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Bendamustine hydrochloride activity against doxorubicin-resistant human breast carcinoma cell lines

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The cytotoxic activity of bendamustine hydrochloride was evaluated against human ovarian and breast carcinoma cell lines including cell lines resistant to cisplatin and doxorubicin *in vitro*. The relative degree of resistance to bendamustine hydrochloride was lower in all cell lines compared with cyclophosphamide, melphalan and BCNU, suggesting only incomplete cross-resistance. Furthermore lower levels of resistance were also observed in all breast cancer cell lines when bendamustine hydrochloride was compared with cisplatin. Bendamustine hydrochloride also presents good activity in cell line MCF 7 AD, which is approximatly 80-fold resistant to doxorubicin compared with MCF 7. Basic glutathione levels and activity of glutathione-S-transferase showed no correlation to the IC_{50} values for bendamustine hydrochloride in the cell lines. When given at equitoxic concentrations, bendamustine hydrochloride consistently induced more DNA double-strand breaks than melphalan, cyclophosphamide or BCNU. In addition, removal of DNA double-strand breaks induced by bendamustine hydrochloride was relatively slow with the majority of DNA double-strand breaks still being detectable after 24 h. These findings indicate differences in the interaction between bendamustine hydrochloride and DNA, and may explain the lack of complete cross-resistance between bendamustine hydrochloride and the other alkylating agents.

Key words: Alkylating agents, bendamustine hydrochloride, breast cancer, drug resistance, ovarian cancer.

Introduction

A variety of agents have demonstrated antitumor activity against ovarian and breast carcinoma including cisplatin, cyclophosphamide, melphalan and doxorubicin.[1–5] Currently, only a few agents are available with documented activity in cisplatin- and doxorubicin-resistant ovarian and breast carcinoma.[6] Bendamustine hydrochloride (4-[5-bis (2-chlorethyl)amino]-1-methyl-2-benzimidazolebutyric acid) is a benzimidazol derivative. Like other nitrogen mustard derivates, the cytotoxic effect of bendamustine hydrochloride is due to the alkylating *N*-lost group (Figure 1). The structure of bendamustine hydrochloride is related to chlorambucil: the benzene of the chlorambucil is replaced by 1-methyl-benzimidazol; consequently, bendamustine hydrochloride becomes water soluble by hydrochloride formation.

Only very few studies on the cytotoxic mechanism of action of bendamustine hydrochloride have been reported. Bendamustine hydrochloride has been in clinical use since 1985. The drug has demonstrated clinical activity in malignant lymphoma and in breast cancer in combination with methotrexate and 5-fluorouracil (5-FU)[7] and in combination with doxorubicin and vincristin,[8] respectively; so far there is no report concerning the clinical activity of bendamustine hydrochloride used as a single agent. Furthermore, no studies concerning the cross-resistance pattern of bendamustine hydrochloride and other alkylators have been reported.

We therefore studied the cytotoxic acitivity of bendamustine hydrochloride in comparison with other nitrogen mustards (cyclophosphamide and melphalan), the *N*-nitrosurea BCNU and cisplatin in human ovarian and breast cancer cell lines including a cisplatin-resistant ovarian and a doxorubicin-resistant breast cancer cell line.

Additionally, mechanistic studies concerning the influence of cellular glutathione content on the sus-

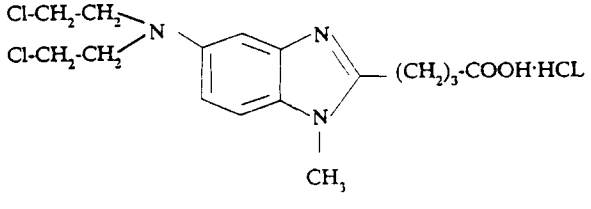

Fig. 1. Structure of bendamustine hydrochloride (4-[5-bis(2-chlorethyl)amino]-1-methyl-2-benzimidazolebutyric acid.

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ceptibility to the various alkylating agents and the different properties of alkylating agents to induce DNA double-strand breaks were evaluated.

