Received: 29 September 2020 | Revised: 11 November 2020 | Accepted: 13 November 2020

DOI: 10.1002/ardp.202000366

FULL PAPER

ARCH PHARM DPhG
Archiv der Pharmazie

Derivatives of nitrogen mustard anticancer agents with improved cytotoxicity

Frauke Antoni | Günther Bernhardt

Institute of Pharmacy, University of Regensburg, Regensburg, Germany

Correspondence
Frauke Antoni, Institute of Pharmacy, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany.
Email: frauke.antoni@ur.de

Abstract

In previous studies, we demonstrated that esters of bendamustine containing a basic moiety are far more cytotoxic anticancer agents than their parent compound and that the substitution of the labile ester moiety by a branched ester or an amide markedly increases stability in the blood plasma. In the current study, we showed that this substitution was bioisosteric. Aiming at increased cytotoxicity, we introduced the same modification to related nitrogen mustards: 6-isobendamustine, chlorambucil, and melphalan. The synthesis was accomplished using the coupling reagents *N*,*N*′-dicyclohexylcarbodiimide or 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate. Cytotoxicity against a panel of diverse cancer cells (carcinoma, sarcoma, and malignant melanoma) was assessed in a kinetic chemosensitivity assay. The target compounds showed cytotoxic or cytocidal effects at concentrations above 1 µM: a striking enhancement over bendamustine and 6-isobendamustine, both ineffective against the selected cancer cells at concentrations up to 50 µM, and a considerable improvement over chlorambucil, showing some potency only against the sarcoma cells. Melphalan was almost as effective as the target compounds—derivatization only provided a small improvement. The novel cytostatics are of interest as model compounds for analyzing a correlation between cytotoxicity and membrane transport and for the treatment of malignancies.

KEYWORDS
bendamustine, chlorambucil, derivatives, melphalan, nitrogen mustards

1 | INTRODUCTION

Cancer is the second leading cause of death worldwide.[1] Surgery and radiation are local therapies and cannot eradicate metastatic cancer, where every organ in the body needs to be reached.[2] By contrast, chemotherapy works systemically and is the most effective treatment for disseminated tumors.[2,3] The era of chemotherapy began in 1942 with the discovery of nitrogen mustards—originally produced as chemical warfare agents—as a remedy for cancer.[2,4] A basic chemical reaction underlies the mechanism of action of

Abbreviations: Boc, *tert*-butyloxycarbonyl; CLL, chronic lymphocytic leukemia; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, dimethyformamide; DMSO, dimethylsulfoxide; FCS, fetal calf serum; HL, Hodgkin lymphoma; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; MeCN, acetonitrile; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

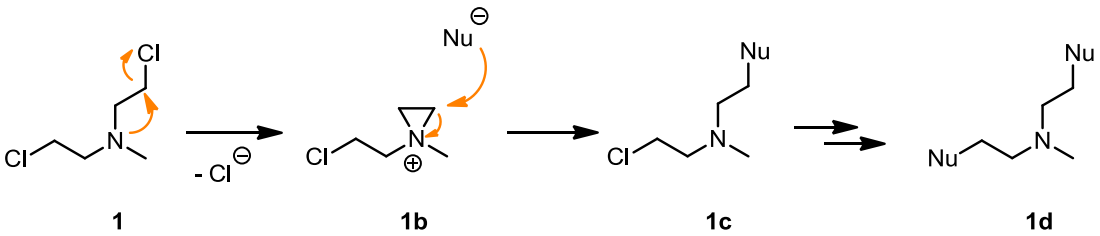


FIGURE 1 The mechanism of the reaction of nitrogen mustards with nucleophiles (Nu^-), here shown for mechlorethamine (**1**)

nitrogen mustards, namely the intramolecular cyclization in a polar solvent to form an aziridnium cation, which reacts readily with bio-nucleophiles (Figure 1), for example nitrogen in the DNA. This reaction leads to the formation of DNA interstrand crosslinks, which prevents cell replication and ultimately causes cell death.[4,5]

Inspired by this success, further, improved DNA-alkylating agents of the nitrogen mustard type were developed as chemotherapeutics, such as chlorambucil, melphalan, or bendamustine.

Chlorambucil (**2**) (Figure 2) was first synthesized by Everett et al.[6] in 1953. The nitrogen mustard moiety (i.e., the bis(2-chloroethyl)amino moiety, also called N-lost moiety) was attached to a phenyl ring, which withdraws electrons from the nitrogen atom, thereby disfavoring aziridinium ion formation, which renders the nitrogen mustard moiety less reactive toward the nucleophilic attack.[7] Therefore, chlorambucil and other aromatic analogs are sufficiently deactivated so that they can reach their target DNA sites before being degraded by reacting with collateral nucleophiles, resulting in reduced toxic side effects and allowing oral administration.[7] The butanoic acid side chain makes the compound hydrophilic.[8] Chlorambucil is sold, among others, under the brand name of Leukeran and is indicated in the treatment of chronic lymphocytic leukemia (CLL), Hodgkin and non-Hodgkin lymphomas (HL and NHL), as well as breast and ovarian carcinomas.[5,9,10]

Melphalan (**3**) (Figure 2) differs from chlorambucil in the length of the alkanoic acid side chain and in the amino group attached to the latter—the amino acid L-phenylalanine is the "carrier" of the N-lost moiety. Melphalan was first synthesized in 1954 by Bergel et al.,[11] aiming at increased tumor selectivity. Indeed, melphalan is imported by amino acid transporters[12] whose expression is upregulated in cancer cells.[13] Melphalan is used for treating various malignancies including multiple myeloma (MM), ovarian cancer, breast cancer, and melanoma, and is sold, among others, under the trade name of Alkeran.[14,15]

Bendamustine (**4**) (Figure 3) was initially synthesized in 1963 by Ozegowski et al.[16,17] in the former German Democratic Republic

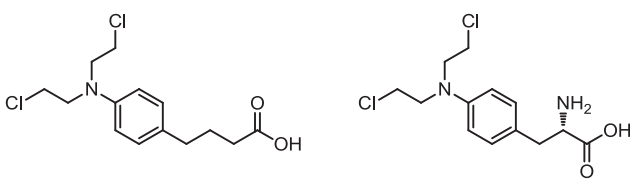


FIGURE 2 Structures of the nitrogen mustard anticancer agents chlorambucil (**2**) and melphalan (**3**)

and was introduced into the market there.[18,19] It distinguishes itself from chlorambucil in the central benzimidazole ring, which is unique to bendamustine and was intended to include antimetabolite properties, which, however, have not yet been confirmed.[17,18] Nevertheless, bendamustine displays a unique mechanism of action, as it inhibits mitotic checkpoints, causes inefficient DNA repair, and induces the expression of the protein p53, a tumor suppressor, which initiates apoptosis.[20,21] Bendamustine was approved in the Federal Republic of Germany after the iron curtain had fallen, and today it is sold, among others, under the brand name of Ribomustin for CLL, indolent NHL, and MM.[19,22] In the United States of America, the drug is marketed, among others, under the brand name of Treanda and is approved for CLL and indolent NHL.[18,19] The assets of bendamustine are a favorable side-effect profile,[18] the lack of cross-resistances, with many other alkylating agents,[19] and its superiority to chlorambucil in previously untreated patients with CLL.[22]

Bendamustine is usually dosed intravenously; however, there have been a couple of patent applications aiming at formulations for oral administration.[23,24] Esterification of the carboxylic acid moiety may increase the chances of oral application and, furthermore, has been reported to enhance the hydrolytic stability of the N-lost moiety (for different reasons).[25–27] The increasing interest in esters of bendamustine prompted our working group to perform a study on the pharmacological properties of the latter. It was revealed that esters of bendamustine are by far more potent cytotoxic agents than the parent compound, especially esters comprising basic moieties, which are charged under physiological conditions, for example, the 2-pyrrolidinoethyl ester **5**[28] (Figure 3). The basic esters show a pronounced cellular accumulation for reasons not yet identified, but transport proteins may be involved.[28] However, the basic esters turned out to be especially prone to hydrolysis at the ester bond in the blood plasma, which was attributed to their similarity to substrates of unspecific cholinesterases.[29] We were able to overcome this obstacle by substituting the linear ester moiety in **5** by a branched ester (compound **6**), which increased the stability to an acceptable level,[29] and so did the replacement of the ester by an amide bond (compound **7**; unpublished data). Bendamustine, its derivatives **5**–**7**, and their respective half-lives in the human blood plasma are depicted in Figure 3. The decomposition of bendamustine is due to the hydrolysis of the carbon–chlorine bonds of the N-lost moiety.

The preparation of **7** and the cytotoxicity analysis of **6** and **7** have not yet been reported, which are a part of the present study.

As other anticancer agents are structurally very similar to bendamustine, it is conceivable that these can be improved by the same

