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ORIGINAL ARTICLE

**Camilla Palumbo · Loredana Albonici · Roberto Bei
Chiara Bocci · Susanna Scarpa · Paolo Di Nardo
Andrea Modesti**

HMBA induces cell death and potentiates doxorubicin toxicity in malignant mesothelioma cells

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Abstract *Purpose*: Malignant pleural mesothelioma (MM), a rare tumor characterized by high local invasiveness and low metastatic efficiency, is poorly responsive to current therapeutic approaches. The aim of the present study was to evaluate the cytotoxic efficacy of the hybrid polar compound hexamethylene bisacetamide (HMBA), either as a single agent or in combination with the anthracycline doxorubicin (DOX), against MM cells. *Methods*: The MM cell lines MM-B1 and MM-E1 were treated with HMBA, DOX or with combinations of the two drugs. Cell survival and death were assessed by the MTS assay and trypan blue staining/TUNEL, respectively. The interactions between drugs were evaluated by the method of Kern et al. Western blot analysis was used to investigate the expression of Bcl-2 family proteins. *Results*: When administered alone, HMBA dose-dependently decreased the number of viable cells and increased the death rate of MM-B1 and MM-E1 cultures. Combinations of HMBA and DOX achieved a synergistic inhibition of MM cell survival, and the simultaneous administration of HMBA counteracted the resistance induced by DOX in MM-E1 cells. HMBA, used at cytostatic concentrations, reduced the ratio between antiapoptotic (Bcl-2, Bcl-X_L) and proapoptotic (Bax) members of the Bcl-2 family of proteins, thus lowering the threshold for MM cell death commitment. *Conclusions*: HMBA has therapeutic potential in MM both as a single agent and through potentiation of DOX toxicity. These results support future investigations on the feasibility of intrapleural chemotherapy with this hybrid polar compound.

Keywords Mesothelioma · HMBA · Doxorubicin · Drug synergism · Intrapleural chemotherapy

C. Palumbo (✉) · L. Albonici · R. Bei · C. Bocci · A. Modesti
Department of Experimental Medicine
and Biochemical Sciences, Tor Vergata University,
Via Montpellier 1, 00133 Rome, Italy
E-mail: camillapalumbo@mclink.it
Tel.: +39-06-72596520
Fax: +39-06-72596506

S. Scarpa
Department of General Pathology,
La Sapienza University, Rome, Italy

P. Di Nardo
Department of Internal Medicine,
Tor Vergata University, Rome, Italy

Introduction

Malignant mesothelioma (MM) is a rare tumor arising from serosal linings, most often occurring at the pleural level [2, 3]. Epidemiological studies have unraveled its frequent association with asbestos exposure, which accounts for the ever-increasing incidence of this neoplasm in industrialized countries [3]. Since MM spreads over serous surfaces and invades locally but has a low metastatic efficiency, most patients die due to extensive tumor growth in the pleural cavity and surrounding tissues, usually leading to respiratory failure [2, 3]. Although some advances have recently been made, the median survival from the diagnosis of MM remains between 4 and 18 months, and more effective treatments are urgently needed [2, 14, 33, 39].

Hexamethylene bisacetamide (HMBA) is a hybrid polar compound with differentiating properties on transformed cells [12, 18, 22, 28]. No evidence has been obtained for a membrane receptor for this compound, whose effects are thought to be triggered by early alterations of membrane surface potential observed after its administration [18]. Recently, it has been demonstrated that HMBA induces apoptotic cell death in colon carcinoma as well as multiple myeloma and leukemic cells in vitro [26, 30]. Prompted by these findings, we aimed to assess the cytotoxic potential of HMBA in MM cell cultures. Furthermore, since treatment with some hybrid polar compounds has been found to increase the sensitivity of cancer cell types to irradiation and chemotherapy [4, 16, 37], we also aimed to investigate the interaction of HMBA with classical antineoplastic drugs in MM cells. For this purpose we chose to combine

HMBA with doxorubicin (DOX), one of the most extensively studied agents in combination chemotherapy trials for this cancer [2, 14, 21, 33].

