,5-dione-3-yl]-1,7-diaminoheptane (**3b**).* *N*-(2-Hydroxyethyl)maleimide (2.12 g, 15 mmol), 1,7-diaminoheptane (651 mg, 5 mmol), column chromatography with chloroform/methanol = 10:1. Yield: 1.64 g (80%) as an orange gum: R_f = 0.22 (chloroform/methanol 10:1). ^{1}H NMR ($CDCl_3$): δ = 1.34 (s, 6H, CH_2 DAHEPT), 1.52 (m, 4H, CH_2 DAHEPT), 2.55–2.74 (m, 4H + 2H, CH_2NH + $CH_{2succinimide}$), 2.93–2.99 (m, 2H, $CH_{2succinimide}$), 3.72 (m, 4H, $CH_2N_{succinimide}$), 3.76–3.82 (m, 4H + 2H, CH_2OH + CH).

*4.2.1.3. N,N′-Bis[1-(2-hydroxyethyl)pyrrolidine-2,5-dione-3-yl]-1,8-diaminooctane (**3c**).* *N*-(2-Hydroxyethyl)maleimide (2.12 g, 15 mmol), 1,8-diaminooctane (721.3 mg, 5 mmol), column chromatography with chloroform/methanol = 20:1. Yield: 1.6 g (80%) as a yellow solid: R_f = 0.25 (chloroform/methanol 10:1); mp: 124–125 °C. ^{1}H NMR ($CDCl_3$): δ = 1.31 (s, 8H, CH_2 DAOCT), 1.50 (m, 4H, CH_2 DAOCT), 2.17 (s, 4H, NH + OH), 2.54–2.63 (m, 4H + 2H, CH_2NH + $CH_{2succinimide}$), 2.94–3.04 (m, 2H, $CH_{2succinimide}$), 3.71 (m, 4H, $CH_2N_{succinimide}$), 3.75–3.81 (m, 4H + 2H, CH_2OH + CH).

*4.2.1.4. N,N′-Bis[1-(2-hydroxyethyl)pyrrolidine-2,5-dione-3-yl]-1,12-diaminododecane (**3d**).* *N*-(2-Hydroxyethyl)maleimide (1.27 g, 9 mmol), 1,12-diaminododecane (601.1 mg, 3 mmol), column chromatography with dichloromethane/methanol = 10:1. Yield: 700 mg (48.3%) as a white solid: R_f = 0.33 (dichloromethane/methanol 10:1); mp: 170 °C. ^{1}H NMR ($CDCl_3$): δ = 1.27 (s, 16H, CH_{2DADC}), 1.49 (quin, ^3J(H,H) = 7.14 Hz, 4H, CH_{2DADC}), 2.13 (s, br, 4H, NH + OH), 2.54–2.72 (m, 4H + 2H, **1** CH_2), 2.93 (d, ^3J(H,H) = 8.28 Hz, 1H, **2** CH), 2.97 (d, ^3J(H,H) = 8.29 Hz, 1H, **2** CH), 3.69–3.73 (m, 4H, $CH_2N_{succinimide}$), 3.77–3.81 (m, 4H + 2H, CH_2OH + CH).

*4.2.2. General procedure for the synthesis of the bendamustine spacer diamines **5a**–**d** (mixture of optical isomers)*

Bendamustine as free base, DMAP (tip of a spatula) and the bismaleimide-adducts (**3a**–**d**) were dissolved in dry dichloromethane at room temperature. DCC, dissolved in dry dichloromethane, was added dropwise to this solution within 1 h, and the mixture was stirred for additional 24 h. The solution was filtered and evaporated *in vacuo*. The residue was chromatographed on a silica gel column to afford the desired product.

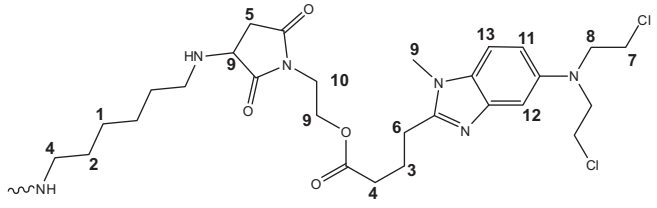


Chart 2. (Supporting scheme for NMR data of compound **5a**).

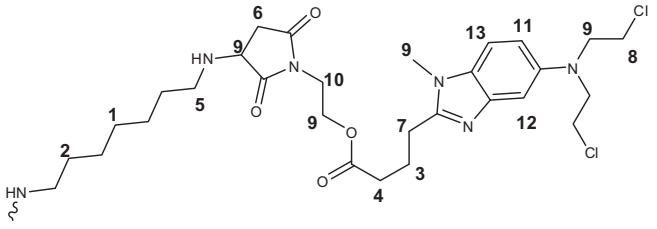


Chart 3. (Supporting scheme for NMR data of compound **5b**).

*4.2.2.1. N,N′-Bis[2-(2,5-dioxopyrrolidin-1-yl)ethyl-4-(5-(bis(2-chloroethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)butanoate)-3-yl]-1,6-diaminohexane (**5a**).* Bendamustine as free base (700 mg, 1.95 mmol), **3a** (394 mg, 0.97 mmol), 80 mL of dry dichloromethane, DCC (450 mg, 2.18 mmol), 80 mL of dry dichloromethane, column chromatography with chloroform/methanol = 20:1 and 10:1. Yield: 250 mg (23.73%) as a brown oil: R_f = 0.4 (dichloromethane/methanol 10:1). ^{1}H NMR ($CDCl_3$): δ = 1.24 (s, 4H, **1** CH_2), 1.32 (s, 4H, **2** CH_2), 2.15 (m, 4H, **3** CH_2), 2.43–2.56 (m, 8H, **4** CH_2), 2.62–2.66 (m, 2H, **5** CH_2), 2.90 (m, 4H + 2H, **5** + **6** CH_2), 3.60–3.65 (m, 11H, **7** + **8** + **9** CH_2), 3.69–3.71 (m, 17H, **7** + **8** + **9** CH_2), 4.22 (m, 4H, **10** CH_2), 6.76 (dd, ^3J(H,H) = 1.73 Hz, 8.64 Hz, 2H, **11** CH), 7.05 (d, ^3J(H,H) = 2.56 Hz, 2H, **12** CH), 7.17 (d, ^3J(H,H) = 8.79 Hz, 2H, **13** CH). ^{13}C NMR ($CDCl_3$): δ = 22.53 (**3** CH_2), 26.18 (**6** CH_2), 26.35 (**1** CH_2), 29.67 (**2** CH_2), 32.85 (CH_3), 32.96 (**4** + **5** CH_2), 37.90 (**9** CH_2), 40.8 (**7** + **8** CH_2), 47.51 (**4** CH_2), 54.78 (**9** CH), 60.86 (**10** CH_2), 103.01 (**12** CH), 109.81 (**11** CH), 110.83 (**13** CH), 128.82 (*C*CH_3), 142.75 (*C*N=C), 143.00 ($C_{aromatic}$), 154.36 (N=*C*), 172.98 (COO), 173.62 ($CO_{succinimide}$), 175.05 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{50}H_{69}N_{10}O_8Cl_4$ 1079.40, found 1079.3947 (Chart 2).