Materials and methods

Drugs and chemicals

Cisplatin and BCNU were from Bristol Arzneimittel (München, Germany); Hydroperoxy-cyclophosphamide was from Asta Medica (Frankfurt a.M., Germany); bendamustine hydrochloride was provided by Ribosepharm (Haan/Rhld, Germany). Melphalan, sulforhodamine B reagent, reduced nicotinamide adenine dinucleotide phosphate (NADPH), NADP, reduced glutathione, glutathione reductase, chlorodinitrobenzoic acid (CDNB) and 5,5-dithiobis-2-nitro-benzoic acid (DTNB) were supplied by Sigma (St Louis, MO); PFGE grade agarose was from Beckman (Palo Alto, CA), proteinase K was from Merck (Darmstadt, Germany); *Saccharomyces pombe* and *S. cerevisiae* PFGE marker chromosomes, TE and TBE buffers were obtained from BioRad (Richmond, CA); low melting agarose (incert-agarose) was supplied by FMC (Rockland, MD), L-15 medium was obtained from Boehringer Mannheim (Mannheim, Germany); RPMI 1640 and Dulbecco's modified Eagle medium (DMEM) from Gibco/Life Technology (Eggenstein, Germany). All drug solutions were prepared fresh immediately before use.

Cell lines

The human ovarian carcinoma cell line A2780 (A2780-WT for 'wild-type') established from a non-pretreated patient with ovarian carcinoma and the cisplatin-resistant variant, designated A2780-CP2, were obtained from R Ozols and T Hamilton (Fox Chase Cancer Center, Philadelphia, PA).[9] A2780-CP2 is about 10-fold cisplatin resistant.[10]

The ovarian cancer cell line EOV2 was isolated from ascitic fluid of a patient with ovarian adenocarcinoma, pretreated with cisplatin and cyclophosphamide by our group at the West German Cancer Center. The ovarian cancer cell line JAT was provided by BT Hill (Institut de Recherche Pierre Fabre, France). All ovarian cancer cell lines were grown as monolayers and were maintained in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine and 25% DMEM.

The breast adenocarcinoma cell lines MB 231 and MCF 7 were isolated from pleural effusions of patients, pretreated with 5-FU, MTX, ADM, CPM and prednisone (MB-231) or radiation and hormone therapy (MCF 7). All breast carcinoma cell lines were grown as monolayers and were maintained in L-15 medium supplemented with 10% heat-inactivated FCS. The cell line MCF 7AD, which is about 80-fold doxorubicin-resistant, was established by KH Cowan (NCI, Bethesda, MD) by repeated exposure of MCF 7 cells to increasing concentrations of doxorubicin. The cell line MCF 7 AD expresses the multidrug resistance phenotype. All breast cancer cell lines were grown as monolayers in L-15 medium supplemented with 10% heat-inactivated FCS, non-essential amino acids and L-glutamine. All cell lines were kept in an atmosphere of 5% CO_2 in air at 37°C.

Cytotoxicity assay

For assessment of cytotoxicity, the sulforhodamine B assay was used.[11] Cells in exponential growth were washed with phosphate-buffered saline (PBS), trypsinated with 0.25% trypsin/EDTA for 2 min at 37°C, counted and seeded as a single-cell suspension at a density of 1000 cells/well into 96-well microtiter plates (Falcon, Heidelberg, Germany), and allowed to attach overnight. Then 100 µl of medium containing appropriate drug concentrations was added for 1 h. The drug-containing medium was aspirated from the plates and fresh medium was added. Control dishes without drug exposure were treated identically. After a total incubation time of 120 h, cells were fixed with 50 µl of 50% trichloroacetic acid (TCA) for at least 1 h at 4°C, washed three times with PBS and stained as originally described. Eight wells were used for each drug concentration and the mean values of three independent experiments were calculated. The drug concentration which inhibited cell growth by 50% (IC_{50}) was obtained from semi-logarithmic dose–response plots.