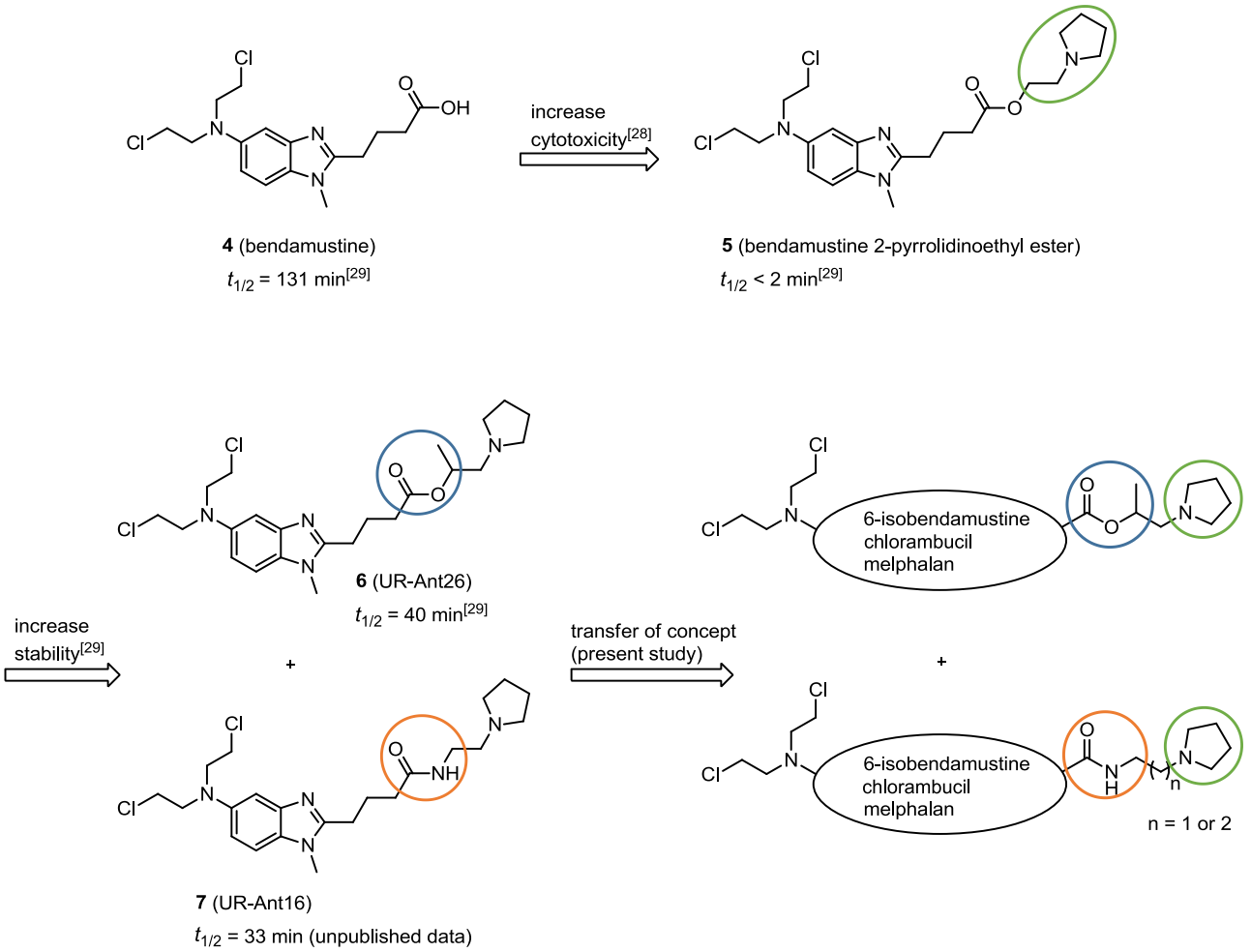


FIGURE 3 Structures of the nitrogen mustard anticancer agent bendamustine (**4**) and its derivatives **5**, **6**, and **7**, as well as their respective half-lives in human blood plasma, and the rationale of the present study

modification. Aiming at increased cytotoxicity, the present study comprises the synthesis and cytotoxicity analysis of basic derivatives of further members of the nitrogen mustard family, especially chlorambucil and melphalan. In addition, we considered an isomer of bendamustine (6-isobendamustine), which we recently described in a patent application.[30,31] Cytotoxicity analyses of 6-isobendamustine are still pending, and this compound serves as another example of increasing the cytotoxicity of nitrogen mustards by derivatization. A pyrrolidinoalkyl chain was introduced into the structure of 6-isobendamustine, chlorambucil, and melphalan via amide or branched ester formation (Figure 3), as these moieties previously proved superior to a linear ester group in terms of stability in the blood plasma.[29]

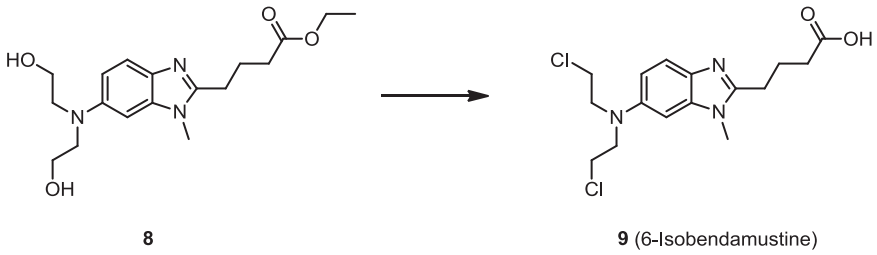
2 | RESULTS AND DISCUSSION

2.1 | Synthesis

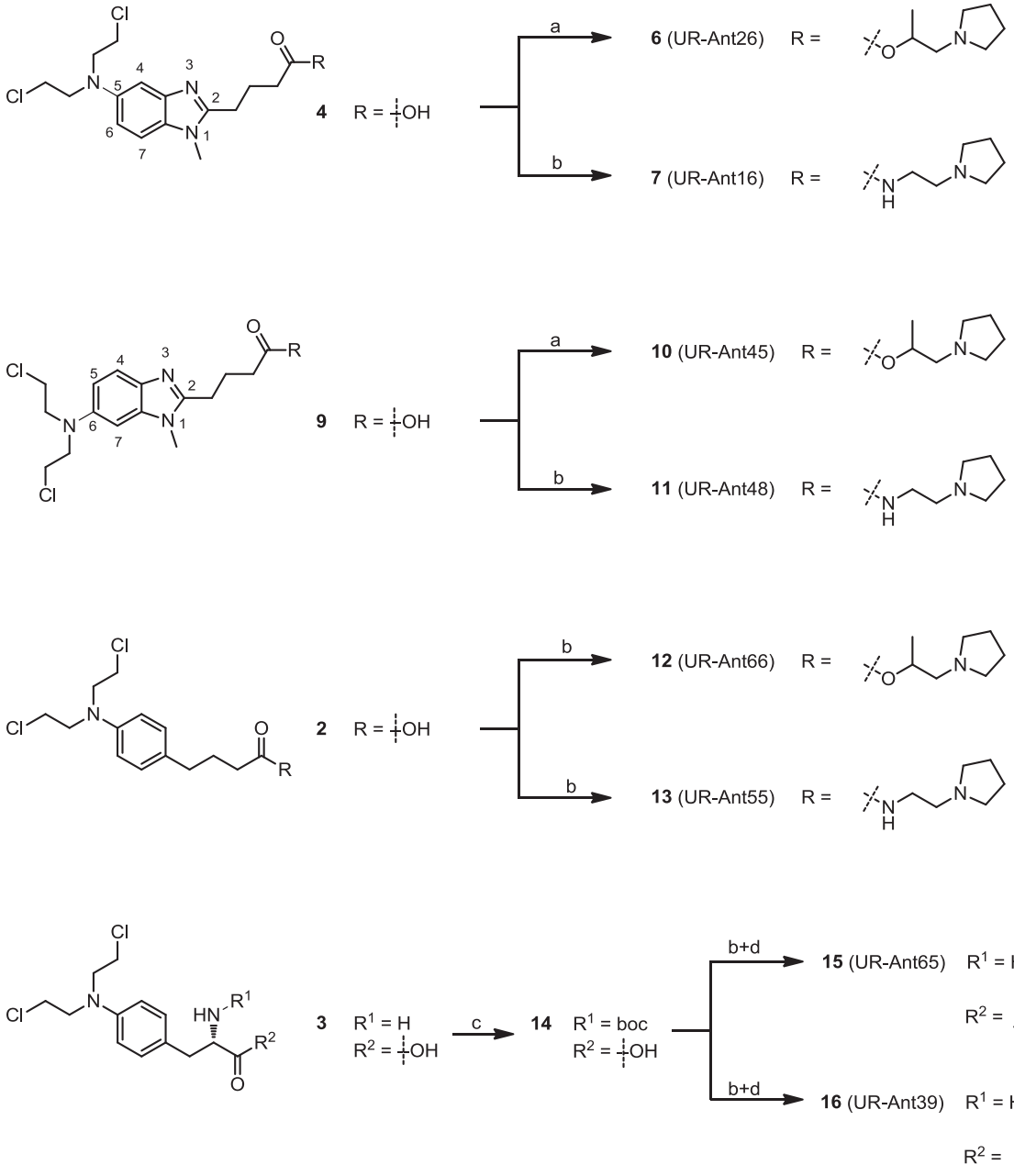
The anticancer agents bendamustine, chlorambucil, and melphalan are marketed drugs. 6-Isobendamustine (**9**)[30,31] (here, isobendamustine), which, in contrast to conventional bendamustine, bears the N-lost moiety in position-6 (instead of position-5) of the benzimidazole ring, is not commercially available. The latter was prepared from 4-(1-methyl-6-bis(2-hydroxyethyl)aminobenzimid azol-2-yl)butyric acid ethyl ester (**8**), which we received as a kind gift from Gemini PharmChem. Compound **8** was first treated with the chlorinating agent thionyl chloride and then with hydrochloric acid to hydrolyze the ester bond (Scheme 1).

The esters and amides were prepared from their respective parent compounds and a pyrrolidinoalkyl alcohol or amine. The bendamustine ester **6** was synthesized with the help of the coupling reagent *N*,*N*′-dicyclohexylcarbodiimide (DCC), as we described previously[29]; for the preparation of the novel bendamustine amide **7**, the coupling reagents 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) and *N*,*N*-diisopropylethylamine (DIPEA) were used (Scheme 2). Isobendamustine was converted to the ester **10** and the amide **11** in the same way as compounds **6** and **7**, respectively (Scheme 2). The chlorambucil ester **12** could not be synthesized with DCC, but coupling with TBTU and DIPEA was successful, and the same

SCHEME 1 The synthesis of 6-isobendamustine (**9**). Reagents and conditions: (I) $SOCl_2$, dichloromethane, reflux, 60 min; (II) 6 M HCl aq., 90°C, 2 h



8 **9** (6-Isobendamustine)



SCHEME 2 The synthesis of the target compounds **6**, **7**, **10**–**13**, **15**, and **16**. Reagents and conditions: (a) 1-(pyrrolidin-1-yl)propan-2-ol, *N*,*N*′-dicyclohexylcarbodiimide, dimethylformamide (DMF), 0°C, 15 min → 80°C (microwave), 30 min; (b) respective alcohol or amine, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate, *N*,*N*-diisopropylethylamine, DMF, rt, overnight or 60–80°C (microwave), 45–120 min; (c) Boc_2O, $NaHCO_3$, tetrahydrofuran/H_2O 1:1, rt, 30 min; (d) trifluoroacetic acid, dichloromethane, rt, 2 h

