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Synergistic effects of chemotherapeutic drugs in lymphoma cells are associated with down-regulation of inhibitor of apoptosis proteins (IAPs), prostate-apoptosis-response-gene 4 (Par-4), death-associated protein (Daxx) and with enforced caspase activation

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Abstract

Cytotoxic drugs mediate apoptotic tumor cell death by influencing key regulator proteins of programmed cell death. In clinical practice cytotoxic drug combinations are desired to potentiate tumor cell kill and to minimize side effects. Nevertheless, the molecular mechanisms underlying synergistic and antagonistic effects on tumor cells are still poorly understood. In order to elucidate these molecular mechanisms we established models of synergistic and antagonistic drug combinations within the same lymphoma cell lines.

By combination index method we demonstrated that bendamustine in combination with either doxorubicin or mitoxantrone caused antagonistic effects on disruption of mitochondrial membrane potential as well as on the rate of apoptosis. In contrast the combination of bendamustine with cladribine acted synergistically on these parameters. By using the IC_{50} (dosages causing 50% rate of apoptosis) the synergistic effect of the combination of bendamustine and cladribine was associated with an enhanced mitochondrial release of cytochrome *c* and Smac/DIABLO, by down-regulation of x-linked inhibitor of apoptosis (XIAP), cIAP1, Par-4 and Daxx as well as by a significantly increased activation of caspases-3, -6, -7, -8 and -9. At the same rate of apoptosis (IC_{50}), the antagonistic combinations did not increase the release of cytochrome *c* or Smac/DIABLO, nor down-regulate the expression of XIAP, cIAP1, Par-4 and Daxx, nor increase the activation of caspases. The role of down-regulation of IAPs and of enforced caspase activation for synergism in this model was supported by the observation, that broad spectrum inhibition of caspases re-established expression of XIAP.

Our study is the first to outline the molecular alterations caused by synergistic and antagonistic drug combinations within the same lymphoma cell model. The above described mechanisms were already assessable at a point where the effects of synergistic or antagonistic combinations could not yet be discriminated quantitatively by the level of apoptosis rate of the lymphoma cells.

Keywords: Bendamustine; Cladribine; Par-4; Daxx; IAPs; Caspases

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Abbreviations: Par-4, prostate-apoptosis-response-gene 4; Daxx, death-associated protein; XIAP, x-linked inhibitor of apoptosis; cIAP, cellular inhibitor of apoptosis; caspases, cysteine proteases that cleave after aspartatic acids; PARP, poly(ADP-ribose)-polymerase; Bcl-2, B-cell lymphoma-gene 2; Bax, Bcl-associated x-protein; Bax, Bak, Bad, Bid, pro-apoptotic members of the Bcl-2 family; Bcl-2, Bcl-xL, anti-apoptotic members of the Bcl-2 family; AIF, apoptosis-inducing factor; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low PI in mouse; PML, promyelocytic leukemia; CI, combination index; IC_{50}, inhibiting concentration 50%; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine-iodide.

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1. Introduction

Induction of apoptosis by cytotoxic drugs is a mechanism by which tumor cells are forced to undergo cell death. The molecular biology of this process has been well characterized during the last decade [1–4]. Other groups as well as ours described a number of drug combinations, resulting in synergistic, additive or antagonistic effects on apoptosis of lymphoma cells [5–9]. However, there is still a lack of information on specific molecular mechanisms induced by synergistic or antagonistic combinations

of cytotoxic drugs, which may explain these different effects.

Two distinct apoptotic signaling pathways have been described. The first signaling pathway is triggered by death receptors (i.e. CD95/Apo-1/Fas or TRAIL), which are members of the tumor necrosis factor receptor family [10–13]. After activation via these receptors procaspase-8 is cleaved, followed by the activation of the downstream effector caspases-3, -6 and -7. Through the cleavage of Bid caspase-8 is also able to activate the second apoptotic pathway, the mitochondrial pathway [2,14], with the subsequent release of cytochrome *c*, AIF [15] and Smac/DIABLO [16]. These are liberated from the intermembrane space into the cytosol, subsequently activating caspase-9, which then induces the execution phase of apoptosis. These events are controlled by the Bcl-2 family members, some of which are located in the mitochondria, either promoting (Bax, Bak, Bad, Bid) or inhibiting (Bcl-2, Bcl-xL) apoptosis [4]. Involved in several steps of apoptotic cell death in lymphatic cells is Par-4, reportedly exhibiting a proapoptotic influence [17]. As mentioned above both pathways are finally characterized by the activation of cysteine proteases called caspases [18,19]. Each caspase is synthesized as an inactive precursor that is converted by proteolytic cleavage into an active heterodimer upon exposure of cells to a variety of stimuli, i.e. chemotherapeutic anticancer or physical agents [20,21]. Activation of caspases is controlled by the inhibitors of apoptosis proteins (IAPs) being able to prevent activation of caspases-3, -7 and -9 [22–26]. Nevertheless, caspase-dependent as well as caspase-independent execution of apoptosis has been described, in particular in association with Daxx [27,28].

We recently identified synergistic and antagonistic drug combinations *in vitro*, containing bendamustine hydrochloride, a new bifunctional alkylating agent characterized by a nitrogen mustard group and an additional purine-like benzimidazol nucleus [29]. Several phase II studies have proven its efficacy in the treatment of low- and high-grade non-Hodgkin's lymphomas (NHL) [30,31].

This study was carried out to elucidate molecular mechanisms responsible for the exhibition of synergistic and antagonistic effects caused by the several drug combinations. This knowledge may enable us to integrate drug combinations in a more specific manner in tumor therapy.

2. Material and methods

2.1. Cell lines

Two follicular lymphoma cell lines (DOHH-2, WSU-NHL; DSMZ) were used to determine the rate of apoptosis and the disruption of mitochondrial membrane potential. The molecular mechanisms of apoptosis were investigated using the DOHH-2 cells.

2.2. Cell preparation and incubation with chemotherapeutic agents

Cells were incubated with drugs at concentrations as specified in RPMI medium (Life Technology) supplemented with 10% fetal calf serum (Greiner), 2% L-glutamine (Life Technology) and 1% penicillin/streptomycin (Bio Whittaker). 1×10^6 cells/mL medium were incubated with the cytotoxic drugs as described in a humified atmosphere at 37°.

2.3. Drug concentrations

Bendamustine hydrochloride (Ribosepharm) was applied at 1–100 μg/mL, cladribine (2-CdA) (Janssen-Cilag GmbH) at 0.01–0.5 μg/mL, doxorubicin (Pharmacia & Upjohn GmbH) at 0.1–1 μg/mL, mitoxantrone (Lederle Arzneimittel GmbH) at 0.01–1 μg/mL.

2.4. Incubation schedules

Cells were incubated with single agents or with drug combinations (applied simultaneously) for 24 and 48 hr. For Western blot analysis of apoptosis-related proteins cells were incubated for 2, 6 (in part 8, 10, 12) and 24 hr. For Western blot analysis of expression of proteins released from mitochondria during apoptosis (cytochrome *c*, AIF and Smac/DIABLO) cells were incubated 6, 12 and 18 hr. To determine caspase activity cells were incubated for 2, 4, 6, 9, 12, 16, 20 and 24 hr.

2.5. Flow cytometry

Apoptotic cell death was analyzed by a FACScan flow cytometer with the CellQuest software package (Becton Dickinson) using the Annexin V-Kit (Bender MedSystems, Vienna/Alexis Corporation). Assays were performed in triplicates. Populations of cells were gated in a forward scatter/side scatter dot plot. The percentage of apoptotic cells was defined by their distribution in a fluorescence (caused by annexin/propidium iodide) dot plot (WinMdi, Version 2.8, Scripps Research Institute).

