In vitro induction of apoptosis of neoplastic cells in low-grade non-Hodgkin's lymphomas using combinations of established cytotoxic drugs with bendamustine

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Background and Objectives. Regulation of apoptotic cell death is being increasingly recognized as a mechanism by which cytostatic agents mediate tumor cell death. Preliminary clinical studies with bendamustine, an alkylating agent with a purine nucleus, provide strong evidence that this drug is a highly effective cytostatic in low grade lymphomas. We, therefore, investigated the *in vitro* activity of bendamustine in combination with other established cytotoxic drugs.

Design and Methods. Two cell lines (DOHH-2, WSU-NHL) and mononuclear cells (MNC) from patients with leukemic low-grade B-non-Hodgkin's lymphoma (NHL) (n=10), T-NHL (n=7) and chronic lymphocytic leukemia (CLL) (n=12). Apoptosis (7-AAD), depolarization of mitochondrial membrane potential (MMP, JC-1), caspase-3-activity (FIENA) and cell proliferation (XTT/WST-1) were determined. Several incubation times and drug dosages (for $IC_{30/50/75/90}$) were studied. Synergistic, additive or antagonistic effects were calculated by a median plot effect and the combination index (CI) method.

Results. In general, combinations of bendamustine with mitoxantrone or doxorubicin resulted in antagonistic effects in the tested cell lines and the MNC from the patients. CI-calculation failed in these cases since there was not a sufficient dose response. On the other hand, the combination of bendamustine with 2-CdA showed synergistic *in vitro* activity on the tested cell lines, neoplastic lymphocytes from patients with peripheral T-cell lymphomas and partially on MNC from patients with CLL and B-NHL. The antagonism of the combination of bendamustine and anthracyclines appeared to be due to inhibition of depolarization of mitochondrial membrane potential and caspase-3-activity during apoptosis of the studied cell lines.

Interpretation and Conclusions. In conclusion, our results suggest that schedules using combinations of bendamustine and anthracyclines should not be recommended for the treatment of low-grade NHL, whereas bendamustine combined with 2-CdA could be considered for the development of future treatment strategies.

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Key words: bendamustine, antagonistic interactions, apoptosis, mitochondrial-membrane potential, caspase-3-activity

Bendamustine hydrochloride is a bifunctional alkylating agent with a nitrogen mustard group and an additional purine nucleus.[1,2] It was developed in East Germany in the late sixties. The nitrogen mustard group in position 5 is linked to a benzimidazole nucleus, with a methyl group in position 1 and a butanic acid residue in position 2.[1] The drug has been shown to be effective in the treatment of breast cancer[3] and other solid tumors.[4,5] In the last 2 years several clinical phase I and II trials on treatment of low-grade non-Hodgkin's lymphoma (NHL) with a bendamustine-containing regimen have been carried out.[6-9] These studies revealed a high activity in patients with refractory or relapsed low-grade NHL, chronic lymphocytic leukemia (CLL) and multiple myeloma.[10-13] The activity of bendamustine in the treatment of low-grade NHL seems to be comparable to that of purine analogs such as fludarabine or cladribine (2-CdA).[14,15] As for other alkylating agents, the toxicity of bendamustine (nausea and vomitimg, alopecia or myelosuppression) is low.[15] Bendamustine induces DNA strand breaks as well as apoptosis.[16] Little is known about its cross-resistance or interactions with other chemotherapeutic drugs. In order to gain a first indication of possible benefits of combination regimes containing bendamustine for the treatment of low-grade NHL, it is feasible to investigate different drug combinations and dose schedules *in vitro*. Mechanisms of drug-induced apoptosis,[17] including its regulation[18,19] or even the effect on inhibition of cell proliferation by cytotoxic drugs may provide information useful for designing more effective treatment strategies[20] by elucidating the main pathways used by the different chemotherapeutic agents.[21] Due to its structure bendamustine may interact as an alkylating agent as well as a purine analog. Several *in vitro* studies have demonstrated synergistic interactions between purine analogs and anthracyclines[22] and between purine analogs and alkylating agents.[23]

Therefore assessment of the in vitro activity of bendamustine in combination with mitoxantrone, doxorubicin and 2-CdA on follicular cell lines and mononuclear cells (MNC) of patients with leukemic low-grade NHL may facilitate the planning of future treatment regimens using bendamustine-containing combinations. Although the value of such in vitro studies in designing clinical protocols has not yet been proven, the results may facilitate general considerations regarding treatment of NHL.