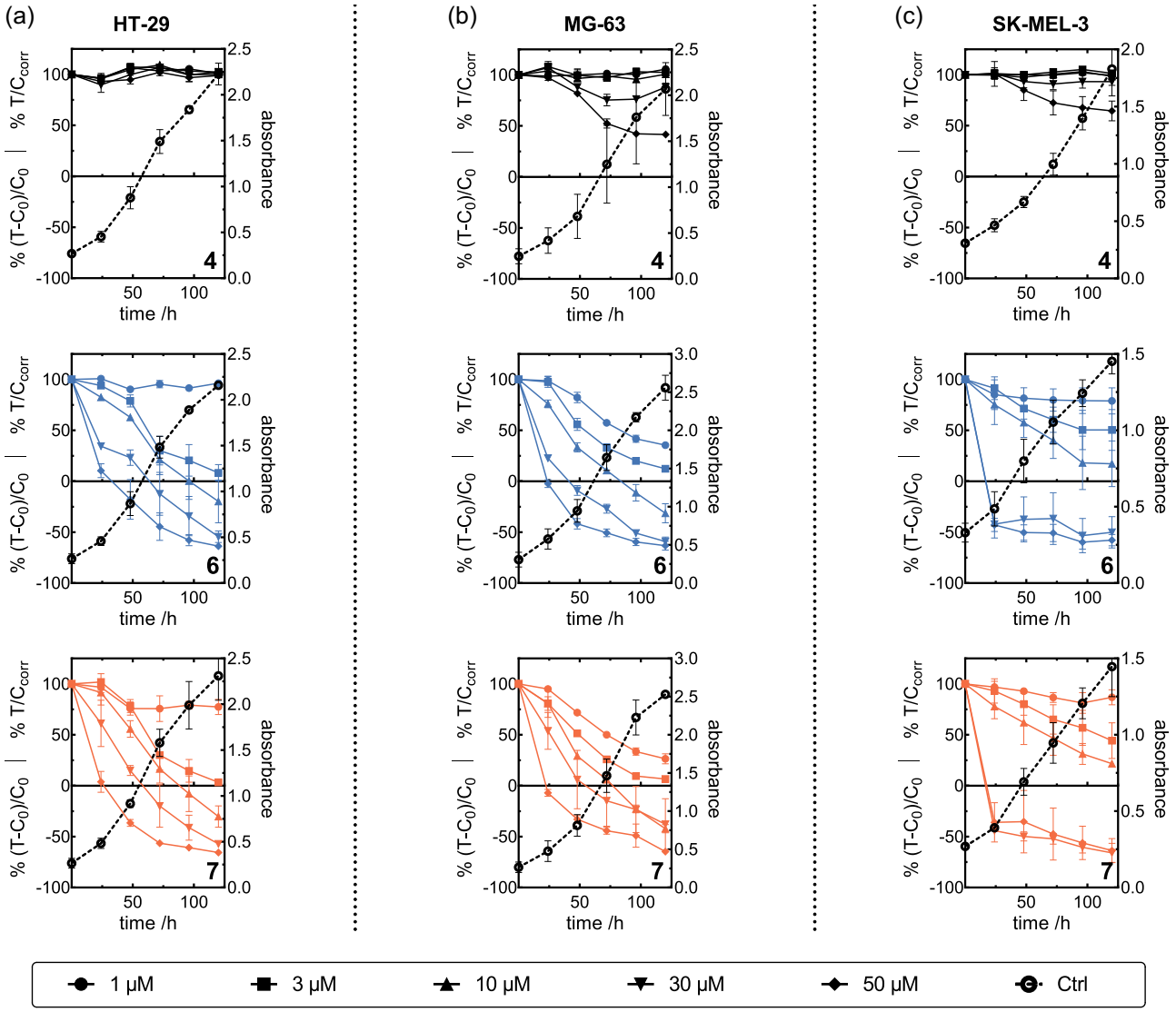


FIGURE 4 Cytotoxicity of bendamustine (**4**), its ester **6**, and its amide **7** against HT-29 cells (a), MG-63 cells (b), and SK-MEL-3 cells (c). Cytotoxic/cytocidal effects correspond to the left *y*-axes. The growth curves of untreated control cells (open circles) correspond to the right *y*-axes. Data are mean values ± SEM of two to four independent experiments, each performed in octuplicate

reagents were used to prepare the chlorambucil amide **13** (which is already known in the literature[32]) (Scheme 2). Melphalan was first *tert*-butyloxycarbonyl (boc)-protected (to compound **14**), then treated with the respective alcohol or amide and the established coupling reagents TBTU and DIPEA, and subsequently deprotected to the melphalan ester **15** or the amide **16** (Scheme 2).

2.2 | Cytotoxicity

The cytotoxicity of the target compounds was investigated in a kinetic chemosensitivity assay, which allows the distinction between cytotoxic, cytostatic, and cytocidal drug effects.[33] The effects against the following three cancer cell lines were determined: a carcinoma (colorectal adenocarcinoma, HT-29), a sarcoma (osteosarcoma, MG-63), and a malignant melanoma (SK-MEL-3).

2.2.1 | Bendamustine and derivatives

In accordance with our previous report,[28] bendamustine proved ineffective against HT-29 carcinoma cells (Figure 4). Also, the chemosensitivity of MG-63 osteosarcoma and SK-MEL-3 melanoma cells was very low with slight cytotoxic effects at concentrations above 30 µM. In contrast, the branched ester **6** and the amide **7** exhibited a distinct increase in cytotoxicity as compared with the parent

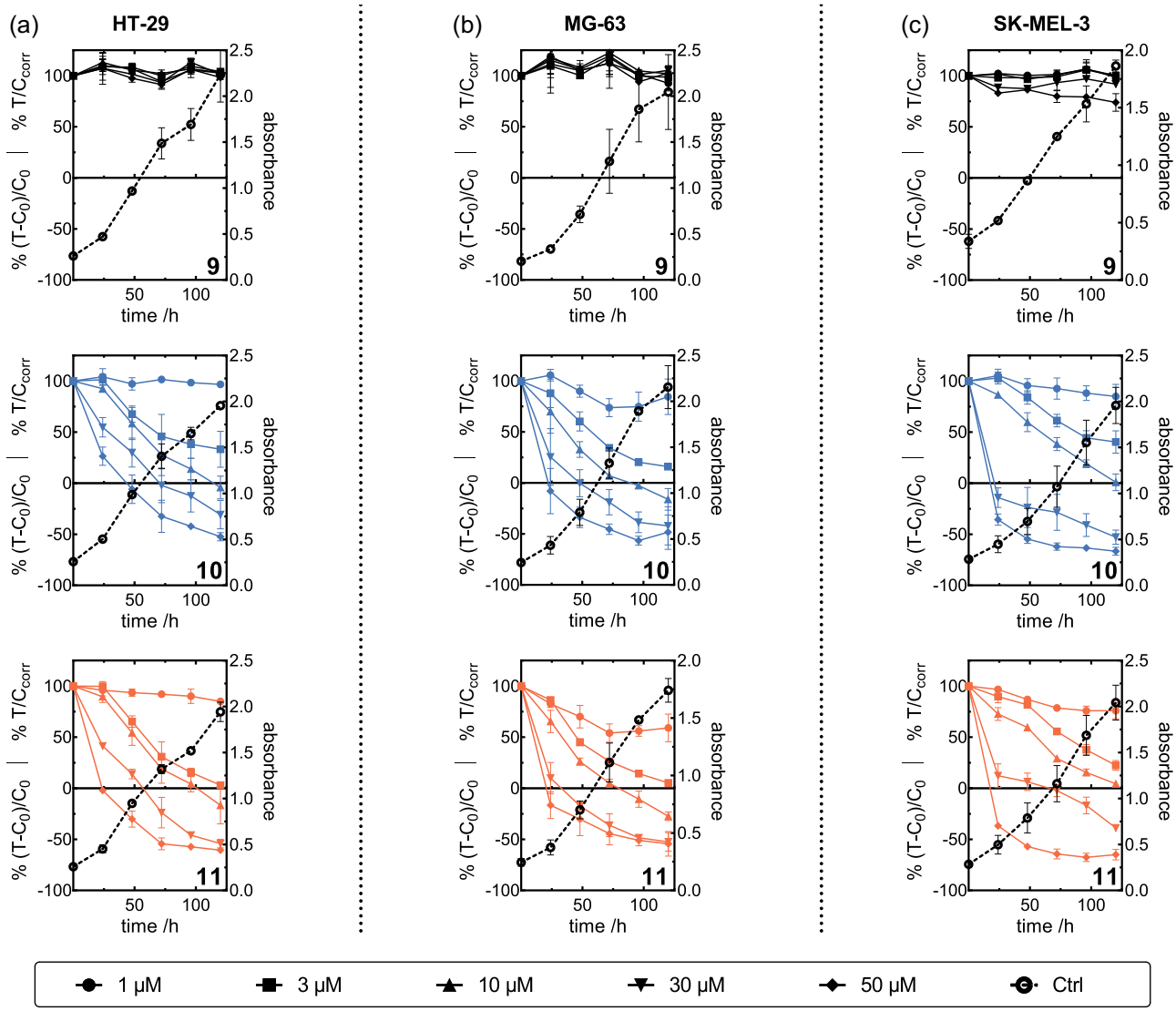


FIGURE 5 Cytotoxicity of isobendamustine (**9**), its ester **10**, and its amide **11** against HT-29 cells (a), MG-63 cells (b), and SK-MEL-3 cells (c). Cytotoxic/cytocidal effects correspond to the left *y*-axes. The growth curves of untreated control cells (open circles) correspond to the right *y*-axes. Data are mean values ± SEM of two to four independent experiments, each performed in octuplicate

compound, showing cytocidal effects against the treated cancer cells at concentrations of 10–30 µM. This is in good accordance with our previously examined linear esters of bendamustine.[28] Thus, the replacement of the linear ester moiety by a branched ester or an amide group did not only increase the stability in human blood plasma but also proved to be bioisosteric.

2.2.2 | Isobendamustine and derivatives

The comportment of isobendamustine in the chemosensitivity assay was almost identical to its isomer bendamustine—all three cell lines were refractory against treatment with isobendamustine (Figure 5). In contrast, the basic derivatives **10** and **11** exerted a pronounced effect against the three cell lines (cytocidal at concentrations between 10 and 30 µM), which is in analogy with the basic derivatives of bendamustine. Hence, the method of increasing the cytotoxicity by the introduction of a basic moiety was successfully applied to the 6-isomer of bendamustine, and it became obvious that the change in the position of the nitrogen mustard moiety at the benzimidazole ring is well tolerated.