Materials and methods

Cell cultures and treatments

The human pleural MM cell lines MM-B1 and MM-E1 were cultured as previously described [5, 27]. HMBA and DOX (Sigma-Aldrich, Milan, Italy) were dissolved in culture medium at 200 m*M* and in distilled water at 1 mg ml^{-1}, respectively [28]. The cells were seeded at low confluence (about 5×10^3 cm^{-2}) and treated 24 h later with HMBA, DOX, or combinations of the two drugs. HMBA was used in the range 1–30 m*M* [30]. DOX was used in the range 0.001–1.000 μ*M*, covering the average range of plasma concentrations achieved and maintained in patients [9]. DOX–HMBA treatments were performed by combining increasing concentrations of DOX with a fixed dose of HMBA (2 or 5 m*M*).

Assessment of cell survival

The cells were grown treated or untreated for 96 h in 96-multiwell plates with 200 μl of medium per well. The viability of triplicate cultures was then colorimetrically determined by the MTS assay (Promega Corporation, Madison, Wis.). Cell survival of treated cultures was determined in terms of optical density expressed as a percentage in relation to the optical density of untreated (control) cultures.

Assessment of cell death

After 96 h of growth with or without drugs, MM cells, both adherent and floating in the medium, were collected. Necrotic and late apoptotic cells that had lost membrane integrity were detected by trypan blue staining and expressed as a percentage of the total cell number [26]. Apoptotic DNA fragmentation was also evaluated onto cytospins by the TUNEL assay, using a fluorescent detection kit (Promega Corporation) following the manufacturer's instructions. Hoechst 33258 (Sigma-Aldrich) was used to counterstain cell nuclei [26]. The percentage of TUNEL-positive nuclei relative to a minimum of 1000 Hoechst-marked nuclei for each sample was determined by counting the cells under a fluorescence microscope.

Analysis of drug interactions

The combined effects of DOX and HMBA were analyzed according to the method of Kern et al. as modified by Romanelli et al. [15, 25, 34]. Several methods have been proposed to evaluate drug interactions in combined cytotoxic treatments [10]. Among these, we chose the method of Kern et al. for its suitability in analyzing biphasic dose-response effects. According to the method of Kern et al., cell survival data were processed to obtain an index (R) defined as follows

$$R = \frac{S_{exp}}{S_{obs}}$$

where S_{exp}, the expected survival, is the product of the percentage survival observed with DOX alone and the percentage survival observed with HMBA alone, and S_{obs}, the observed survival, is the actual percentage survival observed with the DOX–HMBA combination. An R index lower than 1 indicates that the combination exerts a less than additive effect; an R index of 1 indicates that the effect is additive, and any value of R greater than unity indicates a synergistic interaction.

Western blotting

Western blot analysis was performed as described previously [35], resolving 70 μg of proteins by 10–15% SDS-PAGE and probing the membranes with the following monoclonal antibodies: anti-human Bcl-2, anti-human Bcl-X_L, anti-human Bax (all from Santa Cruz Biotechnology, Santa Cruz, Calif.) and anti-β-actin (Sigma-Aldrich). The bands were visualized using Western blotting luminol reagent (Santa Cruz Biotechnology) and quantified using NIH Pro-Image 1.62 software after blot scanning.

Semiquantitative RNA analysis

MM cells were grown either untreated or treated with 5 m*M* HMBA. After 48 h, total RNA was extracted using Trizol (Invitrogen Italia, Milan, Italy) and 2 μg per sample was retrotranscribed using 200 U of Superscript II reverse transcriptase (Invitrogen) in a final volume of 20 μl, according to the supplier's protocol. The cDNAs (1 μl) were amplified in a Perkin Elmer 9600 thermocycler using 1 U of Platinum Taq DNA polymerase (Invitrogen) and 50 pmol of each primer in a final volume of 50 μl. All genes were amplified for 25 cycles (94°C for 30 s, 65°C for 30 s, and 72°C for 30 s) preceded by activation of the enzyme at 94°C for 2 min and followed by a final extension at 72°C for 7 min. The bcl-2 primer pair (*upstream* 5′-GTGGTGGAGGAGCTCTTCAGGGAC-3′; *downstream* 5′-GATTGGC ACCCAGGGTGATGCAAG-3′) generated a 304-bp product, the bcl-X_L primer pair (*upstream* 5′-TTGAACAGGCAGTGGAATGAACTC-3′; *downstream* 5′-GAGTTCCACAAAAGTATCCCAGC-C-3′) generated a 214-bp product, the bax primer pair (*upstream* 5′-GGCCCACCAGTCCTGAGCAGA-3′; *downstream* 5′-GCCACGTGGGCGTCCCAAAGT-3′) generated a 478-bp product, and the GAPDH primer pair (*upstream* 5′-TGTTCGTCATGGGTGTGAACCATG-3′;

downstream 5′-CTGCTTCACCACCTTCTTGATGTC-3′) generated a 404-bp product. Each pair of primers was designed to span different exons to avoid amplification of contaminating genomic DNA. The bcl-X_L downstream primer was designed to exclude coamplification of bcl-X_S [19]. PCR products were confirmed by fragment size and automated sequencing.