*4.2.2.2. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-4-(5-(bis(2-chloroethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)butanoate)-3-yl]-1,7-diaminoheptane (**5b**).* Bendamustine as free base (335 mg, 0.93 mmol), **3b** (193 mg, 0.46 mmol), 25 mL of dry dichloromethane, DCC (211.6 mg, 1.025 mmol), 25 mL of dry dichloromethane, column chromatography with chloroform/methanol = 20:1. Yield: 370 mg (72.5%) as a brown oil: R_f = 0.38 (chloroform/methanol 20:1). ^{1}H NMR ($CDCl_3$): δ = 1.28 (s, 6H, **1** CH_2), 1.45 (s, 4H, **2** CH_2), 2.15 (quin, ^3J(H,H) = 7.15 Hz, 4H, **3** CH_2), 2.45 (m, 4H, **4** CH_2), 2.50–2.57 (m, 4H, **5** CH_2), 2.62 (m, 2H, **6** CH_2), 2.89 (m, 2H + 4H, **6** + **7** CH_2), 3.61 (m, 8H, **8** CH_2), 3.69–3.76 (m, 20H, **9** CH_2), 4.22 (m, 4H, **10** CH_2), 6.77 (dd, ^3J(H,H) = 2.03 Hz, 8.76 Hz, 2H, **11** CH), 7.05 (d, ^3J(H,H) = 1.73 Hz, 2H, **12** CH), 7.16 (d, ^3J(H,H) = 8.75 Hz, 2H, **13** CH). ^{13}C NMR ($CDCl_3$): δ = 22.22 (**3** CH_2), 26.12 (**7** CH_2), 26.98 (**1** CH_2), 29.18 (CH_2), 29.67 (**2** CH_2), 29.95 (CH_3), 32.95 (**6** CH_2), 36.00 (**4** CH_2), 37.91 (**9** CH_2), 40.82 (**8** + **9** CH_2), 47.6 (**5** CH_2), 56.18 (**9** CH), 60.88 (**10** CH_2), 102.4 (**12** CH), 110.03 (**11** CH), 110.96 (**13** CH), 129.00 (*C*CH_3), 142.12 (*C*N=C), 143.02 ($C_{aromatic}$), 154.18 (N=*C*), 172.96 (COO), 175.05 ($CO_{succinimide}$), 177.50 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{51}H_{71}N_{10}O_8Cl_4$ 1093.4186, found 1093.4158; $C_{51}H_{71}N_{10}O_8Cl_4^{2+}$ 546.2139, found 546.2118 (Chart 3).

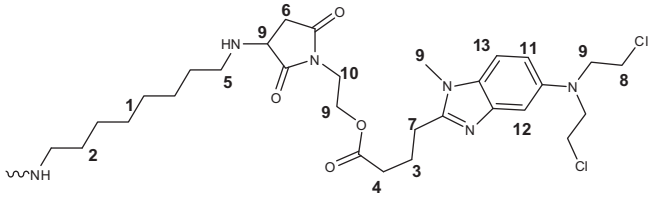


Chart 4. (Supporting scheme for NMR data of compound **5c**).

*4.2.2.3. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-4-(5-(bis(2-chloroethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)butanoate)-3-yl]-1,8-diaminooctane (**5c**).* Bendamustine as free base (335 mg, 0.93 mmol), **3c** (200 mg, 0.46 mmol), 25 mL of dry dichloromethane, DCC (211.6 mg, 1.025 mmol), 25 mL of dry dichloromethane, column chromatography with chloroform/methanol = 20:1 and chloroform/methanol = 10:1. Yield: 180 mg (35%) as a brown oil: $R_f = 0.36$ (chloroform/methanol 10:1). ^{1}H NMR ($CDCl_3$): $\delta = 1.21$ (s, 8H, **1** CH_2), 1.39 (s, 4H, **2** CH_2), 2.07 (quin, ^3J(H,H) = 7.18 Hz, 4H, **3** CH_2), 2.39 (m, 4H, **4** CH_2), 2.44–2.51 (m, 4H, **5** CH_2), 2.55–2.61 (m, 2H, **6** CH_2), 2.82 (m, 2H + 4H, **6** + **7** CH_2), 3.55 (m, 8H, **8** CH_2), 3.62–3.69 (m, 20H, **9** CH_2), 4.14 (m, 4H, **10** CH_2), 6.70 (dd, ^3J(H,H) = 2.15 Hz, 8.77 Hz, 2H, **11** CH), 6.99 (d, ^3J(H,H) = 2.01 Hz, 2H, **12** CH), 7.10 (d, ^3J(H,H) = 8.75 Hz, 2H, **13** CH). ^{13}C NMR ($CDCl_3$): $\delta = 22.23$ (**3** CH_2), 26.25 (**7** CH_2), 27.06 (**1** CH_2), 29.31 (CH_2), 29.87 (**2** CH_2), 32.99 (CH_3 + **6** CH_2), 36.12 (**4** CH_2), 37.86 (**9** CH_2), 40.83 (**8** + **9** CH_2), 47.65 (**5** CH_2), 56.26 (**9** CH), 60.81 (**10** CH_2), 102.84 (**12** CH), 109.90 (**11** CH), 110.88 (**13** CH), 129.28 (*C*NCH_3), 142.80 (*C*N=C + $C_{aromatic}$), 154.30 (N=*C*), 172.95 (COO), 175.07 ($CO_{succinimide}$), 177.65 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{52}H_{73}N_{10}O_8Cl_4$ 1107.4342, found 1107.4327, $C_{52}H_{73}N_{10}O_8Cl_4^{2+}$ 554.2208, found 554.2196 (Chart 4).

*4.2.2.4. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-4-(5-(bis(2-chloroethyl)amino)-1-methyl-1H-benzo[d]imidazol-2-yl)butanoate)-3-yl]-1,12-diaminododecane (**5d**).* Bendamustine as free base (660 mg, 1.86 mmol), **3d** (410 mg, 0.85 mmol), 50 mL of dry dichloromethane, DCC (422 mg, 2.05 mmol), 50 mL of dry dichloromethane, column chromatography with dichloromethane/methanol = 20:1. Yield: 560 mg (56.7%) as a crimson solid: $R_f = 0.46$ (dichloromethane/methanol 20:1); mp: 322 °C. ^{1}H NMR ($CDCl_3$): $\delta = 1.25$ (s, 16H, **1** CH_2), 1.48 (quin, ^3J(H,H) = 7.02 Hz, 4H, **2** CH_2), 2.15 (t, ^3J(H,H) = 7.00 Hz, 4H, **3** CH_2), 2.46 (t, ^3J(H,H) = 6.84 Hz, 4H, **4** CH_2), 2.50–2.60 (m, 4H, **5** CH_2), 2.63–2.70 (m, 2H, **6** CH_2), 2.89–2.96 (m, 2H + 4H, **6** + **7** CH_2), 3.64 (t, ^3J(H,H) = 6.8 Hz, 8H, **8** CH_2), 3.72–3.78 (m, 20H, **9** CH_2), 4.22 (quin, ^3J(H,H) = 5.54 Hz, 4H, **10** CH_2), 6.79 (dd, ^3J(H,H) = 2.3 Hz, 8.81 Hz, 2H, **11** CH), 7.08 (d, ^3J(H,H) = 2.22 Hz, 2H, **12** CH), 7.19 (d, ^3J(H,H) = 8.81 Hz 2H, **13** CH). ^{13}C NMR (DMSO-d6): $\delta = 24.34$ (**3** CH_2), 25.23 (**7** CH_2), 26.18 (CH_2), 28.48 (CH_2), 28.82 (**1** CH_2), 29.87 (**2** CH_2), 32.49 (CH_3), 33.24 (**4** + **6** CH_2), 37.16 (**9** CH_2), 41.18 (**8** + **9** CH_2), 47.38 (**5** CH_2), 52.99 (**9** CH_2), 60.27 (**10** CH_2), 110.68 (**12** CH), 117.62 (**11** + **13** CH), 127.62 (*C*NCH_3), 143.27 (*C*N=C), 146.05 ($C_{aromatic}$), 156.56 (N=*C*), 171.88 (COO), 172.29 ($CO_{succinimide}$), 173.75 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{56}H_{81}N_{10}O_8Cl_4$ 1163.4963, found 1163.4892 (Chart 5).