2.2.3 | Chlorambucil and derivatives

Chlorambucil displays a pronounced structural difference from bendamustine, as it bears a phenyl ring instead of a benzimidazole moiety. Its effect against HT-29 carcinoma cells and SK-MEL-3 melanoma cells was rather weak, whereas it showed a cytocidal effect against MG-63 osteosarcoma cells at a concentration of 30 µM (Figure 6). The conversion into the basic ester **12** or amide **13** markedly increased the cytotoxicity as compared with the parent

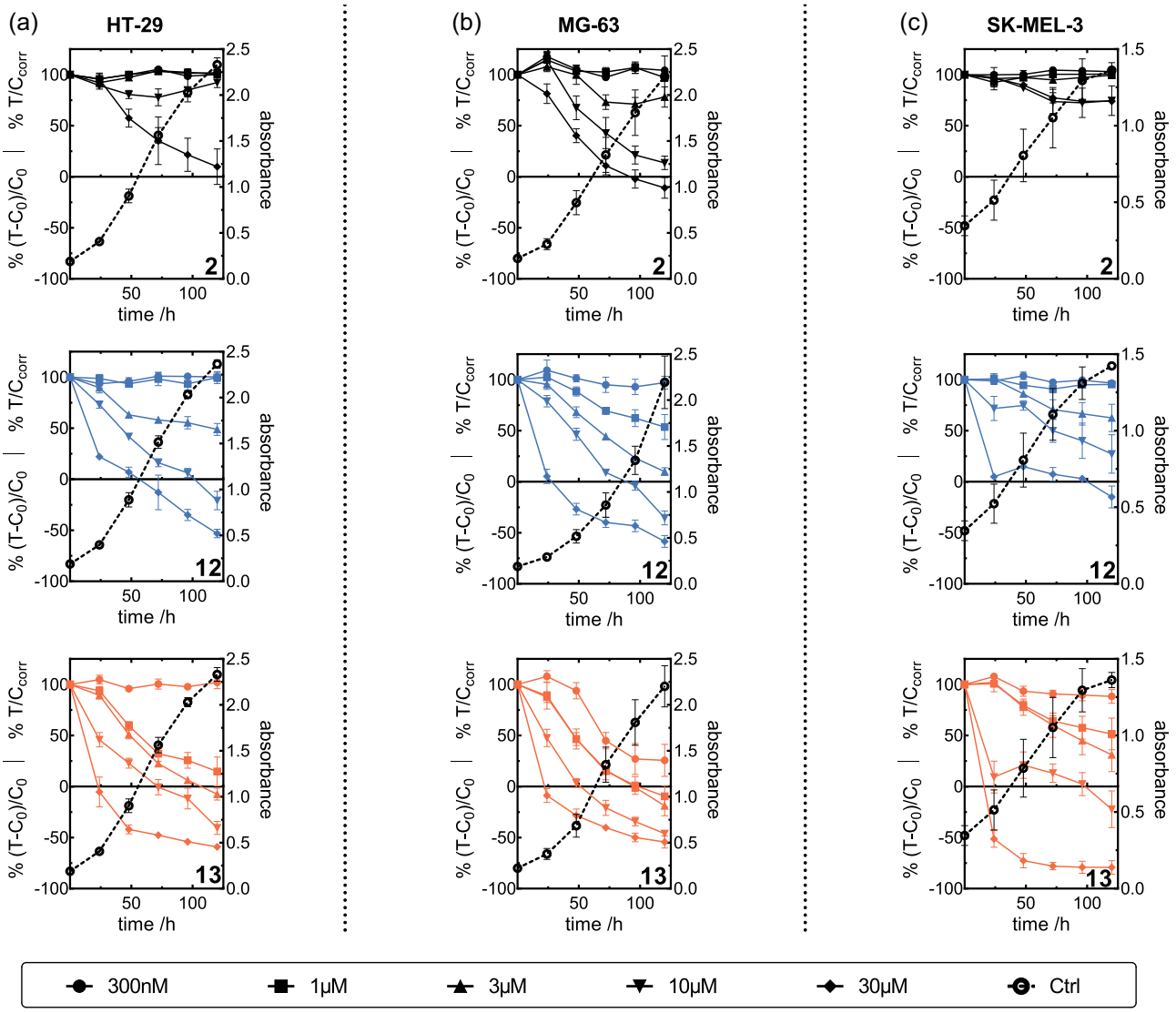


FIGURE 6 Cytotoxicity of chlorambucil (**2**), its ester **12**, and its amide **13** against HT-29 cells (a), MG-63 cells (b), and SK-MEL-3 cells (c). Cytotoxic/cytocidal effects correspond to the left *y*-axes. The growth curves of untreated control cells (open circles) correspond to the right *y*-axes. Data are mean values ± SEM of two to four independent experiments, each performed in octuplicate

compound, both derivatives showing cytocidal effects against HT-29 and MG-63 cells at concentrations of 1–10 µM and against SK-MEL-3 cells, which were refractory against treatment with chlorambucil, at concentrations of 10–30 µM. The chlorambucil amide was more potent than the ester; for instance, the amide exhibited a cytocidal effect against MG-63 osteosarcoma cells at a concentration as low as 1 µM, whereas a concentration of 10 µM of the ester was necessary to achieve the same effect. Taken together, the introduction of a basic moiety via an ester or amide bond turned out an effective means to increase the potency of chlorambucil.

2.2.4 | Melphalan and derivatives

Melphalan, an L-phenylalanine-nitrogen mustard, was more effective against the three cell lines than bendamustine, isobendamustine, or chlorambucil, which may be due to its property as an amino acid transporter substrate, causing increased cellular uptake. Melphalan showed a cytocidal effect on carcinoma HT-29 and osteosarcoma MG-63 cells at concentrations of 30 and 10 µM, respectively (Figure 7). The chemosensitivity of SK-MEL-3 cells was lower, with a cytostatic effect at a concentration of 30 µM. The basic ester **15** and amide **16** showed effects similar to the parent compound against MG-63 cells. However, HT-29 and SK-MEL-3 cells exhibited a higher response upon treatment with **15** and **16** than when treated with melphalan (cytocidal effects at concentrations of 3–30 µM). As in the case of the chlorambucil derivatives, the amide was somewhat more potent than the ester. All in all, only a slight improvement in cytotoxicity of melphalan could be achieved by the introduction of a basic moiety.

Comparing the basic derivatives of bendamustine, isobendamustine, chlorambucil, and melphalan, it becomes apparent that they all display similar potencies—the growth curves of the individual cell lines

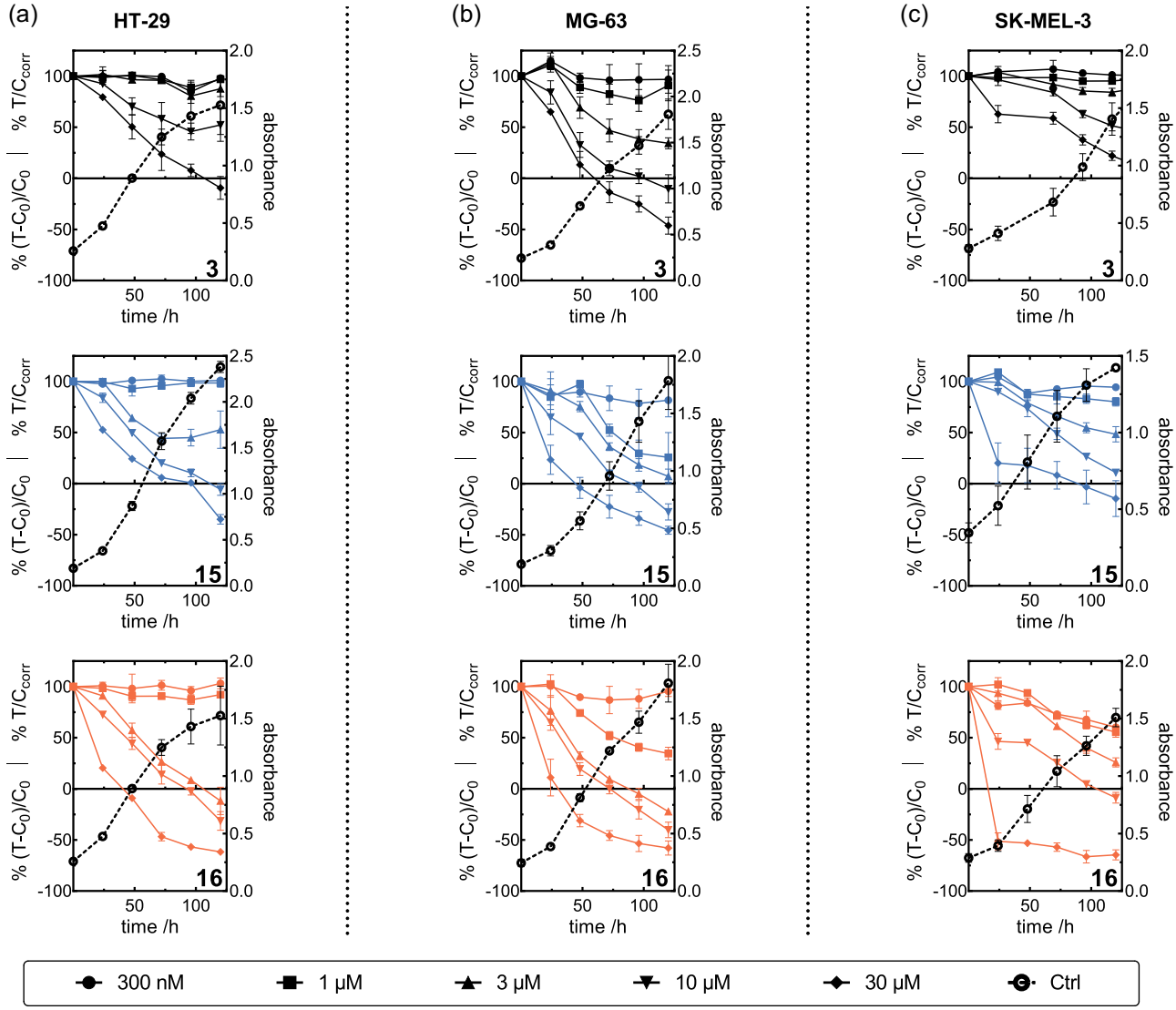


FIGURE 7 Cytotoxicity of melphalan (**3**), its ester **15**, and its amide **16** against HT-29 cells (a), MG-63 cells (b), and SK-MEL-3 cells (c). Cytotoxic/cytocidal effects correspond to the left *y*-axes. The growth curves of untreated control cells (open circles) correspond to the right *y*-axes. Data are mean values ± SEM of two to four independent experiments, each performed in octuplicate

upon treatment with **6**, **7**, **10**–**13**, **15**, and **16** are very much alike. This can be taken as a hint that the enhancement of cytotoxicity is due to the same mechanism, for instance, increased cellular uptake. As the basic pyrrolidine ring is protonated under physiological conditions, the involvement of cation transporters is conceivable. This would also explain the smaller potency difference between melphalan and its derivatives—contrary to bendamustine and chlorambucil, melphalan is imported into the cell by amino acid transporters,[12] which is why the cellular uptake does not leave much room for optimization.

3 | CONCLUSION

Previously, we have shown that esters of bendamustine are by far more cytotoxic anticancer agents than their parent compound, especially those containing a basic moiety. As the latter was rapidly cleaved in the human blood plasma, the labile ester moiety was substituted by a branched ester, resulting in compound UR-Ant26 (**6**) or an amide group, yielding UR-Ant16 (**7**), both of which markedly increased the stability. In the current study, the cytotoxicity of compounds **6** and **7** was examined. By analogy with the previous linear esters, they showed cytocidal effects at concentrations between 10 µM and 30 µM—a striking increase in cytotoxicity as compared with bendamustine, which was practically ineffective against the treated cells. These results verify that the replacement of the labile ester moiety by a branched ester or an amide was bioisosteric.