Design and Methods

Patients

The influence of the various drug combinations on apoptosis was tested on mononuclear cells (MNC) from peripheral blood of patients with leukemic low-grade B-NHL (n=10), T-NHL (n=7) and CLL (n=12). Patients were untreated or had not received treatment within the 6 months prior to the in vitro testing. Diagnoses were confirmed by bone marrow biopsy and immunophenotyping of leukemic cells. The malignant cells represented > 80% of the total MNC.

Entities

Low-grade B-NHL: 3 mantle-cell, 2 marginal, 4 not further classified, 1 immunocytoma; T-NHL: 1 PLL, 1 NK-like, 1 NK-cell, 1$\gamma\delta$, 3 not further classified; CLL: 7 typical, 2 atypical, 3 $CD38^+$.

Cell lines

Two follicular lymphoma cell lines (DOHH-2, WSU-NHL, DSMZ, Braunschweig, Germany) were used.

Cell preparation and incubation with bendamustine ± other drugs

Peripheral mononuclear cells obtained from patients with leukemic low-grade B-NHL, T-NHL or CLL were isolated by Ficoll-gradient sedimentation and washed twice in PBS. Cells were then incubated with drugs at various concentrations (see later) in RPMI medium (LifeTechnology, Paisley, Scotland) supplemented with 10% fetal calf serum (Greiner, Frickenhausen, Germany), 2% L-glutamine (Life Technology), and 1% penicillin/streptomycin (Bio Whittaker, Verners, Belgium). The cell lines and MNC from patients with leukemic low-grade B-NHL, T-NHL or CLL were incubated (1×10^6cells/mL medium) with the cytotoxic drugs as described below in a humified atmosphere at 37°C using different incubation schedules. Apoptosis or cell proliferation was then evaluated.

Incubation periods for malignant cells from patients and cell lines prior to determination of apoptosis or cell proliferation

Simultaneous incubation. Freshly isolated cells were incubated continuously for 24 or 48h at 37°C with drug A ± other drugs.

Consecutive incubation. Freshly isolated cells were treated with drug A for 2h, washed twice and incubated for a further 21h without addition of drugs. Subsequently, drug B was added for a further 24h.

Drug concentrations. Bendamustine hydrochloride (Ribosefarm, Muenchen, Germany) in the range from 1 µg/mL to 100 µg/mL. Cladribine (2-CdA) (Janssen-Cilag GmbH, Neuss, Germany) in the range from 0.01µg/mL to 0.1µg/mL. Doxorubicin (Pharmacia & Upjohn GmbH, Erlangen, Germany) in the range from 0.1 µg/mL to 1 µg/mL. Mitoxantrone (Lederle Arzneimittel GmbH, Wolfratshausen, Germany) in the range from 0.01 µg/mL to 1 µg/mL. Mafosphamide (Asta Medica, Frankfurt, Germany) in the range from 0.15 µg/mL to 6µg/mL. Fludarabine (Medac Schering, München, Germany) in the range from 0.25 µg/mL to 10 µg/mL. Drug dosages were chosen to reach the IC_{30}, IC_{50}, IC_{75} and IC_{95} (data not shown).