Amplified products were resolved on 2% agarose gel, blotted on nylon membranes (Roche Diagnostics, Mannheim, Germany) and hybridized with an internal probe labeled using a DIG oligonucleotide 3′-end labeling kit (Roche Diagnostics) as recommended by the manufacturer. The probe for bcl-2 was 5′-AACAG-AGGCCGCATGCTGGGGCCGTACAGTTCC-3′, the probe for bcl-X_L was 5′-CCTTTTTCTCCTTGC GCGGGGCACTGTGCGTGG-3′, the probe for bax was 5′-TCTCAAGCGCAT CGGGGACG AACTG GACAG-3′, and the probe for GAPDH was 5′-AGA-AGACTGTGGATGGCCCCTCCGGGAAAC-3′. The detection was performed with a DIG luminescent detection kit (Roche Diagnostics).

Statistical analysis

Data from a minimum of three experiments were analyzed by Microsoft Excel software and are expressed as means ± SE. Means were compared through the two-tailed Student's *t*-test, with a significance threshold set at $P < 0.05$ [23]. In the analysis of drug interaction, an interaction was considered synergistic when the expected survival (S_{exp}) was significantly greater ($P < 0.05$) than the observed survival (S_{obs}), meaning that the R index was significantly greater than 1 [8].

Results

The 96-h HMBA treatments induced a dose-dependent decrease in the proportion of viable MM cells (Fig. 1a). In particular, with 2 m*M* HMBA, MM-B1 living cells decreased by 17%, whereas no significant effect was observed in MM-E1 cultures. With 5 m*M* HMBA, the reduction in cell survival was of 38.5% and 26.7% in MM-B1 and MM-E1, respectively, while with 10 m*M* HMBA it was of 80.8% and 75.4%. A significant increase in cell death was observed in cultures treated with HMBA ≥10 m*M* (Fig. 1b), indicating that the reduction in the number of viable cells observed with HMBA < 10 m*M* had to be due to a cytostatic effect. In particular, with 10 m*M* HMBA, trypan blue-stained cells were 64% and 55%, whereas TUNEL-stained cells were 22% and 19%, of the total number of MM-B1 and MM-E1 cells (Fig. 1b).

The dose-dependent effects of 96-h treatments with DOX and DOX–HMBA combinations on MM cell survival are shown in Fig. 2 and summarized in Table 1. DOX–HMBA treatments were made by combining increasing concentrations of DOX with fixed concentrations, either 2 or 5 m*M*, of HMBA. Of note, the DOX dose–response curve of MM-E1 cells had a biphasic shape, highlighting the induction of resistance mechanisms in the cultures treated with drug concentrations around 0.1 μ*M*. As a consequence, two DOX IC_{70} values were obtained for this cell line, with their ratio showing that a greater than sixfold dose was required to regain a 70% cell survival inhibition after the induction of resistance.