2.6. Disruption of mitochondrial membrane potential (MMP)

MMP was measured using a specific fluorescent probe, JC-1 (Alexis Biochemicals) at a concentration of 5 μg/mL for 20 min. After incubation with JC-1 cells were analyzed by FACScan using fluorescence channels FL1 (green) and FL2 (red). JC-1 emits a red fluorescence when sequestered as an aggregate in the mitochondrial membrane of non-apoptotic cells, whereas release of JC-1 into the cytoplasmatic compartment of the cell promotes the monomeric state, thus emitting a green fluorescence. At depolarized (−100 mV) membrane

potentials the JC-1 green monomer emission peaks at 527 nm. Upon hyperpolarization of membrane potentials (−140 mV) the JC-1 aggregates emission shifts towards 590 nm.

2.7. Western blot analysis

10×10^6 cells were washed, pelleted, lysed and fractionated by SDS–PAGE (6–15% gradient gels) and proteins were transferred to a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad) using standard protocols. The membrane was blocked with a 5% nonfat, dry milk overnight and subsequently incubated with the primary antibody (see specified below). Unbound antibody was removed by washing with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20 (TBST). The membrane was then incubated with the secondary antibody (alkaline-phosphatase-conjugated antibody; Promega) for 2 hr at room temperature. After extensive washing with TBST proteins were detected upon addition of the development substrates (BCIP: 5-bromo-4-chloro-3-indolyl-phosphate/NBT: 4-nitro-blue-tetrazolium-chloride; Promega).

2.8. Antibodies

Antibodies for cIAP1, cIAP2, XIAP and survivin were purchased from R&D Systems Inc.; Bcl-2 from DAKO, A/S Denmark; Bcl-xL, Bax, Bak, p53, AIF, Smac/DIABLO, Par-4, Daxx, PML from Santa Cruz Biotechnology, Inc.; Bid, cytochrome *c* from BD PharMingen; Bad from Transduction Laboratories; PARP from Roche; and α/β-tubulin from Dunn.

2.9. Caspase activity

Fluorometric caspase-activity assay kits (Biocat) for caspases-6, -8 and -9 and CaspSELECT caspase immunoassay kits (Fluorometric Immunosorbent Enzyme Assay (FIENA)) for caspases-3 and -7 (Biocat) were used for specific and quantitative determination of caspase-activity. Cells were washed, pelleted and lysed according to the manufacturers instructions and transferred to 96-well microtiter plates (MTP). Samples were then incubated for 2 hr or overnight with the supplied, specific AFC conjugated caspase substrates (VEID-AFC for caspase-6, IETD-AFC for caspase-8, LEHD-AFC for caspase-9 and DEVD-AFC for caspases-3 and 7, samples were previously captured by a monoclonal antibody in anti-caspase-3/-7 (100 μL anti-caspase-3/-7 coating solution/well incubated overnight) coated MTP). Thereby the substrates were cleaved proportionally to the amount of active caspase in the lysate generating free AFC which was measured fluorometrically ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 505$ nm) in a multifunctional reader (Spectrafluor plus Tecan). The broad spectrum caspase inhibitor zVAD-fmk (Enzyme Systems Products) was used at a concentration of 50 μM following the manufacturer's instructions.

2.10. Cytosol/mitochondria fractionating

Cytosolic and mitochondrial fractions were obtained using a cytosol/mitochondria fractionation kit (Biocat). 50×10^6 cells per sample were washed and pelleted according to the manufacturers instructions, resuspended in Cytosol Extraction Buffer Mix containing DTT and protease inhibitors and, after incubation on ice for 10 min, homogenized in an ice-cold dounce tissue grinder. The homogenate was then centrifuged at 700 *g* for 10 min at 4° to remove nuclei and unbroken cells. The supernatant was transferred to a fresh 1.5 mL tube and centrifuged at 10,000 *g* for 30 min at 4°. The supernatant was collected as the cytosolic fraction and the pellet was resuspended in 0.1 mL Mitochondrial Extraction Buffer and saved as the mitochondrial fraction. The two fractions were separated by SDS–PAGE and subjected to Western blot analysis as described above.

2.11. Immunofluorescence-microscopy

Control and treated cells were incubated for 6, 12, 18 and 24 hr. Cells were then washed 1× with Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS), the pellet was resuspended in 150 μL PBS and spread onto SuperFrost Plus Microscope slides (Menzel-Gläser). The slides were air-dried and then fixed and permeabilized with a 1:1 acetone/methanol solution for 15 min.

Having been washed 3× with PBS the slides were incubated with the primary antibody solutions consisting of PBS + 0.1% Tween 20 (PBST), 1% BSA and primary antibodies concentrated at a ratio of 1:50 for 60 min at room temperature in a humidified chamber. Followed by 3× washing with PBS the cells were then incubated with FITC-conjugated and Cy-3-conjugated secondary antibodies concentrated at 1:50 for 60 min in a dark, humidified chamber. The cells were washed 3× with PBS, stained with DAPI (50 ng/mL), air-dried, then standard mounting medium (Sigma Chemical Co.) and coverslips were added. The slides were viewed and photographed with a Carl-Zeiss Axioplan 2ie MOT fluorescence microscope attached to a CCD camera (AxioCam HRc) driven by AxioVision 3.1 Software (Zeiss). The filters were set for Texas red ($\lambda_{ex} = 540-580$ nm/$\lambda_{em} = 600-660$ nm), FITC ($\lambda_{ex} = 465-495$ nm/$\lambda_{em} = 515-555$ nm) and DAPI ($\lambda_{ex} = 310-380$ nm/$\lambda_{em} = 435-485$ nm).

2.12. Statistical analysis

To determine synergistic, additive or antagonistic effects of the drug combinations software calcusyn (Biosoft) was used. Synergism was defined as more than the expected additive effect of drugs, and antagonism as less than the

expected additive effect. According to Chou *et al.* [32–34] the median effect plot and the CI were determined. A general equation for dose–effect was defined as

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m$$

where D is the dose of drug, D_m is median–effect dose signifying the potency, f_a is the fraction affected by the dose, f_u is the fraction unaffected ($f_u = 1 - f_a$), and m is the exponent signifying the sigmoidicity (shape) of the dose–effect curve. It was determined by the slope of the median–effect plot. The dose–effect plot was a plot of