Glutathione assay

The spectrophotometric determination of reduced and oxidized glutathione was carried out as described by Tietze.[12] This assay is based on the reaction of free glutathione with Ellman's reagent (DTNB), producing a change in absorbance at 412 nm. The assay involves enzymatic recycling of oxidized glu-

tathione mediated by glutathione reductase. Cells resuspended in 900 µl PBS were lysed and protein was precipitated with 100 µl ice-cold 100% TCA. After centrifugation at 0°C the supernatant was removed, stored at −20°C and assayed within 1 week. After thawing, the residual protein precipitate was removed from supernatant solutions by extraction with five rinses of 1 ml diethylether. Residual traces of ether were removed by evaporation. The reaction was started by the addition of glutathione reductase and the linear increase in absorbance was compared with that obtained using four standard solutions containing defined concentrations of reduced glutathione. Results were expressed in nmol/mg protein. Evaluation of glutathione-S-transferase activity was performed as published.[13,14]

Measurement of DNA double-strand breaks by CHEF pulsed field gel electrophoresis

Cells were labeled over 48 h with [^{14}C]thymidine (0.05 µCi/ml). After treatment, cells were washed three times with PBS and harvested by trypsinization (0.25% trypsin/EDTA for 2 min at 37°C), counted and seeded as a single-cell suspension at a density of 1×10^6 cells/well in 6-well plates (Falcon, Heidelberg, Germany) and allowed to attach overnight. Thereafter medium containing concentrations of the specific drug which inhibited cell growth by 50% (IC_{50}) was added for 1 h. The drug-containing medium was aspirated from the plates and fresh medium was added. Control dishes without drug exposure were treated identically. After a incubation time of 2, 4 and 24 h, respectively, medium was aspirated from the plates and the cells were washed three times in ice-cold PBS, harvested by a rubber policeman in PBS on ice and sedimented at 1000 r.p.m. for 5 min at 4°C. The pellet was then resuspended in 170 µl of melted agarose (0.5% solution of low melting agarose in PBS) in a 15 ml conical tube maintained at 45°C. The cell–agarose mixture was vortexed, transfered to a gel plug former on ice and refrigerated for 10 min. The plugs were removed and incubated in 5 ml of ESP (0.5 M EDTA, 1% *N*-lauroyl-sarcosine, 1 mg/ml proteinase K, pH 9) for 1–2 h at 4°C then at 45–50°C for 20 h. The plugs were finally washed twice for 1 h at 45°C and thereafter at 6°C in sterile TE buffer (10 mM Tris–HCl, 1 mM Na_2EDTA, pH 7.5) overnight and stored at 4°C in TE buffer (pH 8).

The CHEF assay for resolving double-stranded DNA fragments over a broad range of sizes was articulated as follows. Briefly, gels were cast using 1% agarose in 0.5 × TBE buffer (45 mM Tris base, 45 mM sodium borate, 1.0 mM Na_2EDTA, pH 8.3) and run on a CHEF-DR III (BioRad, München, Germany) operating at 6.0 V/cm. The switch time was linear ramped from 50–90 s for 22 h at 14°C. The reorientation angle was 120°.

At the end of each electrophoretic run, the gel was stained with ethidium bromide, viewed under a UV transilluminator and photographed. The distribution of radioactivity was determined by cutting portions of the gel containing the DNA (well and lane, according to the fragment size). These portions were then melted in plastic scintillation vials in the presence of 50 µl of 1 N HCl and processed for liquid scintillation counting.

Results

Table 1 summarizes the IC_{50} values for bendamustine hydrochloride, melphalan, cyclophosphamide, BCNU and cisplatin in the human ovarian and breast carcinoma cell lines.