*4.2.3. O-{N-tert-Butoxycarbonyl-4-[bis(2-chloroethyl)amino]-L-phenylalanyl}-2-hydroxyethylmaleimide (**8**)*

7 (330 mg, 0.81 mmol), DMAP (tip of a spatula) and *N*-(2-hydroxyethyl)maleimide (441 mg, 3.1 mmol) were dissolved in 30 mL of dry dichloromethane at room temperature. DCC (176.5 mg, 0.85 mmol), dissolved in 25 mL of dry dichloromethane, was added dropwise to this solution within 1 h, and the solution was then stirred for additional 20 h. The solution was filtered and evaporated *in vacuo*. The red residue was chromatographed on a silica gel column (hexane/ethyl acetate = 2:1) to afford the desired product. Yield: 137 mg (32%) as a yellow solid: $R_f = 0.3$ (hexane/ethyl acetate 2:1); mp: 101 °C. ^{1}H NMR ($CDCl_3$): $\delta = 1.29$ (s, 9H, CH_3), 2.62 (m, 1H, CH_2Ar), 2.74 (dd, ^3J(H,H) = 4.46 Hz, 9.44 Hz, 1H, CH_2Ar), 3.62 (t, ^3J(H,H) = 5.28 Hz, 2H, CH_2 maleimide), 3.66 (s, 8H, CH_2CH_2Cl), 4.09 (m, 2H, CH_2O), 4.22 (m, 1H, CH), 6.63 (d, ^3J(H,H) = 8.52 Hz, 2H, Ar*H*), 7.02 (d, 2H + 2H, Ar*H* + $H_{maleimide}$), 7.17 (d, ^3J(H,H) = 8.01 Hz, 1H, NH). ^{13}C NMR ($CDCl_3$): $\delta = 28.31$ (CH_3), 36.64 (*C*H_2Ar), 36.98 (*C*H_2N$_{maleimide}$), 40.46 (*C*H_2NAr + *C*H_2Cl), 54.49 (CH), 62.11 (CH_2O), 79.84 (*C*$(CH_3)_3$), 112.09 ($C_{aromatic}$), 124.8 ($C_{aromatic}$), 130.56 ($C_{aromatic}$), 134.25 ($CH_{maleimide}$), 145.04 ($C_{aromatic}$), 155.07 (COO_{Boc}), 170.29 ($CO_{maleimide}$), 171.75 (COO). MS (EI, 80 eV, 300 °C); *m*/*z* (%): 527.2 (5.93) [$M^+ - 1$], 404.1 (0.36) [$M-C_6H_6NO_2$]$^+$, 230.3 (100) $C_{15}H_{14}NCl_2$.

*4.2.4. O-{4-[Bis(2-chloroethyl)amino]-L-phenylalanyl}-2-hydroxyethylmaleimide hydrochloride (**9**)*

8 (350 mg, 0.66 mmol) was dissolved in 6 mL of THF. To this solution was added 250 μL of hydrochloric acid (*w* = 25%) and the mixture was stirred for 20 h at room temperature. The solvent was removed under reduced pressure. The remained oil was dissolved in methanol and the product was precipitated with diethyl ether, filtered and dried, to afford 245 mg (80%) as a colorless solid; mp: 130 °C. ^{1}H NMR (CD_3OD): $\delta = 3.00$ (dd, ^3J(H,H) = 7.89 Hz, 14.59 Hz, 1H, CH_2Ar), 3.14 (dd, ^3J(H,H) = 5.22 Hz, 14.60 Hz, 1H, CH_2Ar), 3.69 (m, 4H, CH_2Cl), 3.78 (m, 4H + 2H, CH_2CH_2Cl + CH_2maleimide), 4.18 (m, 1H, CH), 4.38 (m, 1H, CH), 6.77 (d, ^3J(H,H) = 8.81 Hz, 2H, Ar*H*), 6.86 (s, 2H, $H_{maleimide}$), 7.11 (d, ^3J(H,H) = 8.75 Hz, 2H, Ar*H*); ^{13}C NMR (CD_3OD): $\delta = 37.55$ ($CH_2N_{maleimide}$), 39.67 (CH_2Ar), 41.07 (CH_2N), 42.6 (CH_2Cl), 53.7 (CH), 60.14 (CH_2O), 119.5 ($C_{aromatic}$), 132.50 ($C_{aromatic}$), 132.54 ($C_{aromatic}$), 132.7 ($C_{aromatic}$), 135.43 ($CH_{maleimide}$), 135.68 ($C_{aromatic}$), 170.33 ($CO_{maleimide}$), 172.3 (COO). MS (ESI): calcd. for $C_{19}H_{24}N_3O_4Cl_2^+$ 428.1138, found 428.1152.

*4.2.5. General procedure for the synthesis of the Boc-protected L-melphalan-spacer-diamines **10a–d** (mixture of optical isomers)*

8 and the respective diamine were dissolved in chloroform and 40 h refluxed. The solvent was removed *in vacuo* and the oily residue was purified by column chromatography (silica gel).

*4.2.5.1. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)propanoate)-3-yl]-1,6-diaminohexane (**10a**).* **8** (255.8 mg, 0.48 mmol), 1,6-diaminohexane (25.6 mg, 0.22 mmol), 10 mL of chloroform, column chromatography

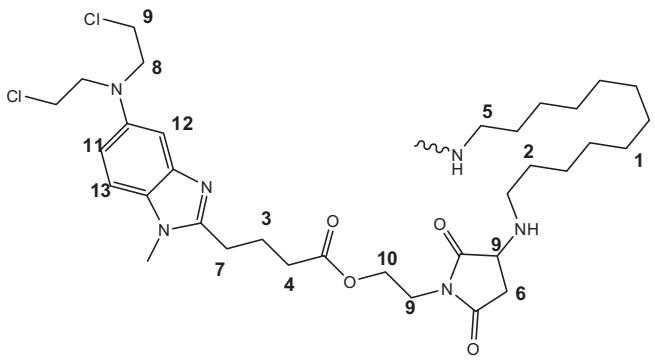


Chart 5. (Supporting scheme for NMR data of compound **5d**).