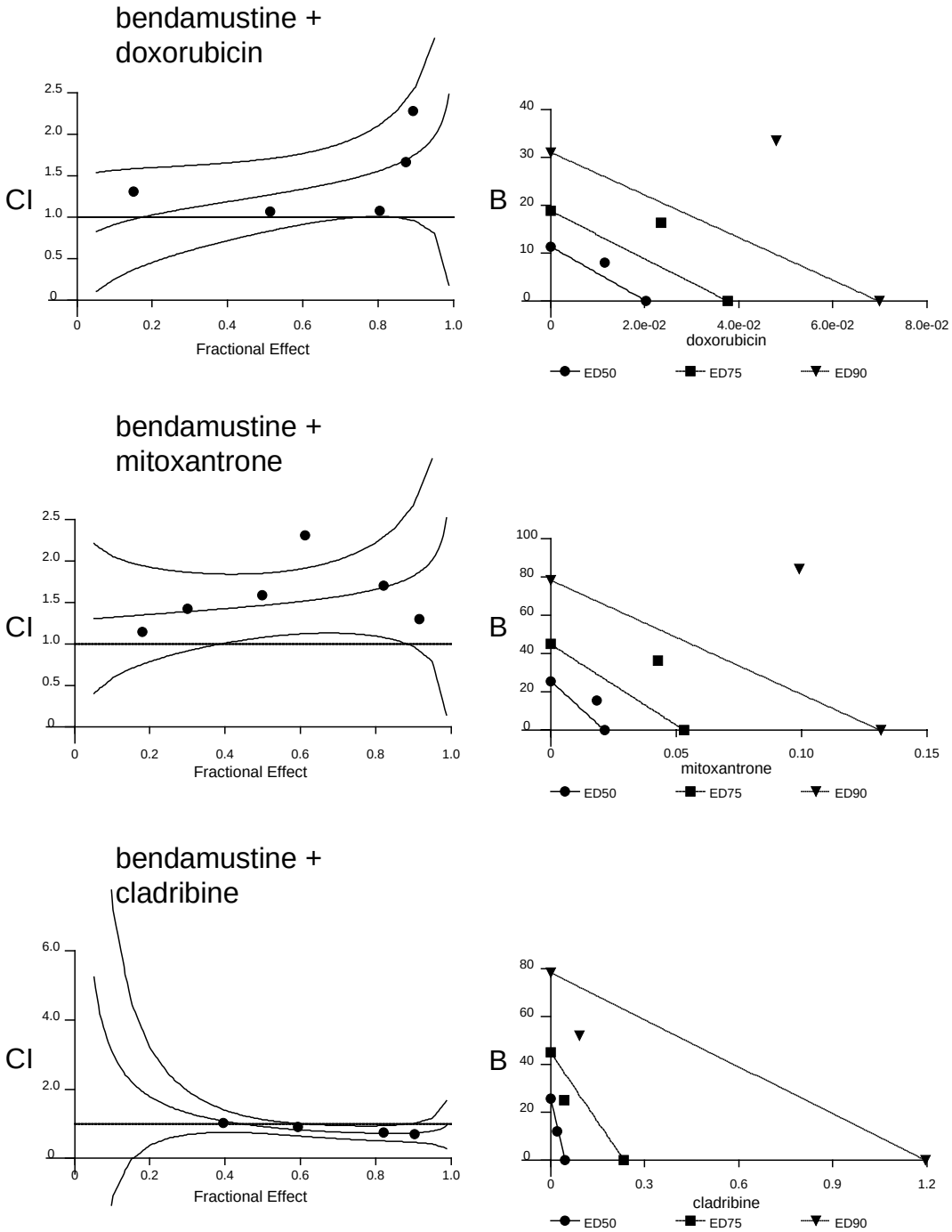


Fig. 1. Bendamustine in combination with cladribine showed synergistic effects on apoptosis in the DOHH-2 cell line after 24 hr. The effects of bendamustine with either doxorubicin or mitoxantrone were antagonistic. Apoptosis was determined by flow cytometry using Annexin V. Graphs on the left side demonstrate the CI analysis, on the right side the corresponding isobologram analysis of IC_{50}/ED_{50}, ED_{75} and ED_{90}. Results are means of triplicates. Similar results were achieved after 48 hr or using the WSU-NHL cell line (data not shown). CI > 1: antagonism, CI < 1: synergism, B: bendamustine. Range of CI B + D at IC_{50}: 1.27–1.66; at IC_{75}: 1.49–2.04; at IC_{90}: 1.77–2.51. Range of CI B + M at IC_{50}: 1.47–1.99; at IC_{75}: 1.61–2.67; at IC_{90}: 1.82–2.64. Range of CI B + C at IC_{50}: 0.92–1.13; at IC_{75}: 0.74–0.85; and at IC_{90}: 0.47–0.79.

$x = \log(D)$ vs. $y = \log(f_a/f_u)$:

$$\log\left(\frac{f_a}{f_u}\right) = m\log(D) - m\log(D_m)$$

This equation has the form of a straight line, $y = mx + b$. The CI for mutually non-exclusive drugs that have different modes of action is defined as

$$\mathrm{CI} = \frac{[D]_1}{[D_x]_1} + \frac{[D]_2}{[D_x]_2} + \frac{[D]_1[D]_2}{[D_x]_1[D_x]_2}$$

where $[D]_1$ is the concentration of drug 1, $[D]_2$ is the concentration of drug 2 and x is the effect x% (rate of apoptosis %).