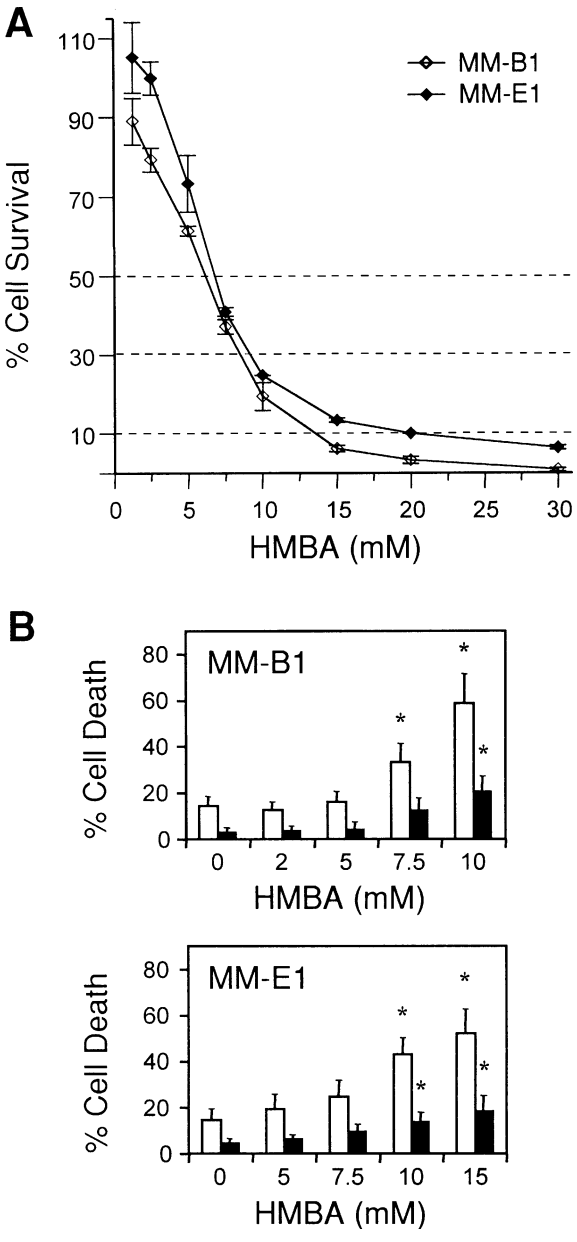

Fig. 1a, b Cell survival and death of MM cultures after 96 h of treatment with increasing concentrations of HMBA. **a** Cell viability evaluated by the MTS assay. **b** Cell death rates evaluated by trypan blue staining (*white bars*) and TUNEL (*black bars*); $*P < 0.05$ vs untreated cells

Several findings emerged from the comparison of the effects of DOX and DOX–HMBA. First, the reduction in cell survival achieved with the combination of DOX

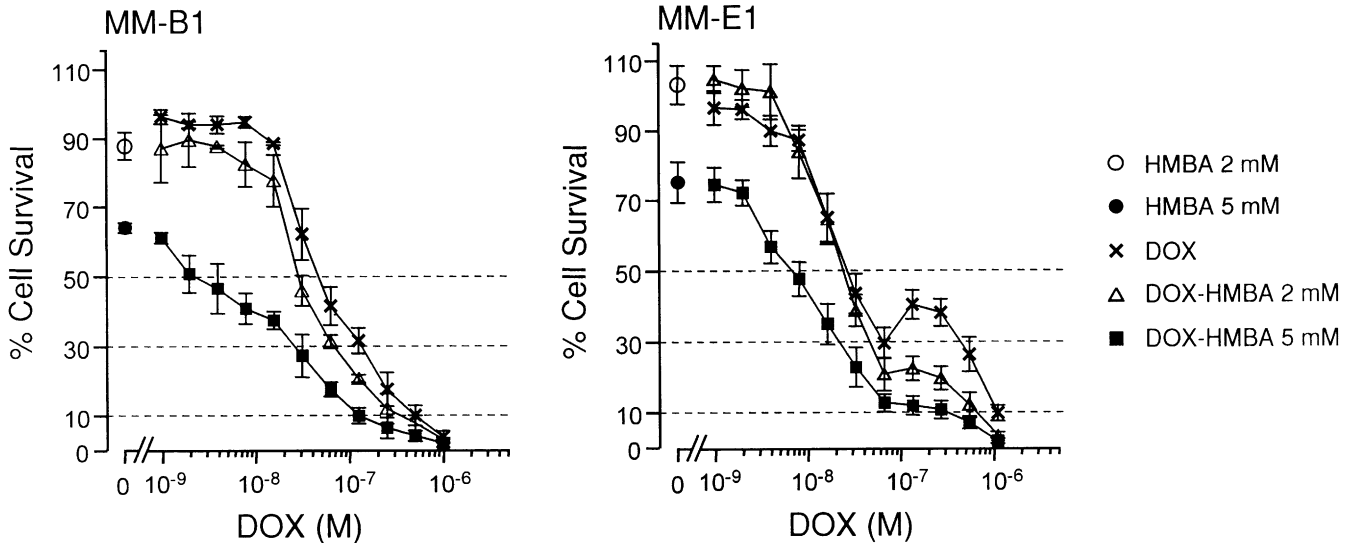

Fig. 2 Dose–response effect of DOX and DOX–HMBA treatments on MM cell survival. DOX–HMBA treatments were made by combining increasing doses of DOX with a fixed dose of 2 or 5 m*M* HMBA. Cell viability was evaluated after 96 h from the administration of: DOX, HMBA 2 m*M*, HMBA 5 m*M*, DOX–HMBA 2 m*M*, DOX–HMBA 5 m*M*