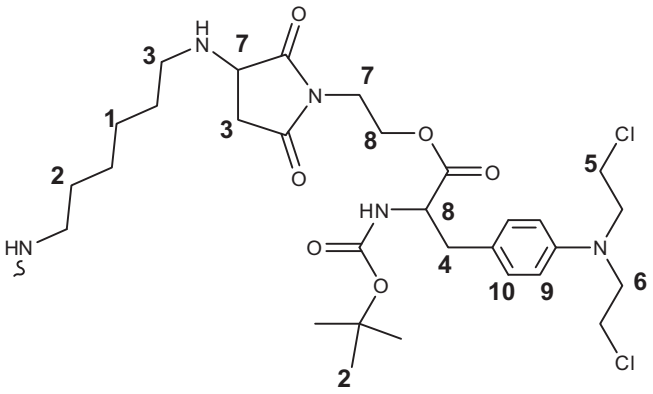


Chart 6. (Supporting scheme for NMR data of compound **10a**).

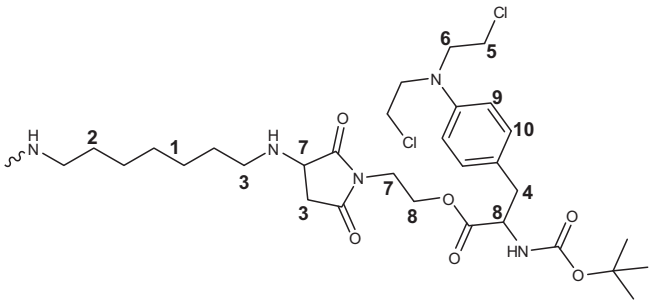


Chart 7. (Supporting scheme for NMR data of compound **10b**).

with chloroform/methanol = 20:1. Yield: 165 mg (64%) as a yellow oil: R_f = 0.45 (chloroform/methanol 20:1). ^{1}H NMR ($CDCl_3$): δ = 1.25 (s, 4H, **1** CH_2), 1.49 (s, 22H, **2** CH_2), 2.46–2.70 (m, 4H + 2H, **3** CH_2), 2.87–3.03 (m, 2H + 4H, **3** + **4** CH_2), 3.62 (t, ^3J(H,H) = 6.29 Hz, 8H, **5** CH_2), 3.69 (t, ^3J(H,H) = 6.35 Hz, 8H, **6** CH_2), 3.76 (m, 6H, **7** CH_2), 4.24 (m, 2H, **8** CH_2), 4.33 (m, 2H, **8** CH_2), 4.45 (m, 2H, **8** CH_2), 4.94 (m, 2H, NH), 6.60 (d, ^3J(H,H) = 8.63 Hz, 4H, **9** CH), 7.00 (d, ^3J(H,H) = 7.88 Hz, 4H, **10** CH). ^{13}C NMR ($CDCl_3$): δ = 26.89 (**1** CH_2), 28.33 (CH_3), 29.69 (**2** CH_2), 33.94 (**3** CH_2), 37.63 (**4** + **7** CH_2), 40.46 (**5** + **6** CH_2), 47.62 (**3** CH_2), 53.48 (**8** CH_2), 56.14 (**7** CH_2), 61.29 (**8** CH_2), 79.86 (C), 112.09 (**9** CH), 124.88 ($C_{aromatic}$), 130.56 ($C_{aromatic}$), 145.08 ($C_{aromatic}$), 155.15 (CO), 171.81 (CO). MS (ESI): calcd. for $C_{54}H_{79}N_8O_{12}Cl_4$ 1173.45422, found 1173.4615 (Chart 6).

*4.2.5.2. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)propanoate)-3-yl]-1,7-diaminoheptane (**10b**).* **8** (485 mg, 0.91 mmol), 1,7-diaminoheptane (60 mg, 0.45 mmol), 25 mL of chloroform, column chromatography, successive with dichloromethane/acetone = 6:1, 3:1, 1:1. Yield: 270 mg (49.72%) as a brown solid: R_f = 0.22 (dichloromethane/acetone 1:1); mp: 120 °C. ^{1}H NMR ($CDCl_3$): δ = 1.32 (s, 6H, **1** CH_2), 1.40 (s, 18H, **Boc** CH_3), 1.49 (s, 4H, **2** CH_2), 2.49–2.70 (m, 4H + 2H, **3** CH_2), 2.86–3.03 (m, 2H + 4H, **3** + **4** CH_2), 3.62 (t, ^3J(H,H) = 6.33 Hz, 8H, **5** CH_2), 3.69 (t, ^3J(H,H) = 6.49 Hz, 8H, **6** CH_2), 3.76 (m, 6H, **7** CH_2), 4.21 (m, 2H, **8** CH_2), 4.33 (m, 2H, **8** CH_2), 4.45 (m, 2H, **8** CH_2), 4.94 (m, 2H, NH), 6.60 (d, ^3J(H,H) = 8.62 Hz, 4H, **9** CH), 7.00 (d, ^3J(H,H) = 8.24 Hz, 4H, **10** CH). ^{13}C NMR ($CDCl_3$): δ = 28.33 (CH_2 + CH_3), 29.27 (CH_2), 36.76 (**3** CH_2), 37.71 (**4** + **7** CH_2), 40.46 (**5** + **6** CH_2), 47.68 (**3** CH_2), 53.48 (**8** CH_2), 54.49 (**7** CH_2), 61.22 (**8** CH_2), 79.86 (C), 112.09 ($C_{aromatic}$), 124.88 ($C_{aromatic}$), 130.56 ($C_{aromatic}$), 145.07 ($C_{aromatic}$), 155.2 (CO), 171.83 (CO). MS (ESI): calcd. for $C_{55}H_{81}N_8O_{12}Cl_4$ 1187.4705, found 1187.4730 (Chart 7).