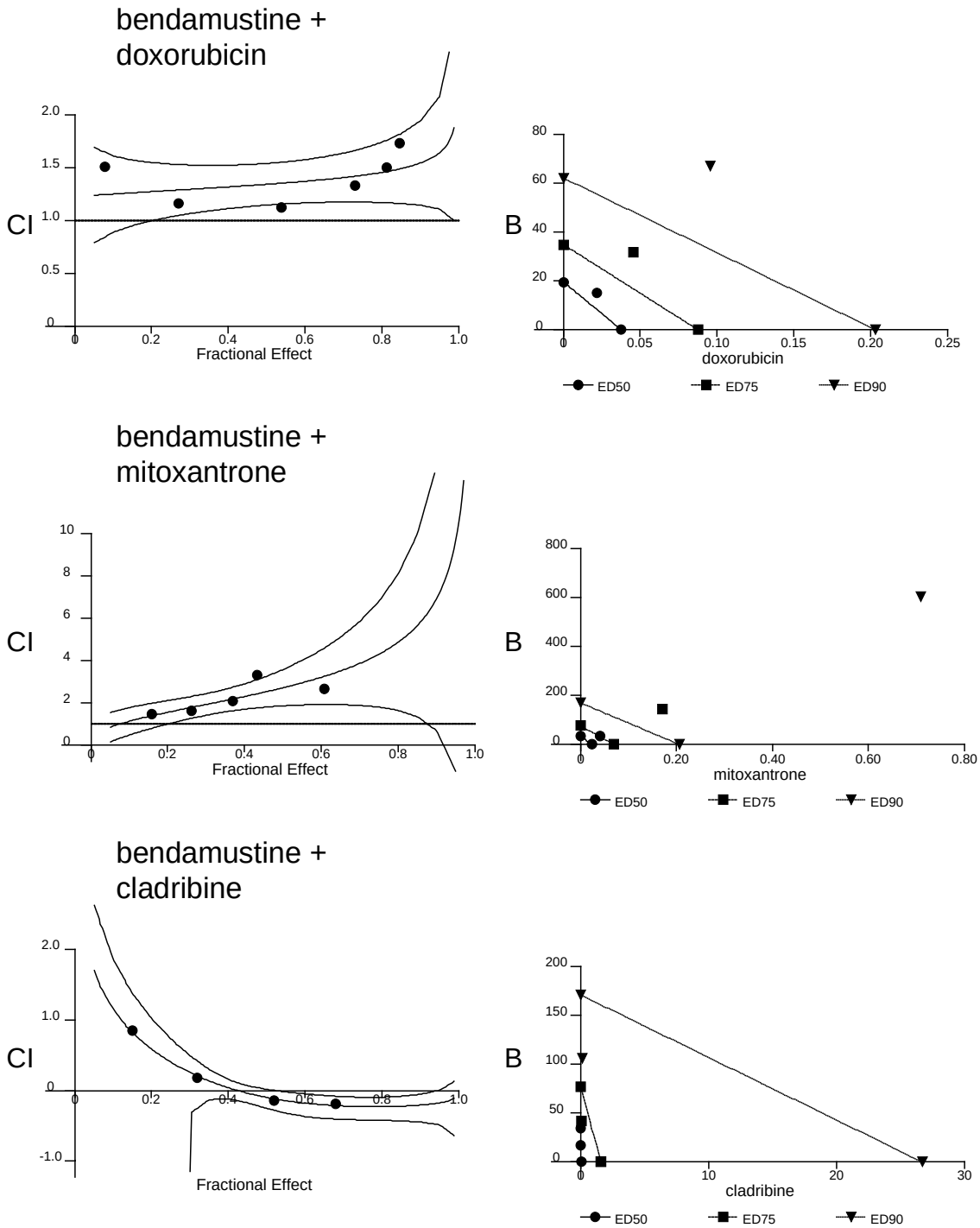


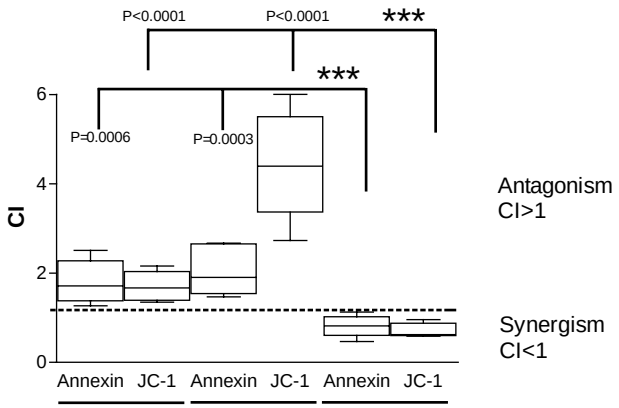
Fig. 2. Bendamustine in combination with cladribine showed synergistic effects on disruption of mitochondrial membrane potential (MMP) in the DOHH-2 cell line after 24 hr. The effects of bendamustine with either doxorubicin or mitoxantrone were antagonistic. Disruption of MMP was determined by flow cytometry using JC-1. Graphs on the left side demonstrate the CI analysis, on the right side the corresponding isobologram analysis of IC_{50}/ED_{50}, ED_{75} and ED_{90}. Results are means of triplicates. Similar results were achieved after 48 hr or using the WSU-NHL cell line (data not shown). CI > 1: antagonism, CI < 1: synergism, B: bendamustine. Range of CI B + D at IC_{50}: 1.35–1.79; at IC_{75}: 1.43–1.91; at IC_{90}: 1.55–2.16. Range of CI B + M at IC_{50}: 2.73–4.47; at IC_{75}: 4.32 to >5; at IC_{90}: >5. Range of CI B + C at IC_{50}: 0.80–0.96; at IC_{75}: 0.59–0.62; and at IC_{90}: 0.63–0.61.

Furthermore, the Wilcoxon-test, two-sided was used. Results were considered to be significant when $P < 0.05$.

3. Results

3.1. On neoplastic lymphocytes bendamustine in combination with doxorubicin or mitoxantrone exhibited antagonistic effects on apoptosis, whereas bendamustine in combination with cladribine exerted synergistic effects

Since bendamustine has proven efficacy in the treatment of NHL, its impact on apoptosis when combined with doxorubicin, mitoxantrone and cladribine, using the lymphoma cell lines DOHH-2 (constitutively expressing high levels of Bcl-2 [35]) and WSU-NHL was assessed. To test whether synergistic effects of the different drugs combined with bendamustine could be achieved we measured the rate of apoptosis induced by these combinations using Annexin V as well as the disruption of MMP. As shown in Figs. 1–3 the combination of bendamustine with the anthracylines doxorubicin or mitoxantrone exhibited antagonistic effects (CI > 1) on the rate of apoptosis as well as on the disruption of MMP. In contrast, when bendamustine was combined with cladribine, synergistic effects (CI < 1) were demonstrated by both methods. Having established models



Dm	8.05	15.19	15.81	35.31	12.01	6.98
m	1.54	1.48	1.31	0.77	1.49	1.19
r	0.96	0.99	0.97	0.98	0.99	0.99

Fig. 3. Comparison of synergistic and antagonistic effects induced by IC_{50}, IC_{75} and IC_{90} of bendamustine in combination with either doxorubicin, mitoxantrone or cladribine on apoptosis (Annexin V) or disruption of mitochondrial membrane potential (JC-1) after 24 hr. CI of the synergistic combination of bendamustine with cladribine differed highly significant (***) from that of the antagonistic combinations. B: bendamustine, D: doxorubicin, M: mitoxantrone, C: cladribine. CI > 1: antagonism, CI < 1: synergism. The table below shows the corresponding median–effect parameters of the different drug combinations obtained by analyses with Annexin and JC-1 measurement of the different drug combinations. D_m: median–effect dose signifying the potency, m: sigmoidicity of the doseeffect curve, r: linear correlation coefficient of the median effect plot.

offering the opportunity to investigate synergistic as well as antagonistic effects of cytotoxic drug combinations within the same cell lines we studied molecular events possibly explaining these differences. Therefore, different molecular levels responsible for the induction and execution of apoptosis were assessed. The studies of the molecular events of apoptosis-related proteins were carried out at the IC_{50} (inhibiting concentration 50% = dosages of the drugs causing a 50% rate of apoptosis) measured with Annexin V. The IC_{50} was calculated for the single drugs and the drug combinations after extensive dose–response experiments. At this point the effects of synergistic or antagonistic combinations could not yet be discriminated quantitatively by the level of apoptosis rate of the lymphoma cells, as the apoptotic rates induced by the different drug combinations were equal.

3.2. The expression levels of Bcl-2 family members were not altered by incubation with the IC_{50} induced bendamustine, doxorubicin, mitoxantrone or cladribine

Bcl-2 members are considered to transmit apoptotic signals and determine their intracellular effects by changes in the expression levels of pro-and anti-apoptotic family members. For that reason we analyzed the expression of Bad, Bak, Bax, Bid, Bcl-2 and Bcl-xL at drug dosages inducing a rate of apoptosis of 50% (IC_{50}) after 24 hr. As illustrated in Fig. 4A neither the IC_{50} of the single drugs (bendamustine, doxorubicin, mitoxantrone and cladribine), nor of the drugs used in combination were able to alter the expression levels of the tested Bcl-2 family members.

3.3. The expression levels of p53 and cleavage patterns of PARP remained unaltered upon incubation with IC_{50} of the combinations of chemotherapeutic drugs as compared to the effects of the single agents

Having not found an explanation for the divergent effects of the drug combinations by assessment of the Bcl-2 family members, we next studied the impact of p53 expression and PARP-cleavage as measures of apoptosis control and execution, respectively. Figure 4B shows that the single agents as well as the drug combinations up-regulated the expression level of p53 and caused PARP-cleavage as compared to the control cells. Nevertheless, there was no detectable difference in expression of p53 or cleavage of PARP between the single agents and the combined drugs.

Since virtually no data exist on molecular events during bendamustine-induced apoptosis, dose-dependent expression of p53 and cleavage of PARP was studied. Figure 4C clearly demonstrates that bendamustine-induced apoptosis is associated with the up-regulation of p53. Furthermore, we observed that dose-dependent PARP-cleavage is accompanied by a progressive down-regulation of apoptosis inhibiting proteins (IAPs, Fig. 6C).