In the hope that this approach of increasing the cytotoxicity would be applicable to other, related nitrogen mustard anticancer agents, basic esters and amides of 6-isobendamustine (compounds UR-Ant45 [**10**] and UR-Ant48 [**11**]), chlorambucil (compounds UR-Ant66 [**12**] and UR-Ant55 [**13**]), and melphalan

(compounds UR-Ant65 [**15**] and UR-Ant39 [**16**]) were synthesized. The reaction of the respective parent compound with a pyrrolidinoalkyl alcohol or amide and the coupling reagent DCC or TBTU yielded the target compounds. Cytotoxicity against carcinoma, sarcoma, and melanoma cells was assessed in a kinetic chemosensitivity assay. The novel derivatives showed cytotoxic or cytocidal effects at concentrations above 1 μM. This constitutes a striking enhancement over 6-isobendamustine, which was ineffective against the selected cancer cells. These results are very similar to bendamustine and its derivatives, which shows that the constitution isomerism, that is, the change in the position of the N-lost moiety, is well tolerated. Also, an ample improvement over chlorambucil was achieved, which only showed a weak potency against the sarcoma cells. Melphalan was almost as effective as the target compounds—derivatization provided only a small increase in cytotoxicity. It can be speculated that the introduction of a basic moiety confers substrate properties of (cation) transporters, thereby increasing cellular uptake and ultimately cytotoxicity. This would explain the comparable meager enhancement achieved by derivatization of melphalan—the latter already exploits membrane transport systems, as it is a substrate of amino acid transporters.[12]

Taken together, the novel nitrogen mustard chemotherapeutics can be considered interesting molecular tools for the analysis of a correlation between cytotoxicity and membrane transport mechanisms. Furthermore, the increased antiproliferative activity suggests higher efficacy in the treatment of malignancies for which the parent compound is approved and a possible extension of the scope of indications.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General experimental conditions

Chemicals and solvents were purchased from commercial suppliers (Sigma Aldrich, Merck, VWR, Thermo Fisher Scientific, and TCI) and used without further purification unless stated otherwise. Bendamustine was a kind gift from Arevipharma. 4-(1-Methyl-6-bis(2-hydroxyethyl)aminobenzimidazol-2-yl)butyric acid ethyl ester was a kind gift from Gemini PharmChem. Reactions requiring anhydrous conditions were carried out in dried reaction vessels under an atmosphere of argon and anhydrous solvents were used. Millipore water was used throughout for the preparation of buffers and high-performance liquid chromatography (HPLC) eluents. Acetonitrile (MeCN) for HPLC (gradient grade) was obtained from Merck.

Microwave reactions were carried out in an Initiator 8 microwave reactor (Biotage).

Thin-layer chromatography (TLC) was performed on TLC Silica gel 60 F_{254} aluminum plates (Merck). Visualization was accomplished by UV irradiation at wavelengths of 254 and 366 nm or by staining with ninhydrin (1.5 g ninhydrin, 5 ml acetic acid, and 500 ml 95% ethanol).

The nuclear magnetic resonance (NMR) spectra (see the Supporting Information) were recorded on an Avance 400 instrument (9.40 T, ^{1}H: 400 MHz, ^{13}C: 101 MHz) or an Avance 600 instrument with a cryogenic probe (14.1 T, ^{1}H: 600 MHz, ^{13}C: 151 MHz) (Bruker) with TMS as an external standard. The high-resolution mass spectrometry (HRMS) analysis (see the Supporting Information) was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies) using an ESI source.

Preparative HPLC was performed on a system from Knauer, consisting of two K-1800 pumps and a K-2001 detector. A Nucleodur 100-5 C18 (5 μm, 110 Å, 250 × 21 mm; Macherey-Nagel) (compound **7**) or a Kinetex® XB-C18 (5 μm, 100 Å, 250 × 21.2 mm; Phenomenex) (all other compounds) served as RP columns at flow rates of 16 ml/min and 15 ml/min, respectively. Mixtures of MeCN and 0.1% aq trifluoroacetic acid (TFA) were used as the mobile phase. The detection wavelength was set to 220 nm throughout. The solvent mixtures were removed by lyophilization using an Alpha 2-4 LD lyophilization apparatus (Christ) equipped with an RZ 6 rotary vane vacuum pump (Vacuubrand).

Analytical HPLC of all compounds, except **12** and **15**, was performed on a system from Thermo Separation Products (Dreieich), composed of an SN400 controller, a P4000 pump, a Degassex DG-4400 degasser (Phenomenex), an AS3000 autosampler, and a Spectra Focus UV–visble detector. A Nucleodur 100-5 C18 (5 μm, 250 × 4.0 mm; Macherey-Nagel) (compound **7**) or a Kinetex® XB-C18 (5 μm, 100 Å, 250 × 4.6 mm; Phenomenex) (all other compounds) served as RP columns at a flow rate of 0.75 ml/min. The oven temperature was set to 30°C throughout. Mixtures of MeCN (A) and 0.05% aq TFA (B) were used as the mobile phase and degassed with helium. The detection wavelength was set to 220 nm throughout. Solutions for injection (100 μM) were prepared in a mixture of A and B, corresponding to the mixture at the start of the gradient. The following linear gradient was applied: 0–30 min: A/B 20:80–95:5, 30–35 min: A/B 95:5. Analytical HPLC of compounds **12** and **15** was performed on a system from Agilent Technologies (Santa Clara) (Series 1100), comprising a G1312A binary pump equipped with a G1379A degasser, a G1329A ALS autosampler, a G1316A COLCOM thermostated column compartment, and a G1314A VWD detector. A Kinetex® C18 (2.6 μm, 100 Å, 100 × 3 mm; Phenomenex) served as an RP column at a flow rate of 0.4 ml/min. The oven temperature was set to 30°C throughout. Mixtures of MeCN (A) and 0.05% aq TFA (B) were used as the mobile phase. The detection wavelength was set to 220 nm throughout. Solutions for injection (100 μM) were prepared in a mixture of A and B, corresponding to the mixture at the start of the gradient. The following linear gradient was applied: 0–12 min: A/B 20:80–95:5, 12–15 min: A/B 95:5. Retention (capacity) factors were calculated from retention times (t_R) according to $k = (t_R - t_0)/t_0$ (t_0 = dead time of the respective HPLC system).

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

4.1.2 | General procedure 1 for ester bond formation

The respective carboxylic acid (1.0 eq) was dissolved in anhydrous DMF (0.2–0.3 M) in a microwave reaction vessel. 1-(Pyrrolidin-1-yl)

propan-2-ol (1.5 eq) was added and the stirred mixture was cooled down to 0°C using an ice bath. DCC (1.1 eq) was added and stirring was continued at 0°C for 15 min and at 80°C in a microwave reactor for 30 min. The mixture was subjected to preparative HPLC (eluent: MeCN/0.1% aq TFA) and the eluate was lyophilized.

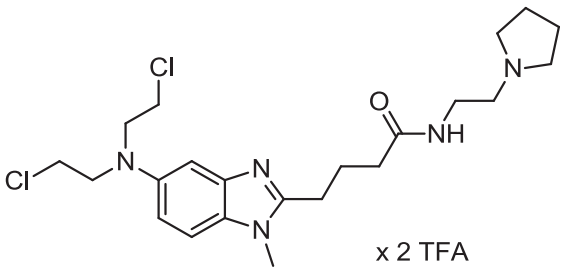
4.1.3 | General procedure 2 for ester bond formation

Under dry conditions, the respective carboxylic acid (1.0 eq) was dissolved in anhydrous DMF (0.2–0.3 M) in a microwave reaction vessel. DIPEA (2.0 eq) and the coupling reagent TBTU (1.0 eq) were added, and the solution was stirred at room temperature for 5 min. 1-(Pyrrolidin-1-yl)propan-2-ol (1.5 eq) was added and stirring was continued for another 45 min at 80°C in a microwave reactor. The mixture was subjected to preparative HPLC (eluent: MeCN/0.1% aq TFA) and the eluate was lyophilized. In the case of boc-protected compounds, work-up and deprotection preceded purification of the final product by preparative HPLC.

4.1.4 | General procedure for amide bond formation

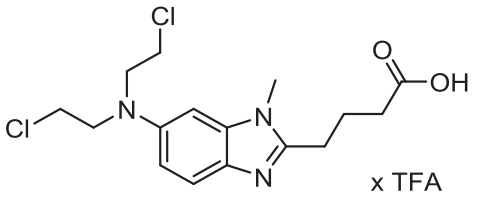
Under dry conditions, the respective carboxylic acid (1.0 eq) was dissolved in anhydrous DMF (0.2–0.3 M) in a microwave reaction vessel or a round-bottom flask. DIPEA (2.0–3.0 eq) and the coupling reagent TBTU (1.0 eq) were added and the solution was stirred at room temperature for 5 min. The appropriate amine (1.0–1.1 eq) was added and stirring was continued for another 45–120 min at 60–80°C in a microwave reactor or at room temperature overnight. The mixture was subjected to preparative HPLC (eluent: MeCN/0.1% aq TFA) and the eluate was lyophilized. In the case of boc-protected compounds, work-up and deprotection preceded purification of the final product by preparative HPLC.

*4-{5-[Bis(2-chloroethyl)amino]-1-methyl-1*H*-benzo[d]imidazol-2-yl}-*N*-[2-(pyrrolidin-1-yl)ethyl]butanamide (isobendamustine 2-pyrrolidinoethyl amide)bis(hydrotrifluoroacetate) (**7**)*



Compound **7** was prepared according to the general procedure for the amide bond formation (in a round-bottom flask with stirring overnight). The reaction was carried out using bendamustine (100 mg, 0.279 mmol, 1.0 eq), TBTU (98.6 mg, 0.307 mmol, 1.1 eq), DIPEA (52.2 µl, 0.307 mmol, 1.1 eq), 2-(pyrrolidin-1-yl)ethan-1-amine (35.4 µl, 0.279 mmol, 1.0 eq), and DMF (1 ml). Preparative HPLC (0–30 min: MeCN/0.1% aq TFA 19:81–55:45, t_R = 13.0 min) yielded **7** as a yellowish resin (97.5 mg, 0.143 mmol, 51%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 11.43 (s, 1H), 8.24 (t, J = 4.9 Hz, 1H), 7.39 (d, J = 9.2 Hz, 1H), 6.98 (d, J = 2.3 Hz, 1H), 6.95 (dd, J = 9.2 Hz, J = 2.3 Hz, 1H), 3.89 (s, 3H), 3.81 (t, J = 6.6 Hz, 6H), 3.67 (t, J = 6.6 Hz, 4H), 3.49 (q, J = 5.4 Hz, 2H), 3.24 (t, J = 5.4 Hz, 2H), 3.19 (t, J = 7.4 Hz, 2H), 2.89 (br s, 2H), 2.39 (t, J = 6.7 Hz, 2H), 2.18 (qi, J = 7.1 Hz, 2H), and 2.08 (br s, 4H). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 173.0, 162.1 (TFA), 161.9 (TFA), 161.6 (TFA), 161.4 (TFA), 151.5, 146.1, 132.8, 125.1, 117.5 (TFA), 115.5 (TFA), 112.9, 112.3, 96.4, 55.0, 54.4 (2C), 54.1 (2C), 40.6 (2C), 35.7, 34.3, 31.0, 24.7, 23.2 (2C), and 22.5. Reversed-phase high-performance liquid chromatography (RP-HPLC) (220 nm): 99% (t_R = 7.8 min, k = 2.3). HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{22}H_{34}Cl_2N_5O^+$: 454.2135, found: 454.2135. $C_{22}H_{33}Cl_2N_5O{\cdot}C_4H_2F_6O_4$ (454.44 + 228.05).