*4.2.5.3. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)propanoate)-3-yl]-1,8-diaminooctane (**10c**).* **8** (485 mg, 0.91 mmol), 1,8-diaminooctane (66 mg, 0.45 mmol), 25 mL of chloroform, column chromatography with dichloromethane/acetone = 3:1 and 1:1. Yield: 360 mg (65.45%) as a brown solid: R_f = 0.22 (dichloromethane/acetone 1:1); mp: 110 °C. ^{1}H NMR ($CDCl_3$): δ = 1.30 (s, 8H, **1** CH_2), 1.40 (s, 18H, **Boc** CH_3), 1.49 (s, 4H, **2** CH_2), 2.58 (m, 4H, **3** CH_2), 2.69 (m, 2H, **4** CH_2), 2.86–3.03 (m, 2H + 4H, **4** + **5** CH_2), 3.62 (t, ^3J(H,H) = 6.33 Hz, 8H, **6** CH_2), 3.69 (t, ^3J(H,H) = 6.50 Hz, 8H, **7** CH_2), 3.76 (m, 6H, **7** CH_2), 4.24 (m, 2H, **9** CH_2), 4.35 (m, 2H, **9** CH_2), 4.45 (m, 2H, **9** CH_2), 4.94 (m, 2H, NH), 6.60 (d, ^3J(H,H) = 8.57 Hz, 4H, **10** CH), 7.00 (d, ^3J(H,H) = 8.05 Hz, 4H, **11** CH). ^{13}C NMR ($CDCl_3$): δ = 26.74 (CH_2), 28.33 (CH_2 + CH_3), 29.27 (CH_2), 31.74 (CH_2), 36.8 (**4** CH_2), 37.66 (**5** + **8** CH_2), 40.45 (**6** + **7** CH_2), 47.84 (**3** CH_2), 53.48 (**9** CH_2), 54.49 (**8** CH_2), 61.26 (**9** CH_2), 79.86 (C), 112.08 ($C_{aromatic}$), 124.84 ($C_{aromatic}$), 130.56 ($C_{aromatic}$), 145.07 ($C_{aromatic}$), 155.16 (CO), 171.82 (CO). MS (ESI): calcd. for $C_{56}H_{83}N_8O_{12}Cl_4$ 1201.4862, found 1201.4890 (Chart 8).

*4.2.5.4. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(tert-butoxycarbonylamino)propanoate)-3-yl]-1,12-diaminododecane (**10d**).* **8** (150 mg, 0.28 mmol), 1,12-diaminododecane (25.8 mg, 0.13 mmol), 5 mL of chloroform, column chromatography with dichloromethane/acetone = 6:1 and 3:1. Yield: 114 mg (70%) as a yellow oil: R_f = 0.64 (dichloromethane/acetone 3:1). ^{1}H NMR ($CDCl_3$): δ = 1.39 (s, 16H, **1** CH_2), 1.33 (s, 18H, **2** CH_2), 1.41 (quin, ^3J(H,H) = 7.04 Hz, 4H, **3** CH_2), 2.40–2.64 (m, 4H + 2H, **4** CH_2), 2.80–2.96 (m, 2H + 4H, **5** + **4** CH_2), 3.54 (t, ^3J(H,H) = 6.27 Hz, 8H, **6** CH_2), 3.61 (t, ^3J(H,H) = 6.47 Hz, 8H, **7** CH_2), 3.69 (m, 6H, **8** CH_2), 4.16 (m, 2H, **9** CH_2), 4.26 (m, 2H, **9** CH_2), 4.37 (m, 2H, **9** CH_2), 4.89 (m, 2H, NH), 6.54 (d, ^3J(H,H) = 8.69 Hz, 4H, **10** CH), 6.93 (d, ^3J(H,H) = 7.26 Hz, 4H, **11** CH). ^{13}C NMR ($CDCl_3$): δ = 26.8 (CH_2), 28.6 (CH_3), 29.27 (**1** CH_2), 31.74 (**3** CH_2), 33.44 (**4** CH_2), 37.66 (**5** + **8** CH_2), 41.35 (**6** + **7** CH_2), 47.10 (**4** CH_2), 54.98 (**9** CH_2), 58.49 (**8** CH_2), 62.26 (**9** CH_2), 80.01 (C), 112.08 (**10** CH), 125.84 ($C_{aromatic}$), 130.47 ($C_{aromatic}$), 145.87 ($C_{aromatic}$), 156.16 (CO), 172.82 (CO). MS (ESI): calcd for $C_{60}H_{91}N_8O_{12}Cl_4$ 1257.54963, found 1257.5461 (Chart 9).

*4.2.6. General procedure for the synthesis of the deprotected L-melphalan-spacer-diamines **11a–d** (mixture of optical isomers)*

10a–d were respectively dissolved in THF. To this solution hydrochloric acid (w = 25%) was added and the mixture was stirred for 3 h at room temperature. The reaction was continuously monitored by TLC. The solvent was then removed under reduced pressure to afford the deprotected product.

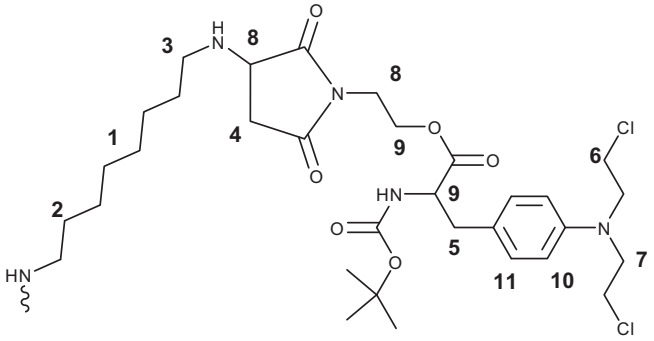


Chart 8. (Supporting scheme for NMR data of compound **10c**).

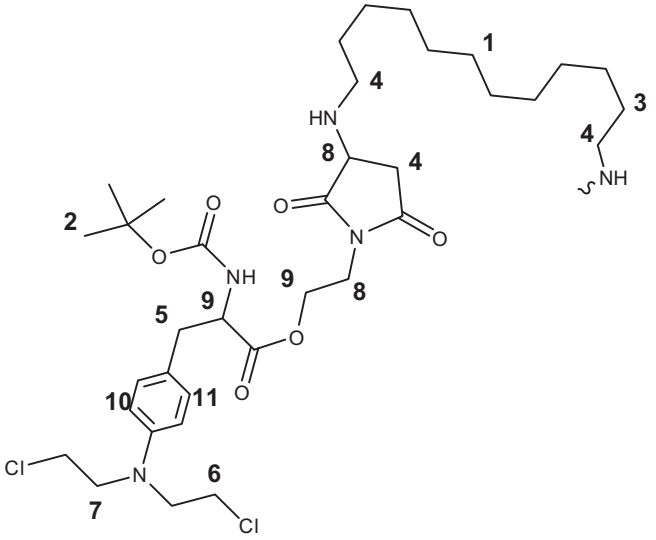


Chart 9. (Supporting scheme for NMR data of compound **10d**).

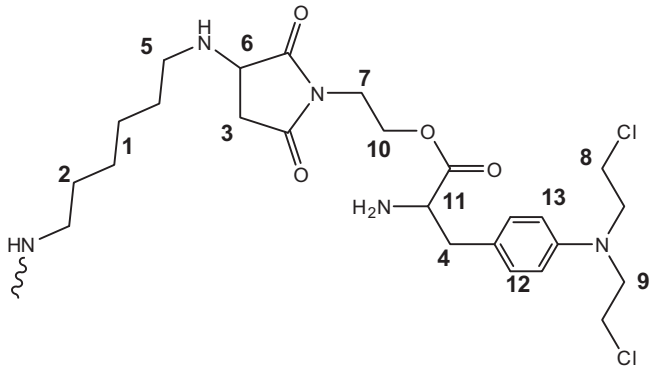


Chart 10. (Supporting scheme for NMR data of compound **11a**).