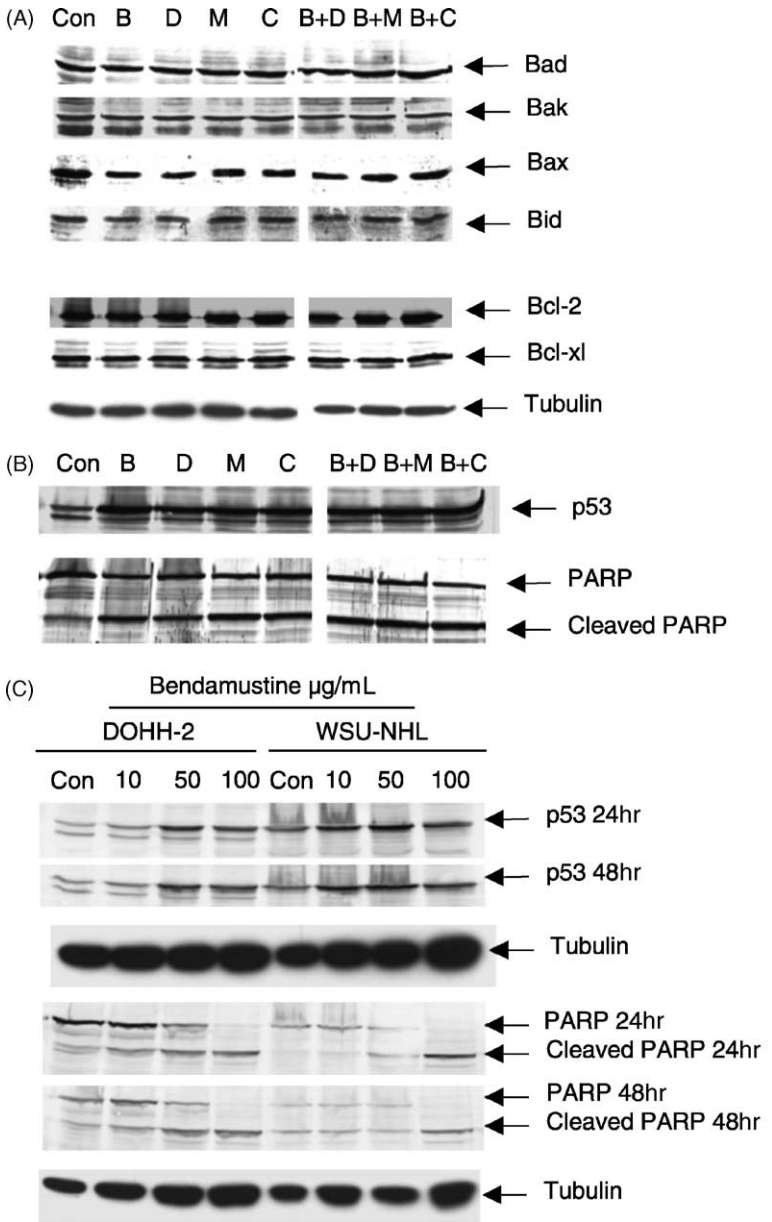


Fig. 4. (A) Expression levels of Bcl-2 family members were not altered by incubation with the IC_{50} dosages of bendamustine, doxorubicin, mitoxantrone or cladribine for 24 hr in DOHH-2 cells. (B) Up-regulation of p53 expression and PARP-cleaving activity induced by the drug combinations did not differ from the expression levels induced by the single drugs after 24 hr in DOHH-2 cells. (C) Bendamustine exerted its apoptotic effects by concentration-dependent up-regulation of p53 as well as PARP cleaving activity in DOHH-2 and WSU-NHL cells after 24 and 48 hr. Con: control without addition of the drugs, B: bendamustine, D: doxorubicin, M: mitoxantrone, C: cladribine. Loading controls were performed by tubulin expression.

3.4. The synergistic combination of bendamustine and cladribine enhanced the release of cytochrome c and Smac/DIABLO from the mitochondria into the cytosol

The release of cytochrome *c*, AIF and Smac/DIABLO from the mitochondria to the cytosol is considered to represent pro-apoptotic events, leading to the activation of executioner caspases. We thus investigated, whether incubation with the synergistic drug combination alters the patterns of cytochrome *c*, AIF and Smac/DIABLO concentrations in the mitochondrion and the cytosol. Figure 5A depicts that the combination of bendamustine and cladribine enforced the release of cytochrome *c* and Smac/DIABLO, whereas the concentrations of AIF remained unaltered in all tested drug combinations. In contrast to these observations doxorubicin induced AIF-liberation in Jurkat cells, chosen as a positive control (Fig. 5B).

3.5. Bendamustine and cladribine exerted their synergistic effects by down-regulation of cIAP1 and XIAP

To further differentiate between effects elicited by the single agents and the effects attributable to the drug

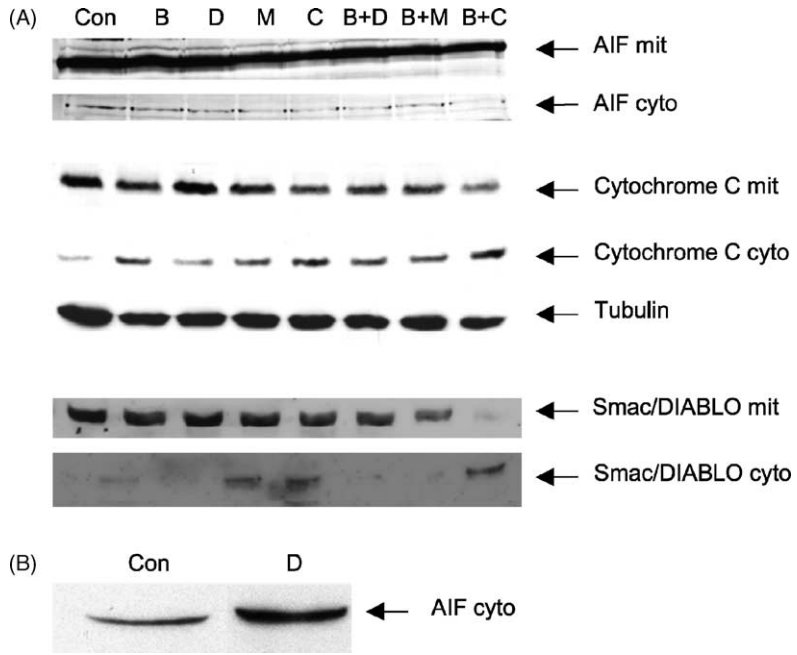


Fig. 5. (A) The synergistic combination of bendamustine and cladribine enhanced the release of cytochrome *c* and Smac/DIABLO from the mitochondria to the cytosol after 18 hr of incubation with the drugs in DOHH-2 cells. Furthermore, Smac/DIABLO-release was detected by incubation with the anthracyclines. The release of AIF was not influenced by the synergistic drug combination. Earlier detection of expression levels did not reveal any differences between the samples incubated with the single drugs or the drug combinations (data not shown). (B) Doxorubicin treated Jurkat cells were chosen as positive control for AIF liberation. Experiments were performed twice and yielded similar results. Loading controls (cytosol) were performed by tubulin expression. Con: control without addition of the drugs, B: bendamustine, D: doxorubicin, M: mitoxantrone, C: cladribine, cyto: cytosol, mit: mitochondria.

combinations we assessed changes in the expression of the IAP-family members. As outlined in Fig. 6A, the synergistic combination of bendamustine and cladribine induced a down-regulation of cIAP1 and XIAP, whereas the levels of survivin remained unaltered. cIAP2 was not detectable in the tested samples (data not shown). Noteworthy, the down-regulation of cIAP1 and XIAP was already detectable after 6–8 hr of incubation with the synergistic drug combination (Fig. 6B). Under inhibition of caspases the expression of IAPs was re-established as described below. The protein levels of IAPs were not influenced by the antagonistic drug combinations (Fig. 6A). Expression of the IAP members after incubation of the two lymphoma cell lines with increasing dosages of bendamustine is shown in Fig. 6C. Both c-IAP1 and XIAP were down-regulated in a dose-dependent manner.

3.6. The synergistic effects of bendamustine and cladribine are associated with down-regulation of Par-4 and Daxx

Reportedly, expression of Par-4 sensitizes cells towards apoptotic stimuli, i.e. increasing the rate of apoptosis upon treatment with different chemotherapeutic agents [17]. In order to test the hypothesis that the synergistic effect of bendamustin and cladribine was associated with an increased expression of Par-4, we assessed its expression before and after incubation of the different chemotherapeutic agents. Noteworthy, upon assessment of Par-4 expression at 50% of tumor cell kill revealed a comparable level in all samples with the exception of the synergistic combination. Contrary to the expected up-regulation of Par-4 upon treatment with bendamustin and cladribine the protein level declined as compared to the control and to all other samples (Fig. 7A). In contrast to the so far only pro-apoptotic role of Par-4, Daxx is considered to be able to act pro- as well as anti-apoptotic [36]. Furthermore, its pro-apoptotic role can be exerted dependent as well as independent of the activation of caspases [27]. Comparable to the described expression of Par-4 the level of Daxx was down-regulated by the synergistic combination of bendamustine and cladribine (Fig. 7A). Additionally, a considerable decrease of Daxx was also caused by bendamustine and doxorubicin when applied as single agents (Fig. 7A).

By immunofluorescence-microscopy we observed that Daxx was not translocated from the cytosol into the nucleus, which is a prerequisite for proapoptotic effects. Daxx was found to be colocalized with PML indicating that it was a part of nuclear bodies in untreated as well as in treated cells. After treatment with bendamustine + cladribine the number and size of the nuclear bodies as well as the Daxx signal decreased (Fig. 8A and B).

Furthermore, Daxx as well as Par-4 was demonstrated by Western blot analyses to be down-regulated in a dose-dependent manner by the tested single drugs (Fig. 7B).