From all cell lines, A2780-WT was the most sensitive line. Therefore the IC_{50} values of this cell line were arbitrarily set to 1 to compare relative degrees of resistance (Table 2). When ordered according to sensitivity to cyclophosphamide, melphalan and BCNU, the relative resistance to bendamustine hydrochloride was lower in all cell lines, suggesting only incomplete cross-resistance. This was especially obvious in cell line EOV 2 and in all breast cancer cell lines (MCF 7, MCF 7 AD and MB 231) where relative degrees of resistance were 5.1, 15.1, 17.3 and 24.5 for cyclophosphamide, 4.5, 28.0, 64.5 and 34.4 for melphalan; 2.8, 10.8, 8.4 and 7.3 for BCNU; but only 1.6, 2.6, 3.5 and 3.6 for bendamustine hydrochloride. Furthermore in all breast cancer cell lines lower levels of resistance were also observed when bendamustine hydrochloride was compared with cisplatin. This lack of cross-resistance was most evident in cell lines MCF 7 and MCF 7 AD, which were 13.2- and 12.6-fold resistant to cisplatin, but only 2.6- and 3.5-fold resistant to bendamustine hydrochloride. Notably the alkylators bendamustine hydrochloride, cyclophosphamide and BCNU showed good activity in cell line MCF 7 AD, which is approximatly 80-fold more resistant to doxorubicin than to MCF 7.

To get more insight into the mechanisms which determine response to bendamustine hydrochloride, the basic glutathione levels and the activity of glutathione-S-transferase of the cell lines were rela-

Table 1. Activity of cisplatin, bendamustine hydrochloride, cyclophosphamide, melphalan and BCNU in human ovarian and breast cancer cell lines

Cell line	CDDP	BEN	CPM	L-PAM	BCNU
A2780-WT	7.1 ± 1.3	53.3 ± -3.2	2.4 ± 0.2	5.10 ± 0.8	28.4 ± 5.3
A2780-CP2	40.9 ± 7.1	156.5 ± 9.4	5.8 ± 1.1	29.6 ± 7.7	67.8 ± 10.9
EOV 2	9.0 ± 1.0	84.5 ± 14.0	12.3 ± 2.9	22.9 ± 2.4	79.8 ± 13.9
JAT	33.0 ± 5.3	393.6 ± 58.4	47.7 ± 10.1	270.8 ± 34.8	237.6 ± 28.3
MCF 7	93.7 ± 8.1	138.3 ± 24.4	36.3 ± 7.3	142.8 ± 47.7	307.1 ± 85.9
MCF 7 AD	89.6 ± 9.1	186.7 ± 24.9	41.6 ± 5.3	328.7 ± 87.8	238.0 ± 79.8
MB 231	73.1 ± 16.3	190.0 ± 87.8	58.9 ± 2.9	175.2 ± 84.7	207.1 ± 30.6

IC_{50} values (μmol/l) (mean value of at least six separate experiments ± SD) for cisplatin (CDDP), bendamustine hydrochloride (BEN), cyclophosphamide (CPM), melphalan (L-PAM) and BCNU in cell lines A2780-WT, A2780 CP2, EOV2, JAT, MB231, MCF 7 and MCF 7AD

Table 2. Relative degrees of resistance in human ovarian and breast cancer cell lines to cisplatin, bendamustine hydrochloride, cyclophosphamide, melphalan and BCNU

Cell line	CDDP	BEN	CPM	L-PAM	BCNU
A2780-WT	1	1	1	1	1
A2780-CP2	5.8	2.9	2.4	5.8	2.4
EOV 2	1.3	1.6	5.1	4.5	2.8
JAT	4.6	7.4	19.8	53.1	8.4
MCF 7	13.2	2.6	15.1	28.0	10.8
MCF 7 AD	12.6	3.5	17.3	64.5	8.4
MB 231	10.3	3.6	24.5	34.4	7.3

Evaluation of individual cell line resistance in relation to the most sensitive cell line A2780-WT, whose resistance is defined as 1.

ted to the IC_{50} for bendamustine hydrochloride. The results are shown as regression analysis in Figures 2 and 3. No correlation could be found between the cellular content of glutathione or activity of glutathione-S-transferase and the sensitivity to bendamustine hydrochloride.