*4.2.6.1. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-aminopropanoate)-3-yl]-1,6-diaminohexane dihydrochloride (**11a**).* **10a** (550 mg, 0.46 mmol), 40 mL of THF, 5.12 mL of hydrochloric acid. Yield: 450 mg (92%) as green oil after lyophilization from water. ^{1}H NMR (CD_3OD): δ = 1.51 (s, br, 4H, **1** CH_2), 1.79 (m, 4H, **2** CH_2), 2.97 (dd, ^3J(H,H) = 5.43 Hz, 17.97 Hz, 2H, **3** CH_2), 3.07–3.12 (m, 4H, **3** + **4** CH_2), 3.17–3.25 (m, 2H + 4H, **4** + **5** CH_2), 3.64–3.71 (m, 2H + 4H+8H, **6** + **7**+**8** CH_2), 3.83 (t, ^3J(H,H) = 6.68 Hz, 8H, **9** CH_2), 4.19 (t, ^3J(H,H) = 5.43 Hz, 1H, **11** CH), 4.29 (m, 1H, **11** CH), 4.40 (m, 2H, **10** CH_2), 4.49 (m, 2H, **10** CH_2), 6.96 (d, ^3J(H,H) = 8.38 Hz, 4H, **12** CH), 7.24 (d, ^3J(H,H) = 8.46 Hz, 4H, **13** CH). ^{13}C NMR (CD_3OD): δ = 25.52 (**1** CH_2), 31.86 (**2** CH_2), 35.07 (**3** CH_2), 38.67 (**4** + **7** CH_2), 41.32 (**8** + **9** CH_2), 46.24 (**5** CH_2), 53.82 (**11** CH), 55.37 (**6** CH), 57.74 (**10** CH_2), 116.70 (**13** CH), 130.81 (**12** CH), 130.93 ($C_{aromatic}$), 141.54 ($C_{aromatic}$), 169.71 (COO), 172.74 ($CO_{succinimide}$), 172.89 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{44}H_{63}N_8O_8Cl_4$ 973.3495, found 973.3498 (Chart 10).

*4.2.6.2. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-aminopropanoate)-3-yl]-1,7-diaminoheptane dihydrochloride (**11b**).* **10b** (290 mg, 0.24 mmol), 20 mL of THF, 2.67 mL of hydrochloric acid. Yield: 240 mg (93%) as a brown solid after lyophilization from water; mp: 213 °C. ^{1}H NMR (CD_3OD): δ = 1.46 (s, br, 6H, **1** CH_2), 1.76 (m, 4H, **2** CH_2), 2.95 (dd, ^3J(H,H) = 5.59 Hz, 17.98 Hz, 2H, **3** CH_2), 3.04–3.12 (m, 2H, **3** CH_2), 3.15–3.25 (m, 4H + 4H, **4** + **5** CH_2), 3.64–3.70 (m, 2H + 4H + 8H, **6** + **7** + **8** CH_2), 3.83 (t, ^3J(H,H) = 6.65 Hz, 8H, **9** CH_2), 4.17 (m, 1H, **11** CH), 4.27 (m, 1H, **11** CH), 4.40 (m, 2H, **10** CH_2), 4.47 (m, 2H, **10** CH_2), 6.86 (d, ^3J(H,H) = 8.59 Hz, 4H, **12** CH), 7.20 (d, ^3J(H,H) = 8.61 Hz, 4H, **13** CH). ^{13}C NMR (CD_3OD): δ = 25.65 (**1** CH_2), 27.86 (CH_2), 31.87 (**2** CH_2), 35.07 (**3** CH_2), 37.65 (**7** CH_2), 39.00 (**4** CH_2), 41.34 (**8** + **9** CH_2), 46.45 (**5** CH_2), 53.82 (**11** CH), 54.89 (**6** CH), 57.76 (**10** CH_2), 115.83 (**12** CH), 130.70 (**13** CH), 130.81 ($C_{aromatic}$), 142.40 ($C_{aromatic}$), 169.76 (COO), 172.28 ($CO_{succinimide}$), 172.89 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{45}H_{65}N_8O_8Cl_4$ 987.3652, found 987.3879 (Chart 11).

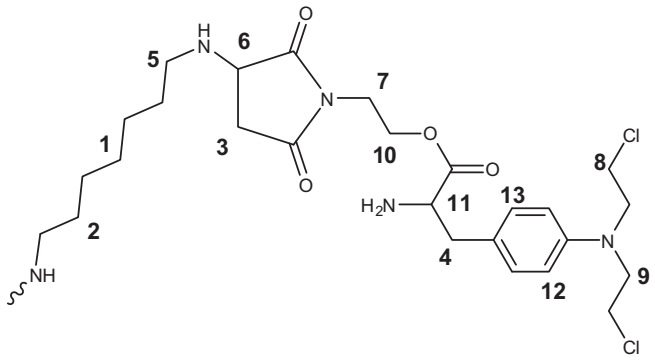


Chart 11. (Supporting scheme for NMR data of compound **11b**).

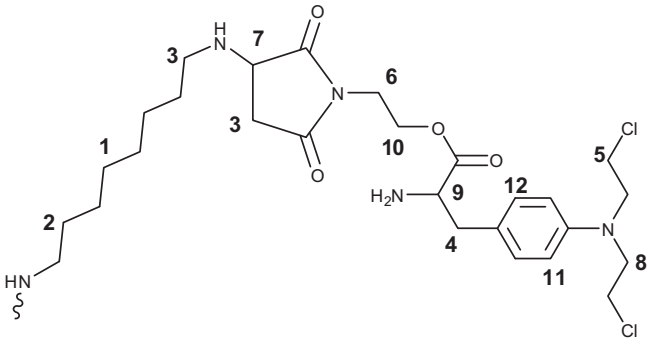


Chart 12. (Supporting scheme for NMR data of compound **11c**).

*4.2.6.3. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-aminopropanoate)-3-yl]-1,8-diaminooctane dihydrochloride (**11c**).* **10c** (330 mg, 0.27 mmol), 25 mL of THF, 3 mL of hydrochloric acid. Yield: 279 mg (95%) as a brown solid after lyophilization from water; mp: 129 °C. ^{1}H NMR (CD_3OD): δ = 1.43 (s, br, 8H, **1** CH_2), 1.75 (m, 4H, **2** CH_2), 2.91–3.24 (m, 4H + 4H + 2H, **3** + **4** CH_2), 3.36 (m, 2H, **4** CH_2), 3.67 (m, 8H + 6H + 2H, **5** + **6** + **7** CH_2), 3.83 (m, 8H + 2H, **8** + **9** CH_2), 4.40 (m, 2H, **10** CH_2), 4.47 (m, 2H, **10** CH_2), 6.78 (d, ^3J(H,H) = 8.55 Hz, 4H, **11** CH), 7.16 (d, ^3J(H,H) = 8.55 Hz, 4H, **12** CH). ^{13}C NMR (CD_3OD): δ = 25.81 (**1** CH_2), 28.23 (CH_2), 31.85 (**2** CH_2), 35.05 (**3** CH_2), 38.72 (**6** CH_2), 38.85 (**4** CH_2), 41.29 (**5** + **8** CH_2), 46.47 (**3** CH_2), 53.79 (**9** CH), 55.41 (**7** CH), 57.71 (**10** CH_2), 116.66 (**11** CH), 130.83 (**12** CH), 130.95 ($C_{aromatic}$), 141.59 ($C_{aromatic}$), 169.73 (COO), 172.27 ($CO_{succinimide}$), 172.93 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{46}H_{67}N_8O_8Cl_4$ 1001.3809, found 1001.4197 (Chart 12).