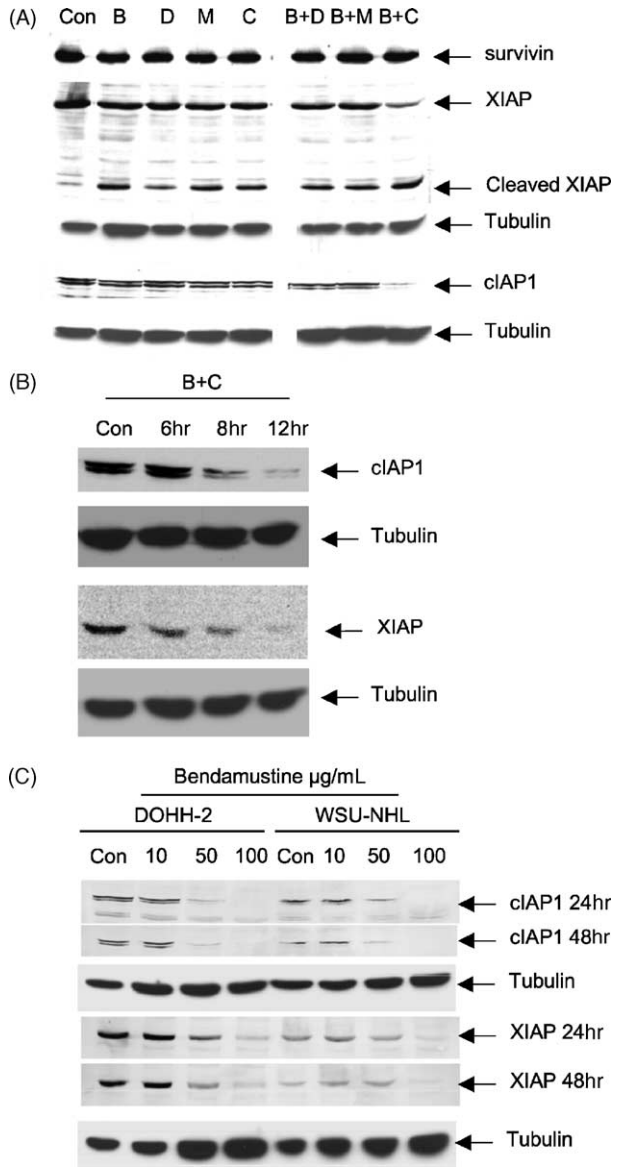


Fig. 6. (A) Bendamustine and cladribine exerted their synergistic effects by down-regulation of cIAP1 and XIAP in DOHH-2 cells. (B) Down-regulation of cIAP1 and XIAP was detected for the first time after 6–8 hr of incubation with the synergistic drug combination of bendamustine and cladribine in DOHH-2 cells. Expression levels of survivin remained unaltered by any of the tested drugs. cIAP2 was not detectable in the tested samples (data not shown). (C) Bendamustine exerted its apoptotic effects by concentration-dependent down-regulation of inhibiting apoptosis proteins (IAPs) in DOHH-2 and WSU-NHL cells after 24 and 48 hr. Con: control without addition of the drugs. Data are representative for results obtained in three independent experiments. B: bendamustine, D: doxorubicin, M: mitoxantrone, C: cladribine. Expression levels of tubulin were assessed as loading controls.

3.7. The synergistic effect of bendamustine and cladribine is due to an increased activation of caspases-3, -6, -7, -8 and -9

We hypothesized that the down-regulation of cIAP1 and XIAP by combination of the IC_{50} dosages of bendamustine and cladribine resulted in activation of caspases. To provide evidence for this hypothesis we investigated whether this drug combination causes a different activation of caspases-3, -6, -7, -8 or -9. As depicted in Fig. 9 upon induction of apoptosis with bendamustine in combination with cladribine activation of all caspases was stronger than the activation of caspases elicited by the antagonistic combinations and the single agents, despite the fact that the synergistic and antagonistic combinations caused the same rate of apoptosis.

Of note is the prolonged linear activation of all caspases by the synergistic combination during the first hours of incubation of the lymphoma cells. Activity of caspases-3 and -7 induced by bendamustine in combination with cladribine remained above the activity induced by the antagonistic combinations or the single drugs throughout the observed time period. The activity of caspases-6, -8 and -9 peaked after 8 hr of incubation with the synergistic drug combination and thereafter declined to the levels induced by the other conditions (Fig. 9).

By addition of broad spectrum caspase-inhibitor zVAD-fmk to the synergistic combination the activation of all caspases was completely inhibited (Fig. 9). The corresponding rate of apoptosis was considerably decreased (data not shown). The role of caspases was further supported by the observation that broad-spectrum inhibition of caspases re-established the expression level of XIAP, whereas the expression of Par-4—considered to be located upstream of caspase activation—remained unaltered (Fig. 10).

4. Discussion

The continuing improvement in the treatment of NHL is mainly based on the combination of established cytotoxic agents with new anticancer drugs. The combination of different drugs aims to achieve additive or synergistic effects on tumor cell kill resulting in an increased clinical efficacy. Major targets of cytotoxic drugs are the signaling events of programmed cell death [1,37]. The induction of CD95/CD95L and the death inducing signaling complex (disc) [11], the disruption of mitochondrial membrane potential and the activation of caspases [38] by chemotherapeutic drugs have been demonstrated as key events. As there is paucity of information about molecular mechanisms by which cytotoxic drug combinations influence apoptotic events which may result in synergistic or antagonistic effects, we investigated the role of expression patterns of apoptosis-associated molecules, of protein release from mitochondria and of enzyme activities of caspases upon induction of synergistic and antagonistic effects. In order to study these mechanisms, we took advantage of our observation that in two lymphoma cell lines the combination of bendamustine with either doxorubicin or mitoxantrone exerted an antagonistic effect on the rate of apoptosis and on the disruption of mitochondrial membrane potential, while in contrast, the combination of bendamustine with cladribine resulted in synergism on

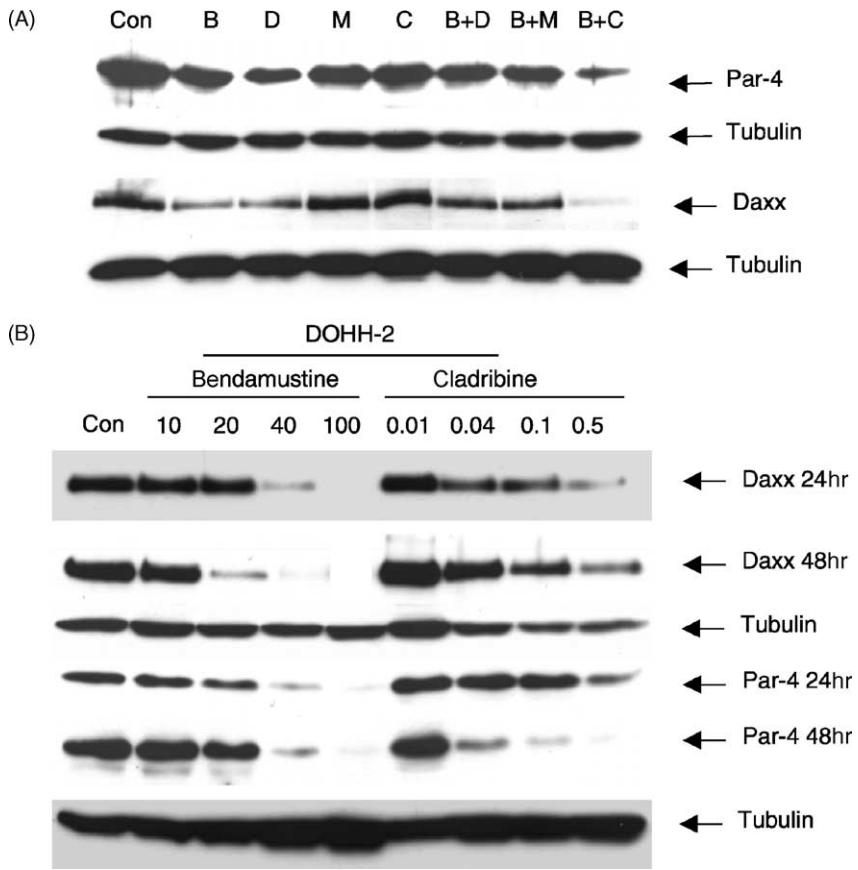


Fig. 7. (A) The synergistic effects of bendamustine and cladribine were associated with a down-regulation of Par-4 and Daxx in DOHH-2 cells. Data are representative for results obtained in three independent experiments. (B) Expression of Daxx as well as of Par-4 was down-regulated in a dose-dependent manner by all tested single drugs; i.e. bendamustine and cladribine. Con: control without addition of the drugs, B: bendamustine, D: doxorubicin, M: mitoxantrone, C: cladribine. Loading controls were performed by tubulin.