Since DNA is the common target for all alkylating drugs and the induction of DNA double-strand breaks is regarded to be an important determinant for response, the ability to induce DNA double-strand breaks was evaluated by means of pulsed field gel electrophoresis for bendamustine hydrochloride, cyclophosphamide, melphalan and BCNU. The results are summarized in Figure 4. When given at equitoxic doses (IC_{50}) melphalan and bendamustine hydrochloride induced significantly more DNA double-strand breaks than cyclophosphamide and BCNU. Furthermore, removal of DNA double-strand breaks induced by bendamustine hydrochloride was relatively slow with the majority of DNA double-strand breaks still being detectable after 24 h.

Discussion

Alkylating agents represent a heterogenous class of anticancer drugs which share the common ability to form DNA intra- or interstrand cross-links and cause DNA single- and double-strand breaks, usually by forming covalent adducts with the DNA bases.[16,17] Alkylating agents belong to the most widely used antitumor drugs. They show high activity in a variety of tumor types and have recently attracted additional interest because they can easily be used in high-dose chemotherapy protocols in combination with autologous bone marrow or stem cell reinfusion.[18–21]

Bendamustine hydrochloride is not really a new anticancer drug since it has been in clinical use since the 1980s, primarily in East Germany.[7,8] However, until now there are no detailed studies regarding the pattern of cross-resistance of bendamustine hydrochloride or the mechanisms of action. We therefore have evaluated this anticancer drug in a panel of established human ovarian and breast

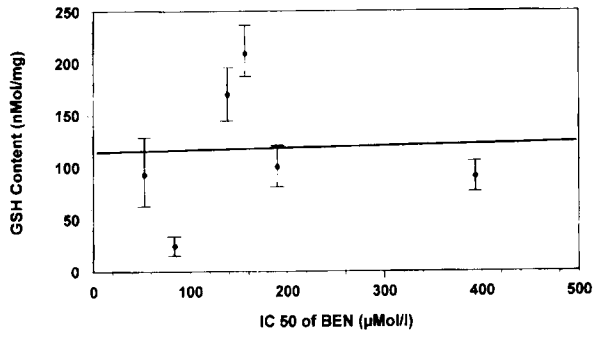

Fig. 2. The IC_{50} values for bendamustine hydrochloride in µmol/l related to the cellular glutathione (GSH) content (mean value of at least six separate experiments ± SD) in nmol/mg protein in the cell lines A2780-WT, A2780-CP2, EOV2, JAT, MB231 and MCF 7 ($p = 0.937$).

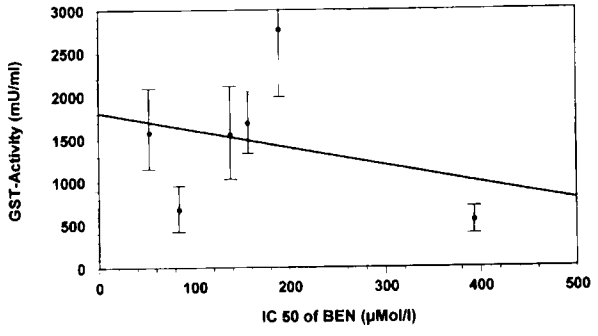

Fig. 3. The IC_{50} values for bendamustine hydrochloride in µmol/l related to the activity of glutathione-S-tranferase (GST) (mean value of at least six separate experiments ± SD) in mU/ml in the cell lines A2780-WT, A2780-CP2, EOV2, JAT, MB231 and MCF 7 ($p = 0.603$).