*4.2.6.4. N,N′-Bis[(2-(2,5-dioxopyrrolidin-1-yl)ethyl-3-(4-(bis(2-chloroethyl)amino)phenyl)-2-aminopropanoate)-3-yl]-1,12-diaminododecane dihydrochloride (**11d**).* **10d** (280 mg, 0.22 mmol), 20 mL of THF, 2.43 mL of hydrochloric acid. Yield: 245 mg (98%) as a brown solid after lyophilization from water; mp: 194–196 °C. ^{1}H NMR (CD_3OD): δ = 1.32 (s, 16H, **1** CH_2), 1.77 (m, 4H, **2** CH_2), 3.05 (m, 2H, **3**

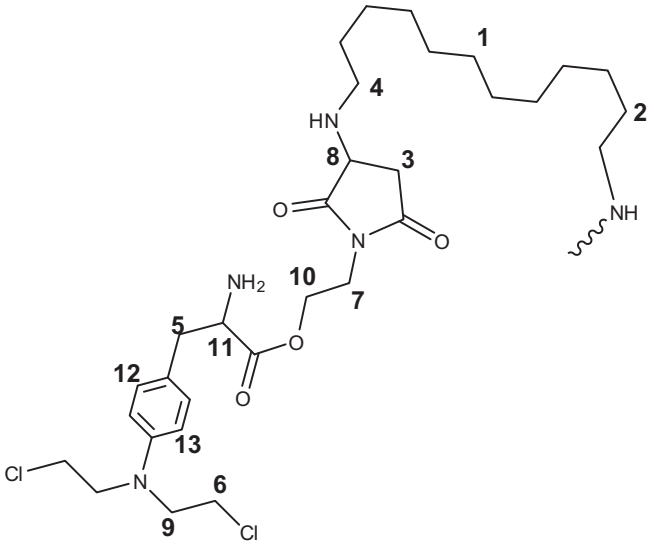


Chart 13. (Supporting scheme for NMR data of compound **11d**).

CH_2), 3.17 (m, 2H, **3** CH_2), 3.30 (s, 4H, **4** CH_2), 3.38 (m, 4H, **5** CH_2), 3.68 (m, 8H + 4H, **6** + **7** CH_2), 3.87 (m, 2H, **8** CH), 4.07 (m, 8H, **9** CH_2), 4.34 (m, 2H, **10** CH_2), 4.43 (m, 2H, **10** CH_2), 4.55 (m, 1H, **11** CH), 4.64 (m, 1H, **11** CH), 7.56 (m, 4H, **12** CH), 7.61 (m, 4H, **13** CH). ^{13}C NMR (CD_3OD): $\delta = 27.50$ (CH_2), 30.15 (CH_2), 30.41 (**2** CH_2), 33.24 (**3** CH_2), 36.24 (**7** CH_2), 36.50 (**5** CH_2), 40.91 (**9** CH_2), 42.73 (**6** CH_2), 48.00 (**4** CH_2), 53.67 (**11** CH), 55.24 (**8** CH), 59.17 (**10** CH_2), 116.26 (**13** CH), 131.99 ($C_{aromatic}$), 132.09 ($C_{aromatic}$), 145.09 ($C_{aromatic}$), 170.50 (COO), 173.67 ($CO_{succinimide}$), 174.15 ($CO_{succinimide}$). MS (ESI): calcd. for $C_{50}H_{74}N_8O_8Cl_4$ 1057.4436, found 1057.4456 (Chart 13).

4.3. Biological methods

4.3.1. Cell culture

The human MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). The MCF-7 breast cancer cell line originated from a 69-year-old Caucasian woman and is a well-characterized estrogen receptor (ER) positive control cell line (cells are positive for cytoplasmic estrogen receptors). The human cell line MDA-MB-231 is a prototype for the study of hormone-independent breast cancer. Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay [48]. Both cell lines were maintained in L-glutamine and sodium pyruvate containing DMEM High Glucose (4.5 g/L) supplemented with 5% fetal calf serum (FCS, Gibco) using 25 cm^2 culture flasks in a humidified atmosphere (5% CO_2) at 37 °C. The cell lines were passaged weekly after treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (EDTA, 0.02%, Boehringer). Mycoplasma contamination was regularly monitored and only mycoplasma-free cultures were used.

4.3.2. In vitro chemosensitivity assays

Briefly, 100 μL of a cell suspension of 7500 cells mL^{-1} culture medium were plated into each well of a 96-well microtiter plate and incubated at 37 °C for 3 days in a humidified atmosphere (5% CO_2). By adding an adequate volume of a stock solution of the appropriate compound (solvent: DMF or methanol) to the medium, the desired test concentration was obtained. After the proper incubation time (0, 48, 72, 96, 120, 144 h) the medium was removed, and the cells were fixed with a glutardialdehyde solution and stored under phosphate-buffered saline (PBS) at 4 °C. Cell biomass was determined by a crystal violet staining technique described previously [49,50]. The *in vitro* cytotoxicity test was performed 3 times for each substance. In the diagrams, the vertical error bars on data points represent the standard error of the mean.

The efficiency of the compounds is expressed as corrected %T/C_{corr} values according to the following equations:

$$\text{Cytostatic effect} : \%T/C_{corr} = [(T - C_0)/(C - C_0)]100 \quad (1)$$

$$\text{Cytocidal effect} : \%\tau = [(T - C_0)/C_0]\,100 \quad (2)$$

in which T (test) and C (control) are the optical density values at 578 nm of the crystal violet extract of the cells in the wells (that is the chromatin-bound crystal violet extracted with 70% ethanol), and C_0 is the density of the cell extract immediately before treatment. A microplate reader at 590 nm (Flashscan Analytik Jena AG) was used for the automatic estimation of the optical density of the crystal violet extract in the wells. The calculated %T/C values can be interpreted as follows:

$T/C_{corr} > 80\%$: no antiproliferative effect;
$80\% > T/C_{corr} > 20\%$: antiproliferative effect;
$20\% > T/C_{corr} > 0\%$: cytostatic effect;
$\tau = T/C_{corr} < 0\%$: cytocidal effect.

Acknowledgments

The presented study was supported by grants Gu285/4-1, Gu285/5-1 and Gu285/5-2 as well as the SFB 765 from the Deutsche Forschungsgemeinschaft.

Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.02.008.