both events. The latter combination was furthermore associated with a subsequent increase of the mitochondrial release of cytochrome *c* and Smac/DIABLO. This stimulus translated in enforced caspase activation and down-regulation of IAPs accompanied by a decrease of Daxx and Par-4 expression. The enhanced disruption of MMP prior to the increased release of cytochrome *c* and Smac/DIABLO may be considered to be an early sign of the observed synergism. In this context bendamustine may directly interfere directly with the mitochondrial membranes, as has been reported for other alkylating agents [39]. It is of particular interest that these qualitative changes in protein expression and caspase activation were already observed at a point (IC_{50}) before synergistic or antagonistic effects could be discriminated quantitatively by the apoptosis-rate of the lymphoma cells.

Noteworthy, despite the previously described role of Par-4 as a pro-apoptotic protein [17], the level of expression of Par-4 was exclusively down-regulated upon induction with the synergistic drug combination, whereas Par-4 levels under the single drugs and the antagonistic combinations remained unaltered as compared with the control cells. Comparable to these results are the changes observed in the expression of Daxx, but in contrast to Par-4 Daxx may exhibit a pro- as well as an anti-apoptotic role depending on the cellular context [27,28]. So far down-regulation of Daxx has been reported only in cells treated with histone deacetylase inhibitors [28] and down-regulation of Par-4 in context with apoptosis induction was not yet known. Therefore, our results may indicate that these proteins are involved in apoptosis caused by synergistic drug combinations.

These data on the functional consequences are in accordance with results obtained by Genini *et al.* [40], demonstrating that purine analogues such as cladribine used as single agents are able to exert their cytotoxic effects via the mitochondrial pathway with the release of cytochrome *c* and AIF, as well as the activation of caspases-3 and -9. In contrast to the mentioned study solely assessing the mode of action of cladribine, we show that the combination of cladribine with bendamustine did not cause an increased AIF-release, which may be possibly explained by data of Arnoult *et al.* [41], showing AIF being a downstream effector of cytochrome *c* release, requiring subsequent caspase activation.

Other investigators [16,42] showed that Smac/DIABLO is released along with cytochrome *c* during apoptosis and promotes cytochrome *c*-dependent activation of the executioner phase of apoptosis. Since mature Smac/DIABLO is liberated only during apoptosis, the pivotal regulatory step

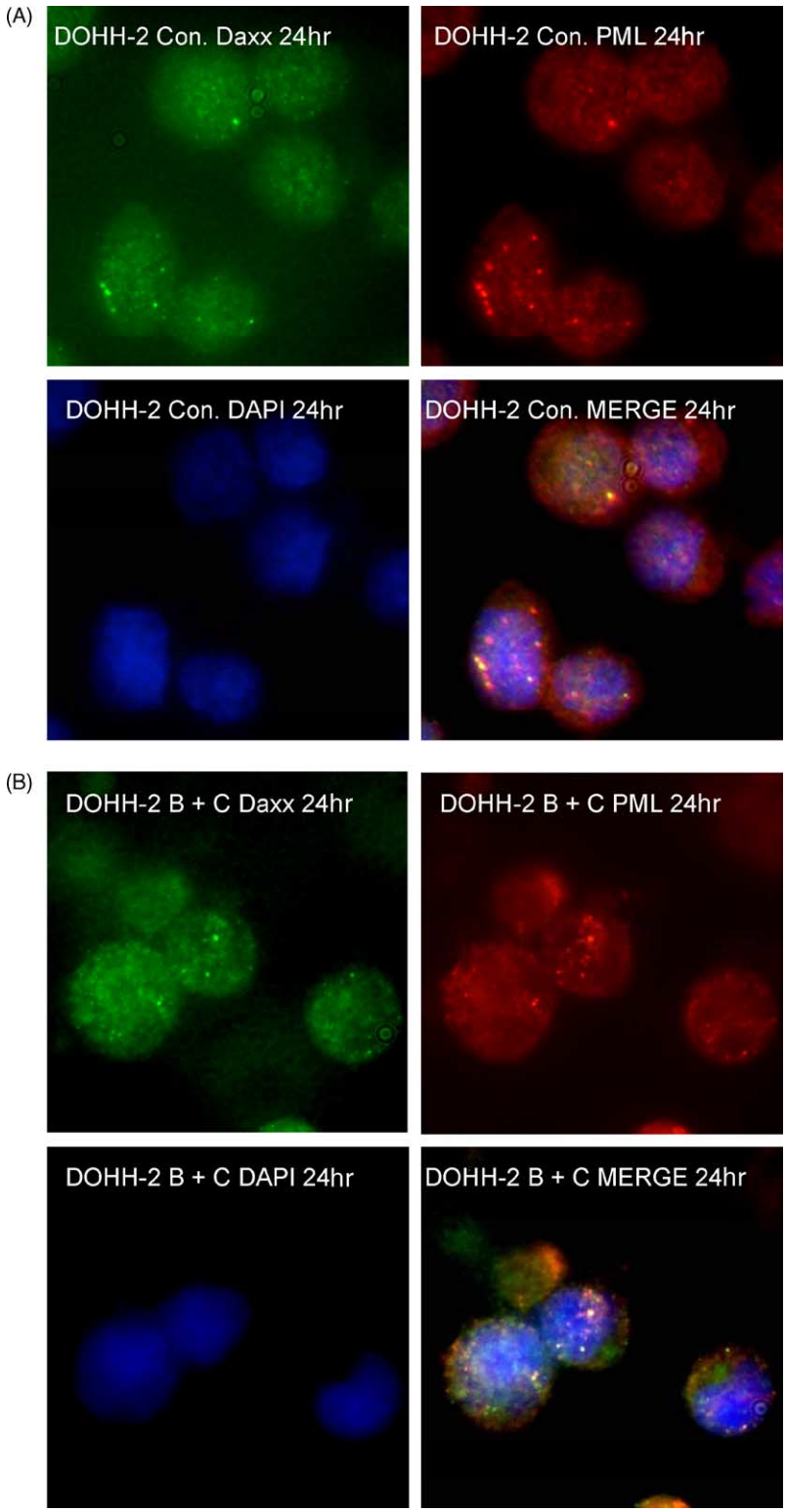


Fig. 8. (A) Daxx was not translocated from the cytosol into the nucleus as demonstrated by immunofluorescence-microscopy. Daxx was found to be colocalized with PML indicating that it was a part of nuclear bodies in untreated as well as in treated cells. (B) After treatment with bendamustine + cladribine the number and size of the nuclear bodies as well as of the Daxx signal decreased.

for this protein is the release from mitochondria, a process that is likely to be controlled by the Bcl-2 family [4]. Nevertheless, changes in the expressional pattern of the major Bcl-2 family members were not detectable in our experiments. Our data are in accordance with results by Schwanen *et al.* [43], showing unaltered levels of mRNA of Bcl-2 family members in synergistic effects on apoptosis of CLL cells caused by bendamustine and fludarabine.