Analysis of apoptosis by flow cytometry

Apoptotic cell death was analyzed by a FACScan flow cytometer with the Lysis II software package (Becton Dickinson, Heidelberg, Germany). The rate of apoptosis was assessed using 7-aminoactinomycin D (7-AAD, Sigma, Deisenhofen, Germany). Assays were performed in triplicate. The populations of leukemic cells were gated in the forward side scatter/side side scatter dot plot. The percentage of apoptotic cells of these populations was defined by their distribution in a fluorescence (caused by 7-AAD) dot plot (Lysis II, Becton Dickinson).[22]

Disruption of mitochondrial membrane potential $\Delta\Psi_m$: was measured using a specific fluorescent probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Alexis Biochemicals, Gruenberg, Germany) which was incubated at 37°C with the cells at a concentration of 5 µg/mL for 20 min. After incubation with JC-1, cells were analyzed by FACScan flow cytometry in fluorescence channels FL1 and FL2. JC-1 emits a red fluorescence (JC-1 aggregates, high $\Delta\Psi_m$) when sequestered in the mitochondrial membrane of healthy cells and emits a green fluorescence (JC-1 monomers, low $\Delta\Psi_m$) when released into the cytoplasmic compartment of the cell.[24] At depolarized (–100 mV) membrane potentials JC-1 green monomer emission peaks are around 527 nm. At hyperpolarized membrane potentials (-140 mV) the emission of the JC-1 aggregates shifts towards 590 nm.[25-27]

Caspase-3-like activity

A fluorometric immunosorbent enzyme assay (FIENA) for specific and quantitative determination of caspase-3 activity (Roche, Mannheim, Germany) was used. Caspase-3 from cellular lysates (previously incubated with

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single drugs and drug combinations) was captured by a monoclonal antibody in anti-caspase-3 coated (100 µL anti-caspase-3 coating solution/well incubated over night) microtiter plates (MTP). Following the washing steps, carried out according to the manufacturer's instructions, substrate solution (100 µL/well incubated for 2 hours) was added. The substrate was cleaved proportionally to the amount of activated caspase-3. Due to the proteolytic cleavage of the substrate, free fluorescent Ac-DEVD-AFC was generated. Free AFC was determined fluorometrically using a multifunctional reader (Tecan, Crailsheim, Germany) at λ_{max} = 505 nm. The developed fluorochrome was proportional to the concentration of activated caspase-3.

Cell proliferation

Proliferation/metabolic activity was detected using the tetrazolium-based assays, XTT and WST-1 (Roche). Cells were incubated with the drugs at a total volume of 100 µL/well in 96-well MTP. Cell-proliferation agents, XTT (50 µL/well) or WST-1 (10 µL/well), were added and incubated for 4 hours (XTT) or 30 min (WST-1) at 37°C in a humidified atmosphere. Absorbance was measured using an multifunctional reader (Tecan) at 450-500 nm (XTT) or 420-480 nm (WST-1) with a reference wave length >650 nm (XTT) or >600 nm (WST-1).

Statistical analysis

To determine synergistic, additive or antagonistic effects of the drug combinations, Calcusyn software (Biosoft, Cambridge, UK) was used. Synergism was defined as more than the expected additive effect of drugs, and antagonism as less than the expected additive effect. In accordance with the suggestions of Chou et al.,[28-30] the median effect plot and the combination index (CI) were determined. A general equation for dose-effect was defined as:

$$f_a/f_u = (D/D_m)^m$$

where D = dose of drug, D_m = median-effect dose signifying the potency, f_a= fraction affected by the dose, f_u= fraction unaffected ($f_u = 1-f_a$), and m= exponent signifying the sigmoid shape of the dose-effect curve. It was determined by the slope of the median-effect plot. The median-effect plot was a plot of x = log (D) against y = log (f_a/f_u):

$$\log (f_a/f_u) = m \log (D) - m \log (D_m)$$

This equation has the form of astraight line, y = mx + b. The combination index (CI) for mutually non-exclusive drugs that have different modes of action is defined as:

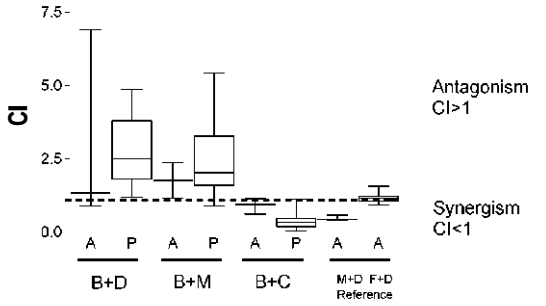

Figure 1. Combination index (CI) values obtained from experiments (24/48 hours) using the lymphatic cell lines, DOHH-2 and WSU-NHL. B=bendamustine, D=doxorubicin, M=mitoxantrone, C=cladribine (2-CdA), M=mafosfamide, F=fludarabine, A=apoptosis determined by 7AAD or JC-1, P=proliferation determined by XTT or WST-1. To control the results of our cell line model we repeated previously published experiments,[23,31] using other drug combinations (F+D, M+D, Reference).