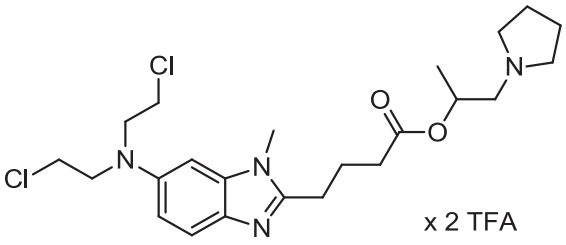
*4-{6-[Bis(2-chloroethyl)amino]-1-methyl-1*H*-benzo[d]imidazol-2-yl}-butanoic acid (6-isobendamustine) hydrotrifluoroacetate (**9**)* [30,31]



4-(1-Methyl-6-bis(2-hydroxyethyl)aminobenzimidazol-2-yl)-butyric acid ethyl ester (800 mg, 2.29 mmol, 1.0 eq) was dissolved in dichloromethane (DCM) and the chlorinating agent thionyl chloride (498 µl, 6.87 mmol, 3.0 eq) was added dropwise. The mixture was refluxed for 60 min and the volatiles were removed under reduced pressure. The ester bond was hydrolyzed with 6 M HCl aq (4 ml) 90°C for 2 h. The mixture was washed with DCM to remove lipophilic impurities and isobendamustine hydrochloride was precipitated by adding 10 M NaOH aq until a pH of 0–1 was reached. Recrystallization in isopropanol yielded isobendamustine free base as a brownish solid (346 mg, 0.966 mmol, 42%). For further purification, 50 mg of the substance was subjected to preparative HPLC (gradient: 0–30 min: MeCN/0.1% aq TFA 25:75–55:45, t_R = 13.0 min) and the eluate was lyophilized, yielding compound **9** as a brownish resin (61.8 mg, 0.131 mmol, 94%). Analytical and pharmacological characterization was performed with the HPLC-purified substance. ^{1}H NMR (600 MHz, deuterated dimethyl sulfoxide [DMSO-d_6]): δ (ppm) = 14.70 (br s, 1H), 12.26 (br s, 1H), 7.58 (d, J = 9.03 Hz, 1H), 7.11 (d, J = 2.28 Hz, 1H), 7.08 (dd, J = 9.03 Hz, J = 2.28 Hz, 1H), 3.90 (s, 3H), 3.86 (t, J = 6.57 Hz, 2H), 3.80 Hz (t, J = 6.66 Hz, 4H), 3.15 (t, J = 7.74 Hz, 2H), 2.41 (t, J = 7.1 Hz, 2H), and 2.00 (qi, J = 7.56 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6): δ (ppm) = 173.7, 158.5 (TFA), 158.3 (TFA), 158.1 (TFA), 157.8 (TFA), 151.8, 145.4, 134.3, 121.9, 117.5 (TFA), 115.6 (TFA), 114.5, 112.7, 94.0, 52.4 (2C), 41.2 (2C), 32.5,

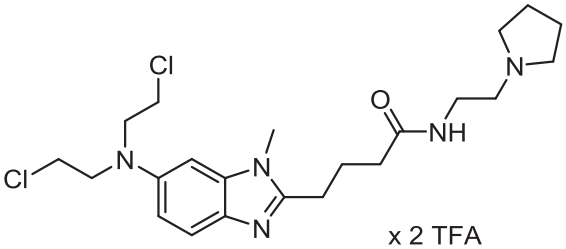
30.8, 24.2, and 21.4. RP-HPLC (220 nm): 98% (t_R = 11.7 min, k = 2.9). HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{16}H_{22}Cl_2N_3O_2^+$: 358.1084, found: 358.1104. $C_{16}H_{21}Cl_2N_3O_2{\cdot}C_2HF_3O_2$ (358.26 + 114.02).

*1-(Pyrrolidin-1-yl)propan-2-yl 4-{6-[bis(2-chloroethyl)amino]-1-methyl-1H-benzo[d]imidazol-2-yl}butanoate (isobendamustine 1-methyl-2-pyrrolidinoethyl ester) bis(hydrotrifluoroacetate) (**10**)*



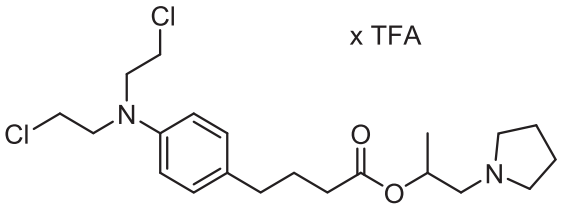
Compound **10** was prepared according to the general procedure 1 for the ester bond formation. The reaction was carried out using isobendamustine (75 mg, 0.209 mmol, 1.0 eq), 1-(pyrrolidin-1-yl)propan-2-ol (40.6 mg, 0.314 mmol, 1.5 eq), DCC (47.5 mg, 0.230 mmol, 1.1 eq), and DMF (0.5 ml). A yellow coloration and a white precipitate could be observed. Preparative HPLC (0–30 min: MeCN/0.1% aq TFA 20:80–55:45, t_R = 14.5 min) yielded **10** as a brownish resin (32.8 mg, 0.047 mmol, 22%). More than one diastereoisomer was evident in the NMR spectra. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 15.03 (br s, 1H), 10.18 (s, 1H), 7.59 (d, J = 9.13 Hz, 1H), 7.12 (d, J = 2.23 Hz, 1H), 7.08 (dd, J = 9.13 Hz, J = 2.23 Hz, 1H), 5.13 (m, 1H), 3.91 Hz (s, 3H), 3.86 (t, J = 6.58 Hz, 4H), 3.80 (t, J = 6.58 Hz, 4H), 3.54 (br s, 2H), 3.38 (m, 2H), 3.16 (t, J = 7.56 Hz, 2H), 3.10 (br s, 2H), 2.55 (m, 2H), 2.04 (m, 2H), 1.92 (br s, 4H), and 1.18 (d, J = 6.25 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6): δ (ppm) = 171.7, 158.8 (TFA), 158.5 (TFA), 158.3 (TFA), 158.1 (TFA), 151.7, 145.4, 134.3, 122.0, 117.4 (TFA), 115.5 (TFA), 114.6, 112.7, 94.0, 66.5, 57.4, 54.9 and 53.3 (the two carbons adjacent to the pyrrolidine nitrogen yielded two signals), 52.4 (2C), 41.2 (2C), 32.4, 30.8, 24.1, 22.6 (2C), 21.1, and 17.7. RP-HPLC (220 nm): 96% (t_R = 10.2 min, k = 2.4). HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{23}H_{35}Cl_2N_4O_2^+$: 469.2132, found: 469.2137. $C_{23}H_{34}Cl_2N_4O_2{\cdot}C_4H_2F_6O_4$ (469.45 + 228.05).

*4-{6-[Bis(2-chloroethyl)amino]-1-methyl-1H-benzo[d]imidazol-2-yl}-N-[2-(pyrrolidin-1-yl)ethyl]butanamide (isobendamustine 2-pyrrolidinooethyl amide) bis(hydrotrifluoroacetate) (**11**)*



Compound **11** was prepared according to the general procedure for the amide bond formation (in the microwave). The reaction was carried out using isobendamustine (100 mg, 0.279 mmol, 1.0 eq), TBTU (89.6 mg, 0.279 mmol, 1.0 eq), DIPEA (97.2 µl, 0.558 mmol, 2.0 eq), 2-(pyrrolidin-1-yl)ethan-1-amine (38.9 µl, 0.279 mmol, 1.0 eq), and DMF (1 ml). Preparative HPLC (0–30 min: MeCN/0.1% aq TFA 15:85–55:45, t_R = 14.2 min) yielded **11** as a yellow resin (77.2 mg, 0.113 mmol, 41%). ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 14.98 (br s, 1H), 10.02 (s, 1H), 8.24 (t, J = 5.69 Hz, 1H), 7.58 (d, J = 9.07 Hz, 1H), 7.11 (d, J = 2.25 Hz, 1H), 7.08 (dd, J = 9.07 Hz, J = 2.25, 1H), 3.91 (s, 3H), 3.86 (t, J = 6.51 Hz, 4H), 3.80 (t, J = 6.51 Hz, 4H), 3.57 (br s, 2H), 3.36 (m, 2H), 3.17 (s, 2H), 3.13 (t, J = 7.71 Hz, 2H), 2.99 (br s, 2H), 2.27 (t, J = 7.24 Hz, 2H), 2.01 (m, 2H), and 1.92 (m, 4H). ^{13}C NMR (151 MHz, DMSO-d_6): δ (ppm) = 171.9, 158.8 (TFA), 158.6 (TFA), 158.4 (TFA), 158.1 (TFA), 151.9, 145.4, 134.3, 122.0, 117.6 (TFA), 115.6 (TFA), 114.6, 112.7, 94.0, 53.3 (2C), 53.1, 52.4 (2C), 41.2 (2C), 35.0, 33.7, 30.8, 24.3, 22.5 (2C), and 21.8. RP-HPLC (220 nm): 96% (t_R = 9.0 min, k = 2.0). HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{22}H_{34}Cl_2N_5O^+$: 454.2135, found: 454.2135. $C_{22}H_{33}Cl_2N_5O{\cdot}C_4H_2F_6O_4$ (454.44 + 228.05).