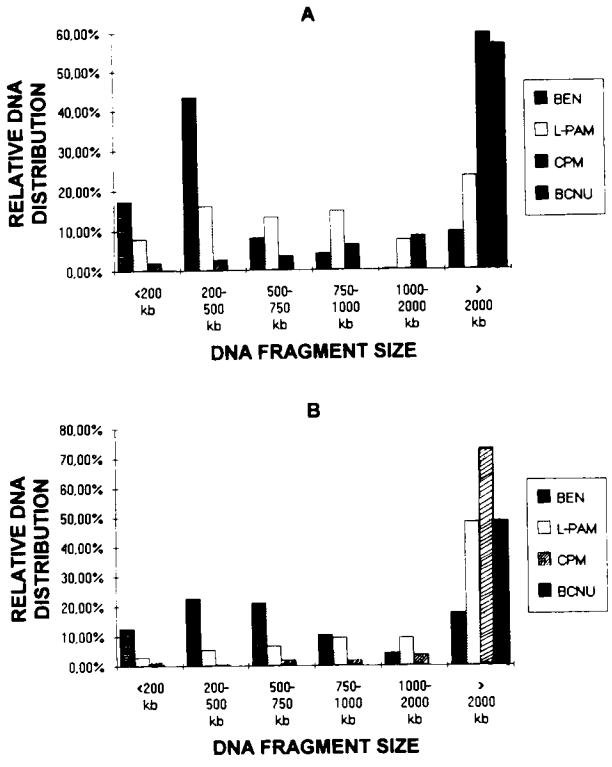

Fig. 4. Bar graph showing the distribution of radiolabeled DNA released from the wells according to fragment size. Incubation with bendamustine hydrochloride (BEN), melphalan (L-PAM), cyclophosphamide (CPM) and BCNU has been performed in equitoxic concentrations (individual IC_{50}) as indicated in Materials and methods. Panels (A) and (B) present results 2 and 24 h after drug incubation. Each lane of the gel has been cut into discrete areas according to the migration pattern of the selected standard chromosomes. Results are expressed as the ratio between the radioactivity in each of the regions and the total radioactivity in the lane, and represent the mean value of three separate experiments.

cancer cell lines and compared its activity to established alkylating agents. It could be demonstrated that bendamustine hydrochloride shows only partial cross-resistance to cyclophosphamide, cisplatin, melphalan or BCNU. This lack of cross-resistance between alkylating agents has also been demonstrated by other investigators.[19,21–27] In particular, the comparatively low level of cross-resistance in ovarian carcinoma cell lines with aquired or natural resistance to cisplatin appears to be promising.

Bendamustine hydrochloride seems not to undergo significant cytosolic detoxification by either cellular glutathione or by glutathione-S-transferase since neither the cellular glutathione level nor the activity of glutathione-S-transferase correlated with the sensitivity of the cell lines to this drug.

Furthermore, bendamustine hydrochloride showed a different capability to induce DNA double-strand breaks when compared with cyclophosphamide or BCNU. When used at equitoxic *in vitro* concentrations (IC_{50}), bendamustine hydrochloride and melphalan induced a significant amount of DNA double-strand breaks as assessed by pulsed field gel electrophoresis. Furthermore, these double-strand breaks appeared to be durable since only a small amount was found to be removed after 24 h. This was in contrast to BCNU or cyclophosphamide, where at the individual IC_{50} significantly less DNA double-strand breaks could be observed. Two explanations are possible; either there are other critical lesions formed by cyclophosphamide or BCNU (e.g. DNA cross-links or DNA–protein links) which contribute to the cytotoxic activity of BCNU or cyclophosphamide or these two agents produce DNA strand breaks in critical, most likely actively transcribed regions of the genome whereas the

majority of the strand breaks produced by bendamustine hydrochloride might appear in not described regions of the DNA.

Nitrogen mustards such as bendamustine hydrochloride are known to cause the formation of cross-links between the paired strands of the DNA double helix. The ability of bendamustine hydrochloride to form cross-links with DNA has been investigated by renaturation experiments. Renaturation efficiency was analyzed by melting measurements after alkaline denaturation. From these results only negligible cross-link formation can be inferred for bendamustine hydrochloride.[28] Further studies which address the formation of DNA cross-links or DNA–protein links are needed to assess this issue.

Conclusion

In summary, these results show that bendamustine-hydrochloride is an alkylating agent which differs in several properties from the classical alkylators like cyclophosphamide or melphalan. It might therefore be a useful addition to our armentarium of cytotoxic drugs since in long clinical experience it could demonstrated that bendamustine hydrochloride shows a favorable toxicity profile and demonstrates activity in breast cancer and malignant lymphoma. Therefore, further preclinical and clinical development of this new alkylator appears to be justified.

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