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1(D)_2/(D_x)_1(D_x)_2$$

where $(D)_1$= drug 1, $(D)_2$= drug 2 and $_x$= effect $_x$ % (rate of apoptosis %).

In the majority of cases a strong inhibitory effect was achieved on the MNC of patients when bendamustine was applied with mitoxantrone or doxorubicin. CI-calculation failed in these cases since a sufficient dose response was not present. Since synergism is defined as more than the expected additive effect of the drugs when used as single agents, in cases of drug combination, we compared actually measured effects with expected effects.[22,23] A Wilcoxon's two-sided test was used. Results were considered to be statistically significant when $p<0.05$.

Results

Bendamustine + other drugs in cell lines

To investigate the interactions between bendamustine and other drugs we first investigated the influence of the drug combinations on two lymphatic follicular cell lines, DOHH-2 and WSU-NHL. Figure 1 presents the assessed combination index (CI) values determined by apoptosis (7AAD) as well as by proliferation assays (XTT/WST-1) of all investigated simultaneously applied drug combinations containing bendamustine + either mitoxantrone, doxorubicin or cladribine after 24 and 48 hours. The combination of bendamustine with doxorubicin was strongly antagonistic on induction of apoptosis as well as on inhibi-

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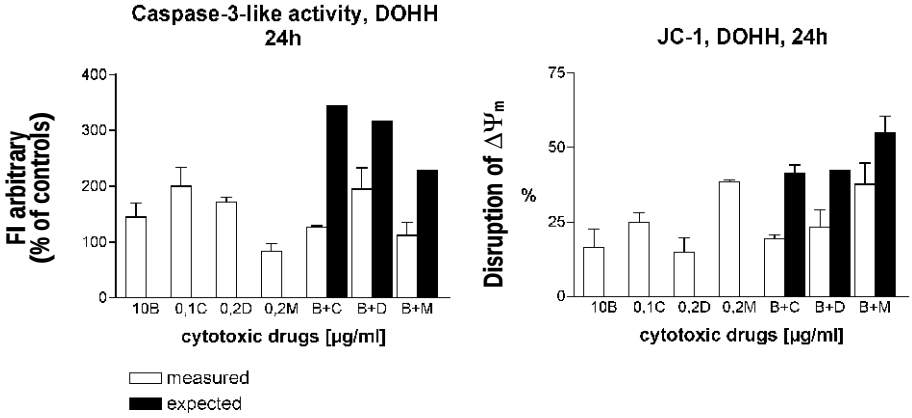

Figure 2. Caspase-3-like activity and disruption of mitochondrial membrane potential (MMP) $\Delta\Psi_m$ determined in cell line DOHH-2 after 24 hours. B=bendamustine, D=doxorubicin, M=mitoxantrone, C=cladribine (2-CdA). The data are shown as % increase of control.