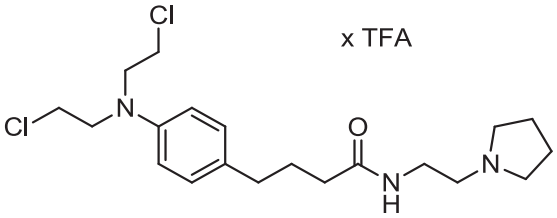
*1-(Pyrrolidin-1-yl)propan-2-yl 4-{4-[bis(2-chloroethyl)amino]phenyl}-butanoate (chlorambucil 1-methyl-2-pyrrolidinoethyl ester) hydrotrifluoroacetate (**12**)*



Compound **12** was prepared according to the general procedure 2 for the ester bond formation. The reaction was carried out using chlorambucil (80 mg, 0.263 mmol, 1.0 eq), TBTU (84.4 mg, 0.263 mmol, 1.0 eq), DIPEA (89.4 µl, 0.526 mmol, 2.0 eq), 1-(pyrrolidin-1-yl)propan-2-ol (51.0 µl, 0.394 mmol, 1.5 eq), and DMF (1 ml). Preparative HPLC (0–30 min: MeCN/0.1% aq TFA 33:67–69:31, t_R = 15.5 min) yielded **12** as a brownish resin (49.2 mg, 0.093 mmol, 35%). More than one diastereoisomer was evident in the NMR spectra. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) 9.99 (br s, 1H), 7.02 (d, J = 8.6 Hz, 2H), 6.67 (d, J = 8.8 Hz, 2H), 5.13 (m, 1H), 3.69 (m, 8H), 3.59–3.45 (m, 2H), 3.44–3.30 (m, 2H), 3.12–3.99 (m, 2H), 2.49–2.45 (m, 2H), 2.38–2.27 (m, 2H), 1.99 (m, 2H), 1.86 (m, 2H), 1.77 (m, 2H), and 1.20 (d, J = 6.3 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 172.3, 158.6 (TFA), 158.3 (TFA), 158.1 (TFA), 157.9 (TFA), 144.5, 129.4, 129.3 (2C), 117.5 (TFA), 115.6 (TFA), 111.9 (2C), 66.1, 57.4, 54.9 and 53.3 (the two carbons adjacent to the pyrrolidine nitrogen yielded two signals), 52.2 (2C), 41.2 (2C), 33.2, 33.0, 26.3, 22.7 and 22.5 (the two pyrrolidine carbons not adjacent to the nitrogen yielded two signals), and 17.7. RP-HPLC (220 nm): 92% (t_R = 9.2 min, k = 6.2) (the second peak in the chromatogram is not due to

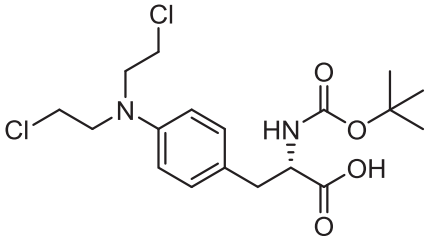
impurity but due to decomposition in the aqueous HPLC eluent). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{21}H_{33}Cl_2N_2O_2^+$: 415.1914, found: 415.1924. $C_{21}H_{32}Cl_2N_2O_2{\cdot}C_2HF_3O_2$ (414.18 + 114.02).

*4-{4-[Bis(2-chloroethyl)amino]phenyl}-*N*-[2-(pyrrolidin-1-yl)ethyl]-butanamide (chlorambucil 2-pyrrolidinooethyl amide) hydrotrifluoroacetate (**13**)*[32]



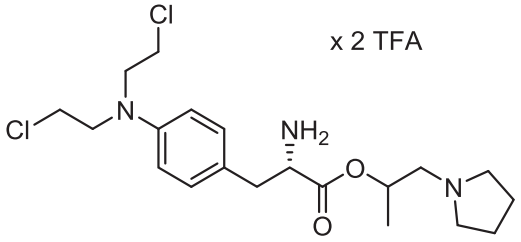
Compound **13** was prepared according to the general procedure for the amide bond formation (in the microwave). The reaction was carried out using chlorambucil (100 mg, 0.329 mmol, 1.0 eq), TBTU (105.5 mg, 0.329 mmol, 1.0 eq), DIPEA (111.8 µl, 0.657 mmol, 2.0 eq), 2-(pyrrolidin-1-yl)ethan-1-amine (45.8 µl, 0.362 mmol, 1.1 eq), and DMF (1 ml). Two-fold purification by preparative HPLC (0–30 min: MeCN/0.1% aq TFA 28:72–68:32, t_R = 13.5 min) yielded **13** as a yellowish resin (75.5 mg, 0.147 mmol, 45%). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) 9.80 (br s, 1H), 8.07 (m, 1H), 7.01 (d, *J* = 8.6 Hz, 2H), 6.66 (d, *J* = 8.6 Hz, 2H), 3.69 (m, 8H), 3.57 (m, 2H), 3.36 (q, *J* = 6.1 Hz, 2H), 3.18 (q, *J* = 6.0 Hz, 2H), 3.00 (m, 2H), 2.44 (t, *J* = 7.7 Hz, 2H), 2.10 (t, *J* = 7.4 Hz, 2H), 1.99 (m, 2H), 1.85 (m, 2H), and 1.74 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 172.8, 158.6 (TFA), 158.4 (TFA), 158.2 (TFA), 158.0 (TFA), 144.4, 129.8, 129.3 (2C), 117. 6 (TFA), 115.6 (TFA), 111.9 (2C), 53.4 (2C), 53.3, 52.2 (2C), 41.2 (2C), 35.0, 34.8, 33.6, 27.0, and 22.5 (2C). RP-HPLC (220 nm): 92% (t_R = 14.3 min, *k* = 3.7) (the second peak in the chromatogram is not due to impurity but due to decomposition in the aqueous HPLC eluent). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{20}H_{32}Cl_2N_3O^+$: 400.1917, found: 400.1925. $C_{20}H_{31}Cl_2N_3O{\cdot}C_2HF_3O_2$ (399.18 + 114.02).

*(S)-3-{4-[Bis(2-chloroethyl)amino]phenyl}-2-[(*tert*-butoxycarbonyl)-amino]propanoic acid (boc-melphalan) (**14**)* [34,35]



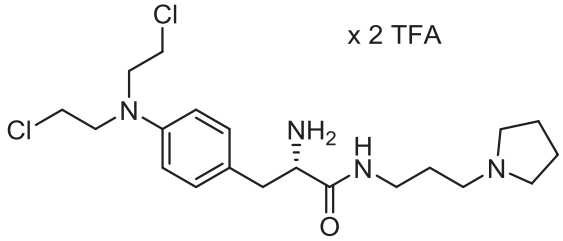
Melphalan (38.0 mg, 0.125 mmol, 1.0 eq) and $NaHCO_3$ (31.4 mg, 0.374 mmol, 3.0 eq) were dissolved in tetrahydrofuran/H_2O 1:1 (1 ml and Boc_2O [di-*tert*-butyl dicarbonate]; 42.9 µl, 0.1787 mmol, 1.5 eq) was added and the mixture stirred at room temperature for 30 min. The suspension was diluted with EtOAc and the organic layer was washed with 1 M HCl aq and brine, dried over Na_2SO_4, and concentrated under reduced pressure to a brown oil, which was used in subsequent reactions without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 9.79 (s, 1H), 7.07 (d, *J* = 7.8 Hz, 2H), 6.62 (d, *J* = 8.6 Hz, 2H), 4.96 (d, *J* = 7.4 Hz, 1H), 4.55 (m, 1H), 3.70 (m, 4H), 3.61 (m, 4H), 3.13–2.96 (m, 2H), and 1.52 (s, 9H). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{22}H_{34}Cl_2N_5O^+$: 454.2135, found: 454.2135. $C_{18}H_{26}Cl_2N_2O_4$ (504.32).

*1-(Pyrrolidin-1-yl)propan-2-yl (2S)-2-amino-3-{4-[bis(2-chloroethyl)-amino]phenyl}propanoate (melphalan 1-methyl-2-pyrrolidinoethyl ester) bis(hydrotrifluoroacetate) (**15**)*



Compound **15** was prepared according to the general procedure 2 for the ester bond formation. The reaction was carried out using boc-melphalan (133 mg, 0.328 mmol, 1.0 eq), TBTU (105 mg, 0.328 mmol, 1.0 eq), DIPEA (111 µl, 0.655 mmol, 2.0 eq), 1-(pyrrolidin-1-yl)propan-2-ol (63.5 µl, 0.491 mmol, 1.5 eq), and DMF (3 ml). The mixture was diluted with brine (50 ml) and the product was extracted with EtOAc (3 × 50 ml). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. For *N*-boc deprotection, the residue was dissolved in DCM (2 ml), and TFA (0.5 ml) was added and the solution was stirred at room temperature for 2 h. Evaporation of the volatiles and purification by preparative HPLC (0–30 min: MeCN/0.1% aq TFA 21:79–57:43, t_R = 13.5 min) yielded **15** as a brown resin (20.2 mg, 0.031 mmol, 10% for protection, coupling, and deprotection). More than one diastereoisomer was evident in the NMR spectra. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) 10.07 (br s, 1H), 8.57 (m, 3H), 7.08 (m, 2H), 6.72 (m, 2H), 5.16 (m, 1H), 4.33–4.15 (m, 1H), 3.72 (m, 8H), 3.62–3.31 (m, 4H), 3.12–2.91 (m, 4H), 2.05–1.74 (m, 4H), 1.27–1.12 (m, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 168.7, 168.0, 158.8 (TFA), 158.6 (TFA), 158.4 (TFA), 158.2 (TFA), 145.7, 145.7, 130.7, 130.6 (2C), 122.3, 122.0, 117.6 (TFA), 115.6 (TFA), 112.0 (2C), 68.9, 68.6, 57.1, 57.0, 54.8, 53.7, 53.5, 53.3, 53.0, 52.0 (2C), 41.1 (2C), 34.8, 34.8, 22.6 (2C), 17.3, and 17.2. RP-HPLC (220 nm): 98% (t_R = 5.5 min, *k* = 3.4). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $C_{20}H_{32}Cl_2N_3O_2^+$: 416.1866, found: 416.1871. $C_{20}H_{31}Cl_2N_3O_2{\cdot}C_4H_2F_6O_4$ (416.39 + 228.05).

*(S)-2-Amino-3-{4-[bis(2-chloroethyl)amino]phenyl}-*N*-[3-(pyrrolidin-1-yl)propyl]propanamide (melphalan 3-pyrrolidinopropyl amide) bis(hydrotrifluoroacetate) (**16**)*



Compound **16** was prepared according to the general procedure for the amide bond formation (in the microwave). The reaction was carried out using boc-melphalan (64.2 mg, 0.125 mmol, 1.0 eq), TBTU (40.0 mg, 0.125 mmol, 1.0 eq), DIPEA (65.1 µl, 0.374 mmol, 3.0 eq), 3-(pyrrolidin-1-yl)propan-1-amine (47.3 µl, 0.374 mmol, 3.0 eq), and DMF (1 ml). The mixture was diluted with brine (20 ml) and the product was extracted with EtOAc (3 × 20 ml). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. For *N*-boc deprotection, the residue was dissolved in DCM (0.5 ml), and TFA (0.5 ml) was added and the solution was stirred at room temperature for 2 h. Evaporation of the volatiles and purification by preparative HPLC (0–30 min: MeCN/0.1% aq TFA 15:85–55:45, t_R = 14.4 min) yielded **16** as a brownish resin (22.9 mg, 0.036 mmol, 29% for protection, coupling and deprotection). More than one diastereoisomer was evident in the NMR spectra. ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm) 10.08 (s, 1H), 8.59 (t, J = 5.8 Hz, 1H), 8.20 (br s, 3H), 7.06 (d, J = 8.7 Hz, 2H), 6.71 (d, J = 8.8 Hz, 2H), 3.86 (m, 1H), 3.71 (s, 8H), 3.51 (m, 2H), 3.21–3.09 (m, 2H), 3.07–2.98 (m, 2H), 2.95–2.83 (m, 4H), 2.03–1.82 (m, 4H), and 1.79–1.67 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 168.3, 158.8 (TFA), 158.6 (TFA), 158.4 (TFA), 158.2 (TFA), 145.5, 130.5 (2C), 122.8, 117.8 (TFA), 115.9 (TFA), 112.0 (2C), 53.9, 53.3 and 53.2 (the two carbons adjacent to the pyrrolidine nitrogen yielded two signals), 52.1 (2C), 51.8, 41.2 (2C), 36.1, 35.9, 25.4, and 22.6 (2C). RP-HPLC (220 nm): 97% (t_R = 8.9 min, k = 1.9). HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{20}H_{33}Cl_2N_4O^+$: 415.2026, found: 415.2034. $C_{20}H_{32}Cl_2N_4O{\cdot}C_4H_2F_6O_4$ (415.40 + 228.05).