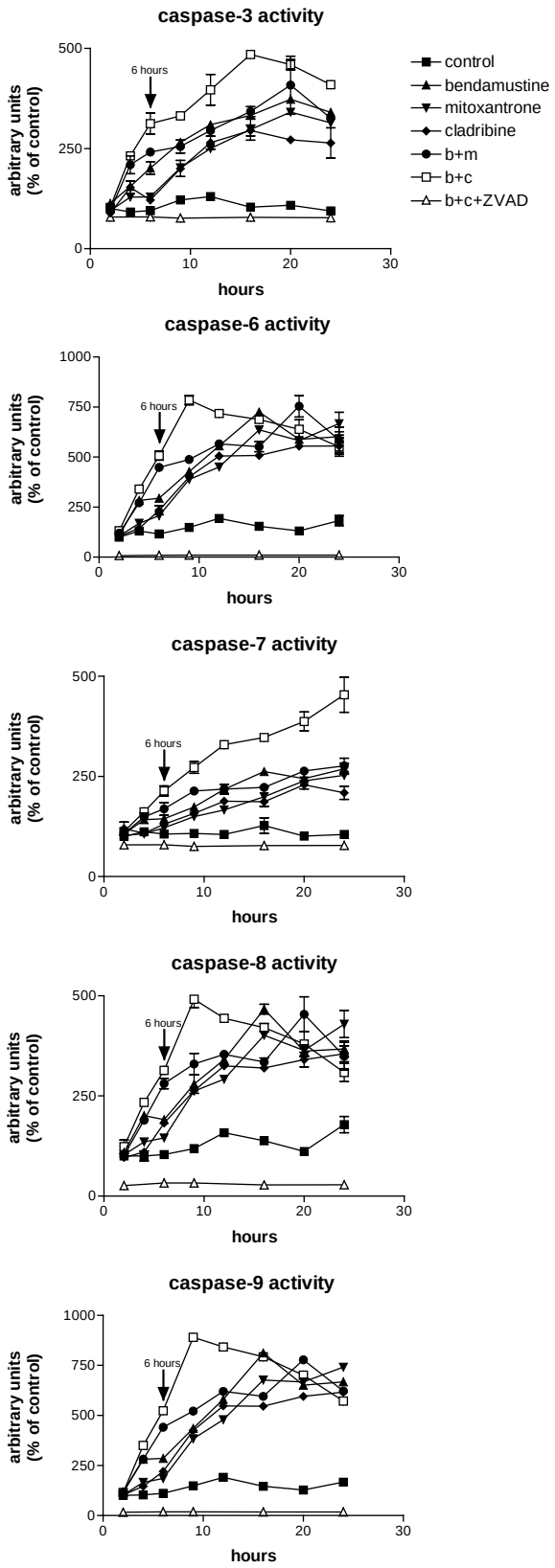


Fig. 9. The synergistic effect of bendamustine and cladribine was due to an increased activation of caspases-3, -6, -7, -8 and -9 in DOHH-2 cells. Caspase activities were determined over time in the range from 2 to 24 hr. A significant increase of the activity of all caspases was induced by the synergistic combination after 6 hr (arrow). The executioner caspases-3 and -7 showed a significantly increased enzyme activity as compared to the single drugs and the antagonistic drug combination between 6 and 24 hr of incubation. Increased activity of caspases-6, -8 and -9 induced by bendamustine and cladribine significantly differed between the 6th and 12th hour of incubation. Addition of the broad spectrum caspase-inhibitor zVAD-fmk to the synergistic combination considerably decreased the activation of all tested caspases. Experiments were performed twice and yielded in similar results.

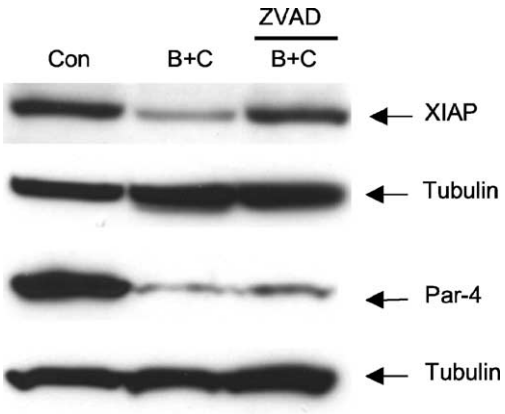


Fig. 10. Expression of XIAP and Par-4 before and upon incubation with the broad-spectrum caspase-inhibitor zVAD-fmk in DOHH-2 cells. Whereas the level of Par-4 remained unaltered by inhibition of caspases, the inhibition of caspases re-established the expression of XIAP to an extent comparable to the one observed in control cells (=Con, i.e. without chemotherapeutics). B: bendamustine, C: cladribine.

Besides Bcl-2 family members, Smac/DIABLO was shown to promote cytochrome *c*-dependent caspase activation by neutralizing IAPs [42]. It functions as a general IAP neutralizer by binding to these proteins. cIAP1, cIAP2, XIAP as well as survivin are able to bind Smac/DIABLO [16,42]. XIAP seems to be the most potent inhibitor within the IAP-family, acting downstream of procaspase-9 cleavage [24,25]. Both, survivin and XIAP mainly inhibit the processing of caspases-3, -7 and -9 [23]. We here provide evidence that the interaction of IAP with caspases may be critically involved in apoptosis of lymphoma cells upon treatment with synergistic drug combinations. While the expression of survivin remained unaffected and c-IAP2 expression was not detectable, down-regulation of cIAP1 as well of XIAP—first detected by Western blotting after 6–8 hr of drug incubation—resulted in an increased activity of the caspases. In particular, an enforced activation of the downstream executioner caspases-3 and-7 over 24 hr was demonstrated, whereas enhanced levels of the executioner caspase-6 and of the upstream inducer caspases-8 and -9 only lasted for 6 hr.

Previous investigations focused on the interaction of XIAP with the executioner caspases-3 and -7 [23,24]. In these structure–function analyses of XIAP the mode of inhibition of caspases-3 and -7 differs: caspase-3 is inhibited competitively, whereas caspase-7 is inhibited in a competitive as well as in a non-competitive manner. According to these results the linker region between BIR1

(baculovirus IAP repeat domain 1) and BIR2 seems to be responsible for the active-side-directed inhibition for caspases-3 and -7, moreover the BIR2 region was shown to be essential for the non-competitive inhibition of caspase-7 [23]. Noteworthy, data of Silke *et al.* [24] demonstrated that XIAP is nevertheless clearly able to block caspase-3, but inhibition of caspase-3 is dispensable for the ability of XIAP to inhibit cell death. These data might explain our findings that caspase-7 activation compared with caspase-3 activation was considerably prolonged after down-regulation of XIAP. The role of cIAP1 in drug induced apoptosis is not well defined, but it is hypothesized that it is able to inhibit the downstream caspases in a same potent manner as XIAP [19,22]. The concomitant down-regulation of XIAP and cIAP1 might also be responsible for the synergistic effects observed in apoptosis.

The role of caspase-8 in drug-induced apoptosis remains controversial: due to its structure with a long pro-domain it is considered to be an upstream caspase enabling it to associate with cell surface death-receptor molecules [44–46]. On the other hand, drug-induced caspase-8 activation in B-lymphoma cells was demonstrated to be independent of the death receptor signaling and is mediated by post-mitochondrial caspase-3 activation [45]. Furthermore, it was concluded that in addition to its proximal role in death receptor signaling, caspase-8 functions as an amplifying executioner caspase [44]. Since our experiments investigated the effects on the mitochondrial pathway, the observed changes in caspase-8 activity may be attributed to its role in the executioner phase of apoptosis.

The results obtained in these cell lines, enables us to study synergistic and antagonistic effects of drug combinations already at a point (IC_{50}) where the effects of synergistic or antagonistic combinations could not yet be discriminated quantitatively by apoptosis-rate of the lymphoma cells. We conclude that on a molecular basis the enforced disruption of MMP followed by enhanced cytochrome *c*- and Smac/DIABLO-release, changes in expression of Par-4, Daxx and IAPs with the resulting increase of caspase activation are critical for the achievement of the synergistic effect.

This is the first study demonstrating the molecular events providing a rationale for the achievement of synergistic effects of a cytotoxic-drug combination as opposed to antagonistic effects.

Acknowledgments

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