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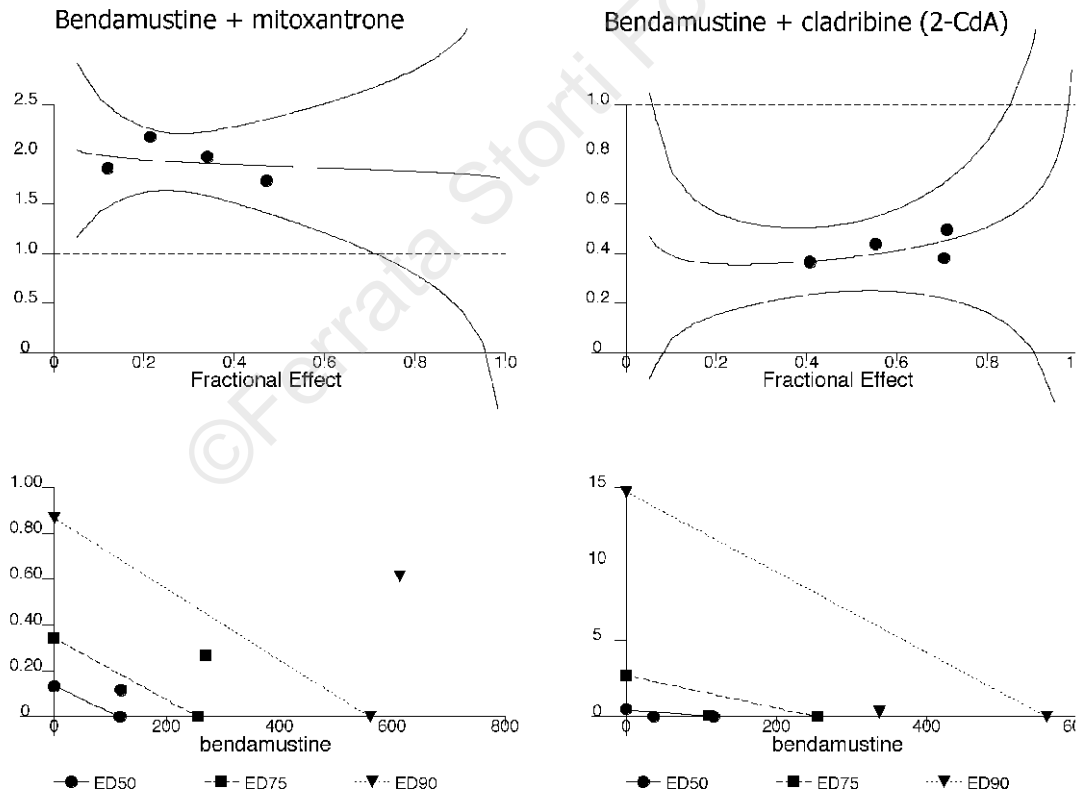

Figure 3. Combination index (CI) values and isobologram graphs measured using MNC from a patient with leukemic low-grade B-NHL after 24 hours. These results were representative of the other experiments. s.d.=standard deviation.

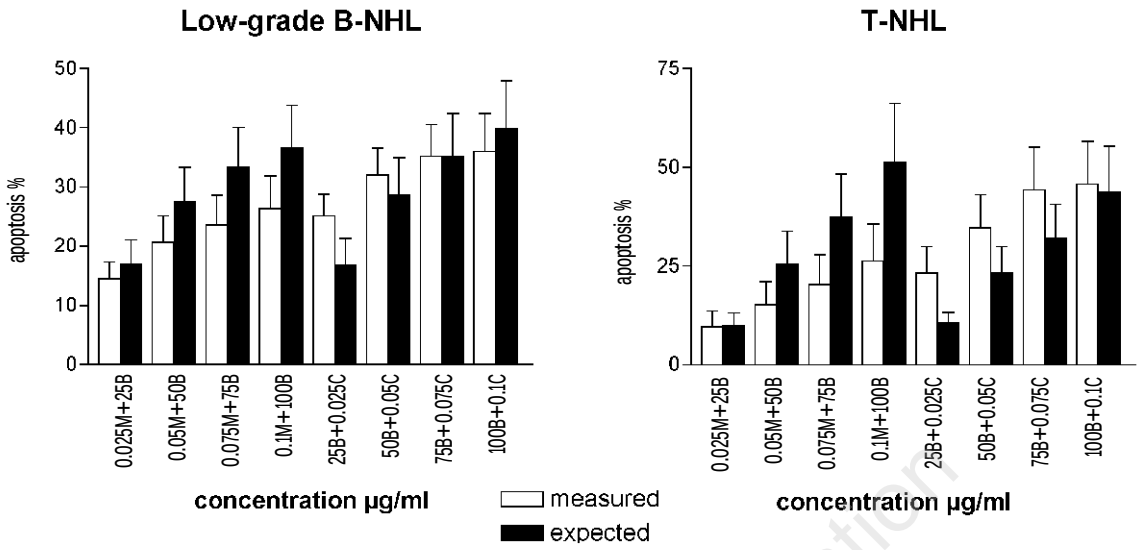

Figure 4. Overview of mean values obtained from experiments on cells from patients with leukemic low-grade B-NHL or T-NHL. Comparisons of expected additive values caused by the single drugs vs. actually measured values caused by the combination of the drugs. B=bendamustine, M=mitoxantrone, C=cladribine (2-CdA).