References

[1] W. Ozegowski, D. Krebs, J. Prakt. Chem. 20 (1963) 178–186.
[2] B.D. Cheson, M.J. Rummel, J. Clin. Oncol. 27 (2009) 1492–1501.
[3] K.U. Chow, S. Boehrer, K. Geduldig, A. Krapohl, D. Hoelzer, P.S. Mitrou, E. Weidmann, Haematologica 86 (2001) 485–493.
[4] A. Forero-Torres, M.N. Saleh, Clin. Lymphoma Myeloma 8 (2007) 13–17.
[5] S. Lonial, Clin. Lymphoma Myeloma 8 (2007) 18–23.
[6] G.L. Plosker, N.J. Carter, Drugs 68 (2008) 2645–2660.
[7] W. Ponisch, M. Rozanski, H. Goldschmidt, F.A. Hoffmann, T. Boldt, A. Schwarzer, U. Ritter, R. Rohrberg, E. Schwalbe, J. Uhlig, T. Zehrfeld, V. Schirmer, A. Haas, U. Kreibich, D. Niederwieser, Biochem. Pharmacol. 143 (2008) 191–200.
[8] D. Schrijvers, J.B. Vermorken, Semin. Oncol. 29 (2002) 15–18.
[9] E. Weidmann, S.Z. Kim, A. Rost, H. Schuppert, G. Seipelt, D. Hoelzer, P.S. Mitrou, Ann. Oncol. 13 (2002) 1285–1289.
[10] M.A. Bergmann, M.E. Goebeler, M. Herold, B. Emmerich, M. Wilhelm, C. Ruelfs, L. Boening, M.J. Hallek, Haematologica 90 (2005) 1357–1364.
[11] A. Ferrajoli, Haematologica 90 (2005) 1300A.
[12] H. Koppler, J. Heymanns, A. Pandorf, R. Weide, Leuk. Lymphoma 45 (2004) 911–913.
[13] M.J. Keating, C. Bach, U. Yasothan, P. Kirkpatrick, Nat. Rev. Drug Discov. 7 (2008) 473–474.
[14] M.H.R. Eichbaum, F. Schuetz, T. Khbeis, I. Lauschner, F. Foerster, C. Sohn, A. Schneeweiss, Anti-Cancer Drugs 18 (2007) 963–968.
[15] K. Hoffken, K. Merkle, M. Schoenfelder, G. Anger, M. Brandtner, K. Ridwelski, S. Seeber, J. Cancer Res. Clin. Oncol. 124 (1998) 627–632.
[16] U. Reichmann, C. Bokemeyer, D. Wallwiener, M. Bamberg, J. Huober, Ann. Oncol. 18 (2007) 1981–1984.
[17] R.L. Furner, R.K. Brown, Cancer Treat. Rep. 64 (1980) 559–574.
[18] R.B. Jones, Cancer Treat. Res. 112 (2002) 305–322.
[19] J.A.B. Balfour, K.L. Goa, Drugs 61 (2001) 631–638.
[20] S. Blumel, A. Goodrich, C. Martin, N.H. Dang, Clin. J. Oncol. Nurs. 12 (2008) 799–806.
[21] S.M. Konstantinov, A. Kostovski, M. Topashka-Ancheva, M. Genova, M.R. Berger, J. Cancer Res. Clin. Oncol. 128 (2002) 271–278.
[22] M. Wickstroem, H. Loevborg, J. Gullbo, Lett. Drug Des. Discov. 3 (2006) 695–703.
[23] F. Bergel, J.A. Stock, J. Chem. Soc. (1960) 3658–3669.
[24] L. Kupczyk Subotkowska, K. Tamura, D. Pal, T. Sakaeda, T.J. Siahaan, V.J. Stella, R.T. Borchardt, J. Drug Target. 4 (1997) 359–370.
[25] U. Beyer, T. Roth, P. Schumacher, G. Maier, A. Unold, A.W. Frahm, H.H. Fiebig, C. Unger, F. Kratz, J. Med. Chem. 41 (1998) 2701–2708.
[26] F. Kratz, U. Beyer, T. Roth, M.T. Schuette, A. Unold, H.H. Fiebig, C. Unger, Arch. Pharm. 331 (1998) 47–53.
[27] Y. Matsumura, H. Maeda, Cancer Res. 46 (1986) 6387–6392.
[28] H. Sinn, H.H. Schrenk, E.A. Friedrich, U. Schilling, W. Maierborst, Nucl. Med. Biol. 17 (1990) 819–827.
[29] T. Kapp, A. Dullin, R. Gust, Bioconjug. Chem. 21 (2010) 328–337.
[30] A.M. Scutaru, M. Wenzel, H. Scheffler, G. Wolber, R. Gust, Bioconjug. Chem. 21 (2010) 1728–1743.
[31] T. Kapp, A. Dullin, R. Gust, J. Med. Chem. 49 (2006) 1182–1190.
[32] S. Okumoto, S. Yamabe, J. Org. Chem. 65 (2000) 1544–1548.
[33] I. Giraud, M. Rapp, J.C. Maurizis, J.C. Madelmont, J. Med. Chem. 45 (2002) 2116–2119.
[34] B. Neises, W. Steglich, Angew. Chem. Int. Edit. 17 (1978) 522–524.
[35] K. Bremer, W. Roth, Tumordiag. Ther. 17 (1996) 1–6.
[36] E.H.L. Chun, L. Gonzales, F.S. Lewis, J. Jones, R.J. Rutman, Cancer Res. 29 (1969) 1184–1194.
[37] I.G. Walker, B.D. Reid, Cancer Res. 31 (1971) 510–515.
[38] S.Y. Chang, D.S. Alberts, D. Farquhar, L.R. Melnick, P.D. Walson, S.E. Salmon, J. Pharm. Sci. 67 (1978) 682–684.
[39] R. Gust, R. Krauser, Monatsh. Chem. 128 (1997) 291–299.
[40] W. Werner, G. Letsch, W. Ihn, Pharmazie 42 (1987) 272–273.
[41] B.A. Teicher, Cancer Principles and Practice of Oncology (1997) 405–418.
[42] M. Wickstroem, K. Viktorsson, L. Lundholm, R. Aesoy, H. Nygren, L. Sooman, M. Fryknas, L.K. Vogel, R. Lewensohn, R. Larsson, J. Gullbo, Biochem. Pharmacol. 79 (2010) 1281–1290.
[43] S. Fuchs, T. Kapp, H. Otto, T. Schoeneberg, P. Franke, R. Gust, A.D. Schlueter, Chem.-Eur. J. 10 (2004) 1167–1192.
[44] W.J. Hopwood, J.A. Stock, Chem. Biol. Interact. 4 (1971) 31–39.

[45] N. Farrell, Y. Qu, M.P. Hacker, J. Med. Chem. 33 (1990) 2179–2184.
[46] T. Kapp, S. Mueller, R. Gust, ChemMedChem 1 (2006) 560–564.
[47] F. Bergel, J.A. Stock, J. Chem. Soc. (1954) 2409–2417.
[48] R.J. Hay, Anal. Biochem. 171 (1988) 225–237.
[49] G. Bernhardt, H. Reile, H. Birnbock, T. Spruss, H. Schoenenberger, J. Cancer Res. Clin. Oncol. 118 (1992) 35–43.
[50] H. Reile, H. Birnboeck, G. Bernhardt, T. Spruss, H. Schoenenberger, Anal. Biochem. 187 (1990) 262–267.