4.2 | Biology

4.2.1 | General experimental conditions

Materials: Commodity chemicals and solvents were purchased from commercial suppliers (Sigma Aldrich, Merck, VWR, Thermo Fisher Scientific, Invitrogen, and Serva). Millipore water was used throughout for the preparation of buffers and aqueous reagent solutions. The pH of buffers was adjusted with NaOH aq or HCl aq. All cell lines were purchased from the ATCC (American Type Culture Collection). Tissue culture flasks were procured from Sarstedt. The RPMI-1640 medium was purchased from Sigma Aldrich. Fetal calf serum (FCS) and trypsin/EDTA solution was purchased from Biochrom. Ninety-six-well microplates (PS, clear, F-bottom, with lid, sterile) were purchased from Greiner Bio-One.

Stock solutions: The test compounds were dissolved in DMSO at 1000 times the final concentrations in the chemosensitivity assay.

Instruments: Absorbance measurements were carried out with a GENios Pro microplate reader (equipped with a Xenon arc lamp; Tecan).

Software: All biological data were analyzed with GraphPad Prism 5 (GraphPad Software).

4.2.2 | Cell culture

All cell lines were cultured in the RPMI-1640 medium containing 110 mg/l sodium pyruvate, 2.4 g/l HEPES, and 2.0 g/l $NaHCO_3$, and supplemented with 10% (v/v) FCS at 37°C in a water-saturated atmosphere containing 5% CO_2. Cells were passaged after treatment with a solution containing 0.05% trypsin and 0.025% EDTA. All cells were routinely monitored for mycoplasma contamination by polymerase chain reaction using the Venor®GeM mycoplasma detection kit (Minerva Biolabs), which were found to be negative.

4.2.3 | Chemosensitivity assay[33]

Cells were seeded into 96-well plates at a density of 1500 cells per well (MG-63 and HT-29 cells) or a density of 3000 cells per well (SK-MEL-3 cells) (100 µl/well), and they were allowed to attach to the surface of the microplates in a water-saturated atmosphere containing 5% CO_2 at 37°C overnight. The next day, fresh medium containing the test compounds at two-fold final concentrations was added (100 µl/well; giving a final volume of 200 µl/well). On each plate, vinblastine at a final concentration of 300 nM served as reference cytostatic (positive control); the vehicle DMSO (0.1%) served as a negative control to monitor cell growth in the absence of a drug. Each concentration was measured in octuplicate and the negative control in a 16-fold replication. Growth of the cells was stopped after different periods of time by removal of medium and fixation with 2% (v/v) glutardialdehyde in phosphate-buffered saline (100 µl/well). All the plates were stored at 4°C until the end of the experiment and afterward stained with 0.02% crystal violet in water (100 µl/well) for 20 min. Excess dye was removed by rinsing the plates with water three times. Crystal violet bound by the fixed cells was redissolved in 70% ethanol (180 µl/well) while shaking the microplates for 2–3 h. The absorbance (580 nm) as a parameter proportional to the cell mass was measured using a GENios Pro microplate reader.

Cytotoxic effects were expressed as corrected T/C values according to

$$T/C_{corr}[\%] = \frac{T - C_0}{C - C_0} \times 100,$$

where T is the mean absorbance of the treated cells, C is the mean absorbance of the negative controls, and C_0 is the mean absorbance of the cells at the time of compound addition (t_0). When the absorbance of treated cells T was lower than at the beginning of the experiment (C_0), the extent of cell killing was calculated as cytocidal effect according to

$$\textit{Cytocidal effect}[\%] = \frac{T - C_0}{C_0} \times 100.$$

ACKNOWLEDGMENTS

The authors thank Maria Beer-Krön for excellent technical assistance, Marlies Antoni for skillful linguistic support, and Christian and Helmut Schickaneder for helpful discussions. Open Access funding enabled and organized by ProjektDEAL.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

Frauke Antoni conceived the project with input from Günther Bernhardt. Frauke Antoni performed the synthesis, the cytotoxicity assays, and the data analysis with Günther Bernhardt as a supervisor. Frauke Antoni wrote the manuscript with input from Günther Bernhardt.

ORCID

Frauke Antoni http://orcid.org/0000-0001-8382-6315
Günther Bernhardt http://orcid.org/0000-0001-6491-9874

REFERENCES

[1] H. Nagai, Y. H. Kim, *J. Thorac. Dis.* **2017**, *9*(*3*), 448.
[2] B. A. Chabner, T. G. Roberts, *Nat. Rev. Cancer* **2005**, *5*(*1*), 65.
[3] M. M. Gottesman, T. Fojo, S. E. Bates, *Nat. Rev. Cancer* **2002**, *2*(*1*), 48.
[4] A. Gilman, F. S. Philips, *Science* **1946**, *103*(*2675*), 409.
[5] R. K. Singh, S. Kumar, D. N. Prasad, T. R. Bhardwaj, *J. Med. Chem.* **2018**, *151*, 401.
[6] J. L. Everett, J. J. Roberts, W. C. J. Ross, *J. Chem. Soc.* **1953**, *0*, 2386.
[7] S. Neidle, D. E. Thurston, *Nat. Rev. Cancer* **2005**, *5*(*4*), 285.
[8] D. Godenèche, J. C. Madelmont, M. F. Moreau, R. Plagne, G. Meyniel, *Cancer Chemother. Pharmacol.* **1980**, *5*(*1*), 1.
[9] S. Genka, J. Deutsch, U. H. Shetty, P. L. Stahle, V. John, I. M. Lieberburg, F. Ali-Osmant, S. I. Rapoport, N. H. Greig, *Clin. Exp. Metastasis* **1993**, *11*(*2*), 131.
[10] D. Steinritz, A. Schmidt, T. Simons, M. Ibrahim, C. Morguet, F. Balszuweit, H. Thiermann, K. Kehe, W. Bloch, B. Bölck, *Chem. Biol. Interact.* **2014**, *219*, 143.
[11] F. Bergel, J. A. Stock, *J. Chem. Soc.* **1954**, (*0*), 2409.
[12] D. T. Vistica, *Biochim. Biophys. Acta, Biomembr.* **1979**, *550*(*2*), 309.
[13] T. B. Salisbury, S. Arthur, *Int. J. Mol. Sci.* **2018**, *19*(*8*), 2373.
[14] I. D. Davies, J. P. Allanson, R. C. Causon, *Chromatographia* **2000**, *52*(*1*), 92.
[15] U. D. Bayraktar, Q. Bashir, M. Qazilbash, R. E. Champlin, S. O. Ciurea, *Biol. Blood Marrow Transplant.* **2013**, *19*(*3*), 344.
[16] W. Ozegowski, D. Krebs, *J. Prakt. Chem.* **1963**, *20*(*3-4*), 178.
[17] W. Ozegowski, D. Krebs, *Zbl. Pharm.* **1971**, *110*(*10*), 1013.
[18] M. Kalaycio, *Cancer* **2009**, *115*(*3*), 473.
[19] L. M. Leoni, *Semin. Hematol.* **2011**, *48*(*Suppl 1*), 4.
[20] L. M. Leoni, B. Bailey, J. Reifert, H. H. Bendall, R. W. Zeller, J. Corbeil, G. Elliott, C. C. Niemeyer, *Clin. Cancer Res.* **2008**, *14*(*1*), 309.
[21] L. M. Leoni, J. A. Hartley, *Semin. Hematol.* **2011**, *48*(*Suppl 1*), 12.
[22] B. D. Cheson, M. J. Rummel, *J. Clin. Oncol.* **2009**, *27*(*9*), 1492.
[23] J. Colledge, (Astellas Deutschland GmbH), *Germany WO2010063493A1*, **2010**.
[24] R. Y. LaBell, P. R. Patel, (Cephalon, Inc.), *USA US20120157505A1*, **2012**.
[25] I. Pencheva, A. Bogomilova, N. Koseva, D. Obreshkova, K. Troev, *J. Pharm. Biomed. Anal.* **2008**, *48*(*4*), 1143.
[26] A. M. Scutaru, M. Wenzel, H. Scheffler, G. Wolber, R. Gust, *Bioconjugate Chem.* **2010**, *21*(*10*), 1728.
[27] R. P. Bakale, P. D. Brown, J. Chen, A. S. Drager, R. Y. LaBell, R. E. McKean, P. R. Patel, R. C. Roemmele, (Cephalon, Inc.), *USA WO2014075035A1*, **2015**.
[28] S. Huber, J. P. Huettner, K. Hacker, G. Bernhardt, J. König, A. Buschauer, *PLOS One* **2015**, *10*(*7*), e0133743.
[29] S. Huber, F. Antoni, C. Schickaneder, H. Schickaneder, G. Bernhardt, A. Buschauer, *J. Pharm. Biomed. Anal.* **2015**, *104*, 137.
[30] H. Schickaneder, C. Schickaneder, A. Buschauer, G. Bernhardt, S. Huber, M. Limmert, M. Lubbe, H. Hofmeier, *WO189847A1*, **2013**.
[31] H. Schickaneder, C. Schickaneder, A. Buschauer, G. Bernhardt, S. Huber, F. Antoni, *WO091827A1*, **2015**.
[32] M. Wolf, U. Bauder-Wüst, A. Mohammed, F. Schönsiegel, W. Mier, U. Haberkorn, M. Eisenhut, *Melanoma Res.* **2004**, *14*(*5*), 353.
[33] G. Bernhardt, H. Reile, H. Birnböck, T. Spruß, H. Schönenberger, *J. Cancer Res. Clin. Oncol.* **1992**, *118*(*1*), 35.
[34] K.-H. Hsieh, G. R. Marshall, *J. Med. Chem.* **1981**, *24*(*11*), 1304.
[35] B. E. Toki, P. D. Senter, (Seattle Genetics, Inc.), *USA WO070457A1*, **2005**.

SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article.

How to cite this article: Antoni F, Bernhardt G. Derivatives of nitrogen mustard anticancer agents with improved cytotoxicity. *Arch Pharm.* 2020;e2000366. https://doi.org/10.1002/ardp.202000366