tion of proliferation (apoptosis: CI_{max}=6.9, proliferation CI_{max}=4.8). Similar results were obtained when bendamustine was combined with mitoxantrone. The CI_{max} of apoptosis was 2.3, the CI_{max} of proliferation measured by tetrazolium-based XTT or WST-1 assays was 4.4 (Figure 1). Synergistic effects were demonstrated when bendamustine was combined with the purine analog cladribine. The combination index of highest synergy of the combination of bendamustine with cladribine determined by apoptosis assay was 0.8 and that measured by inhibition of proliferation was 0.03 (Figure 1). To check our results by confirming previously published data,[23,31] the cell lines were also incubated with fludarabine + doxorubicin and mafosfamide + doxorubicin. The combination of fludarabine with doxorubicin resulted in additive effects, whereas mafosfamide and doxorubicin were synergistic (Figure 1).

Caspase-3-like activity and mitochondrial membrane potential

In order to deepen the analysis of the synergistic and antagonistic interactions of bendamustine combined with other chemotherapeutic drugs, we measured caspase-3-like activity and determined the disruption of the membrane potential, $\Delta\Psi_m$. Figure 2 shows the activation of caspase-3 induced by bendamustine, cladribine, doxorubicin and mitoxantrone as well as the combination of bendamustine with

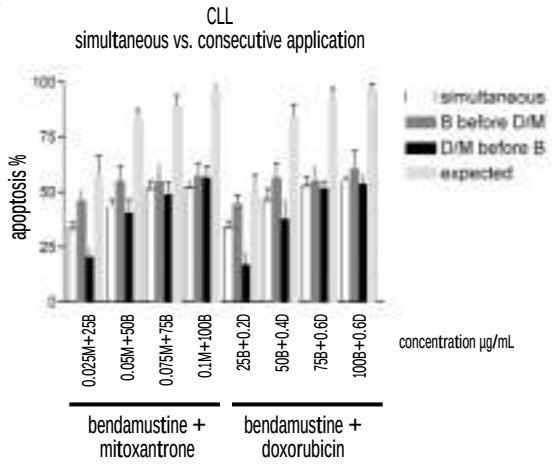

Figure 5. Mean values of experiments on CLL cells using different incubation schedules. Comparisons of expected additive values caused by the single drugs vs. actually measured values caused by the combination of the drugs. B=bendamustine, D=doxorubicin, M=mitoxantrone.

each drug after 24 hours in the DOHH-2 cell line. Of the tested single drugs, cladribine induced the highest caspase-3 activity (200.38 FI-abitrary units % increase of control (FI%)), while mitoxantrone exihibited the lowest effect on caspase-3 activation (83.35 FI % increase of control). In order to determine any inhibitory effect on capase-3 activation the actu-

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ally measured values were compared with the expected additive values caused by the drugs alone. Caspase-3 activity was inhibited when bendamustine was applied with mitoxantrone (expected 227.9 FI%, actually measured 112.28 FI%), doxorubicin (expected 316.91 FI%, actually measured 194.75 FI%) as well as with cladribine (expected 344.92 FI%, actually measured 127.1 FI%, all shown as percentage increase over control, Figure 2). Similar results were obtained determining the disruption of the mitochondrial membrane potential (MMP), $\Delta\Psi_m$. When used as a single drug, mitoxantrone induced the highest rate of cells revealing loss of MMP (38.46 % of cells, Figure 2) while doxorubicin showed the lowest rate of disrupted MMP (14.77%, Figure 2). Using combinations of the tested drugs, inhibitory effects on disruption of MMP were revealed in all cases: bendamustine combined with mitoxantrone (expected disruption 54.87%, actually measured disruption 37.71%), doxorubicin (expected 31.18%, actually measured 23.35%) as well as with cladribine (expected 41.26%, actually measured 19.47%, Figure 2).

Effects of drug combinations on MNC of patients

Representive results, demonstrated as combination index and corresponding isobolograms, of the tested drugs on a patient with leukemic low-grade B-NHL are show in Figure 3. As in the tested cell lines, the combination of bendamustine with mitoxantrone clearly exhibited a strong antagonistic interaction, while bendamustine combined with cladribine had synergistic effects (Figure 3). The CI values of the combination bendamustine + mitoxantrone were at the effective dose 50 (ED_{50}) of 1.89, at the ED_{75} of 1.84 and at the ED_{90} of 1.80 (Dm=0.12, m=1.33, r=0.99). Bendamustine with cladribine revealed CI values at ED_{50} 0.39, at ED_{75} 0.48 and at ED_{90} 0.62 (Dm=0.04, m=0.99, r=0.98). Since the combination index method failed due to insufficient dose response slopes caused by the inhibitory effects of the drugs the mean values of the conducted experiments were analyzed as shown in Figure 4. Again the actually measured value of the tested drug combination was compared with the expected additive value caused by the single drugs. Simultaneous applications of the drugs caused antagonistic effects at all tested dosages of bendamustine and mitoxantrone on MNC of low-grade B-NHL as well as T-NHL (measured value<expected value, $p<0.05$). Synergistic effects of bendamustine combined with cladribine on MNC of low-grade B-NHL were only observed for the two lower drug concentrations whereas a synergistic effect of bendamustine + cladribine on MNC of patients with T-NHL was observed at all tested drug concentrations (measured value<expected value, $p<0.05$, Figure 4). The results in patients with CLL were nearly identically to those of the experiments in patients with low-grade B-NHL. Bendamustine combined with mitoxantrone interacted in an antagonistic manner at all dosages (Figure 5). In combination with cladribine, bendamustine showed synergistic effects only when lower dosages were applied (data not shown). When the cytotoxic drugs were applied consecutively (bendamustine before anthracyclines or anthracyclines before bendamustine) on MNC of patients with CLL, significant antagonistic effects were observed at all dosages and with all incubation schedules. There was a tendency for the antagonism to be stronger when anthracyclines were applied before bendamustine (Figure 5), but this trend was not statistically significantly different from the results of the other incubation schedules.

Discussion

The introduction of purine analogs in the treatment of low grade lymphomas has induced major improvement in the last decade in terms of induction of remissions and progression-free survival. However, it is still unclear whether this improvement also applies to overall survival. Recent developments therefore include combinations of a purine analog and anthracyclines[32] or alkylating agents.[23,33] Another effective agent newly recognized for the treatment of NHL is bendamustine. In the last several years initial reports from clinical phase I and II studies demonstrated that bendamustine, used as a single agent, has high activity in the treatment of CLL and multiple myeloma as well as in low-grade non-Hodgkin's lymphomas.[13-15] This cytotoxic drug is of special interest, since it consists of an alkylating nitrogen mustard group and a purine nucleus. It has been speculated that both groups might be active and therefore responsible for the high effect in low-grade lymphomas.

The efficacy of chemotherapeutic agents is a result of induction of apoptosis and inhibition of cell proliferation. One way to improve treatment with drug combinations may be to investigate interactions of combinations of chemotherapeutics in vitro. We have previously shown that purine analogs combined with anthracyclines have synergistic effects on lymphoma cell lines, as well as on cells from patients with low-grade NHL.[22,32]

In the present study we investigated the efficacy of already established drugs for the treatment of low-grade NHL, such as mitoxantrone, doxorubicin or cladribine, in combination with bendamustine on apoptosis on follicular lymphoma cell lines and on

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MNC from patients with low-grade B-NHL, T-NHL and CLL. The cell line model clearly demonstrated synergistic effects of bendamustine combined with cladribine on induction of apoptosis and inhibition of proliferation. When bendamustine was combined with anthracyclines the effects were mainly antagonistic. To clarify these interactions further we investigated two main events of apoptotic cell death after incubation with the drugs: the activation of executioner caspase-3 and the disruption of mitochondrial membrane potential $\Delta\Psi_m$. Interestingly we observed inhibition of caspase-3 activation as well as of disruption of MMP $\Delta\Psi_m$ at all tested drug combinations. It is a logical hypothesis that the combination of bendamustine and anthracyclines, by inhibiting two main events of apoptotic cell death, could have antagonistic interactions. The inhibition at the mitochondrial level as well as at the level of the executioner caspase may also influence the cell cycle and its associated pathway proteins.[20,34] This may explain the antagonistic interactions on inhibition of cell proliferation. Interestingly we also observed this inhibition of caspase-3 activation and inhibition of disruption of MMP when bendamustine was combined with cladribine. The question remains open as to why this particular drug combination results in synergistic interactions on apoptosis. We hypothesize that a further involvement of apoptosis and cell cycle-associated proteins, such as p53 or Rb, beneath the level of caspase activation may be responsible for the synergistic interaction of bendamustine and cladribine. Another reason may be that they target different phases of the cell cycle. While doxorubicin and mitoxantrone act on the S/G2 phase,[35, 36] bendamustine and cladribine can affect the G0 phase.[1,37] A potentiation of G0 phase interaction, in which resting cells or cells of indolent lymphomas arrest could also be a reason for synergistic effects of bendamustine + cladribine.

The results of the experiments on ex vivo cells from patients in our study support the findings of the cell line model. In general the combination of bendamustine with anthracyclines had antagonistic effects while bendamustine with cladribine had synergistic interactions. These are noteworthy results considering the structure of bendamustine. Since bendamustine contains an additional purine nucleus it was hypothesized that it may also interact as a purine analog.[14] As shown in previous studies purine analogs interact synergistically in combination with anthracyclines on cells of patients with CLL or low-grade lymphomas.[22] However, bendamustine clearly has a different mechanism of interaction than that of purine analogs. Our cell line model demonstrated a synergistic effect of mafosfamide + doxorubicin. This adds support to the hypothesis that bendamustine induces apoptosis in a different manner than other alkylating agents.

Preliminary unpuplished data from clinical phase II studies indicate that bendamustine in combination with purine analogs does not increase the incidence of severe infection (M. Herold, personal communications). Therefore this combination may be a treatment option in low-grade lymphomas and possibly also in patients with relapsed hairy cell leukemia.

Another question of our study was whether the demonstrated antagonism between bendamustine and the anthracyclines can be reduced or even abolished by modifying the incubation schedule. As shown previously it is necessary to modify doses and incubation schedules of purine analogs combined with other chemotherapeutic drugs in order to obtain a high in vitro efficacy.[38,39] In these experiments it was not possible to reduce the antagonistic effects by changing the incubation schedule. On the contrary, administering the anthracyclines before bendamustine tended to enhance the antagonistic effect.

In conclusion our study implies that bendamustine combined with anthracyclines may not be a clinically highly effective treatment of low-grade lymphomas. Bendamustine in combination with cladribine, on the other hand, may be considered for clinical investigation.

Contributions and Acknowledgments

KUC performed the in vitro apoptosis studies in CLL and in the cell lines, evaluated the data and wrote the paper. SB carried out the caspase-3 activity assays and took part in the evaluation of the data. KG did the in vitro apoptosis studies in samples from low grade lymphomas and T-cell lymphomas. AK performed the JC-1 assay. DH and PSM critically reviewed the drafts of the manuscript for important intellectual content. EW co-ordinated the work, supervised the study and critically reviewed the drafts of the manuscript for important intellectual content.

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Disclosures

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Manuscript processing

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this paper was taken jointly by Prof. Lo Coco and the Editors. Manuscript received December 29, 2000; accepted March 26, 2001.

Potential implications for clinical practice

Future treatment protocols involving bendamustine in low grade lymphomas could be designed to evaluate the combination of bendamustine with cladribine.

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