and 5 m*M* HMBA was greater than that obtained with DOX alone with nearly the complete range of DOX concentrations tested, in both cell lines. As shown in Table 1, the concentrations of DOX required to achieve 50%, 70% and 90% inhibition of cell survival were reduced severalfold by combination with 5 m*M* HMBA. As a second finding, the biphasic trend of the DOX dose–response curve in MM-E1 cells was completely abolished by the combined treatment, demonstrating that the induction of resistance occurring with the administration of DOX alone was counteracted by the simultaneous administration of 5 m*M* HMBA. The effect of the DOX–HMBA 2 m*M* combination was instead significantly greater than that of DOX alone with selected concentrations of DOX only, i.e., 0.12 μ*M* in MM-B1 and 0.12–1.00 μ*M* in MM-E1 cells. Nonetheless, the biphasic trend of the DOX dose–response curve in MM-E1 cells was still greatly reduced by this drug combination.

The analysis of drug interaction revealed that the combination of HMBA 5 m*M* with most DOX concentrations achieved R index values significantly greater than 1, indicating synergistic effects, in both cell lines (Fig. 3). The most marked synergistic interactions were observed in MM-E1 cells with DOX concentrations in the range associated with induction of resistance. The combination of DOX and 2 m*M* HMBA achieved synergistic effects only with a limited number of DOX concentrations, as expected, and the synergy was less pronounced than that achieved with the combination of DOX and 5 m*M* HMBA.

To evaluate the effect of combined treatments on MM cell death, MM-B1 and MM-E1 cells were treated with either DOX and HMBA alone or with DOX–HMBA combinations, using as representative drug concentrations 5 m*M* HMBA and a dose of DOX cor-

Table 1 Relative cytotoxic efficacy of DOX and DOX–HMBA combinations. DOX–HMBA treatments were made by combining increasing concentrations of DOX with fixed concentrations, either 2 or 5 m*M*, of HMBA. DOX concentrations are $\times 10^{-7}$ *M*. The inhibitory concentrations (IC) at 50%, 70%, and 90% cell survival inhibition were calculated from survival graphs. The fold dose reduction (DR) allowed for DOX by the combined treatments at 50%, 70%, and 90% cell survival inhibition were obtained from the ratio between the DOX IC_x and the DOX–HMBA IC_x, with *x* being a specified level of toxicity

Cell line	Treatment	Inhibitory concentrations			Fold dose reduction		
		IC_{50}	IC_{70}	IC_{90}	DR_{50}	DR_{70}	DR_{90}
MM-B1	DOX	0.45 ± 0.12	1.27 ± 0.15	5.13 ± 1.50			
	DOX–HMBA 2 m*M*	0.28 ± 0.04	0.67 ± 0.08*	3.30 ± 1.56	1.61	1.89	1.55
	DOX–HMBA 5 m*M*	0.03 ± 0.02*	0.38 ± 0.20*	1.42 ± 0.51*	15.00	3.34	3.61
MM-E1	DOX	0.25 ± 0.05	0.64 ± 0.19 (a); 4.10 ± 0.99 (b)[a]	10.12 ± 1.18			
	DOX–HMBA 2 m*M*	0.22 ± 0.03	0.43 ± 0.07**	5.67 ± 1.40*	1.14	1.49 (a); 9.53 (b)	1.78
	DOX–HMBA 5 m*M*	0.06 ± 0.03*	0.21 ± 0.07***	2.76 ± 1.53*	4.17	3.05 (a); 19.52 (b)	3.67

*$P < 0.05$ vs corresponding DOX IC value, **$P < 0.05$ vs DOX IC_{70}(b), ***$P < 0.05$ vs both DOX IC_{70}(a) and DOX IC_{70}(b)
[a]Due to the biphasic viability curve of MM-E1 cells under DOX treatment, two DOX IC_{70} values, IC_{70}(a) and IC_{70}(b), were obtained for this cell line; DR_{70}(a) and DR_{70}(b) were calculated accordingly.

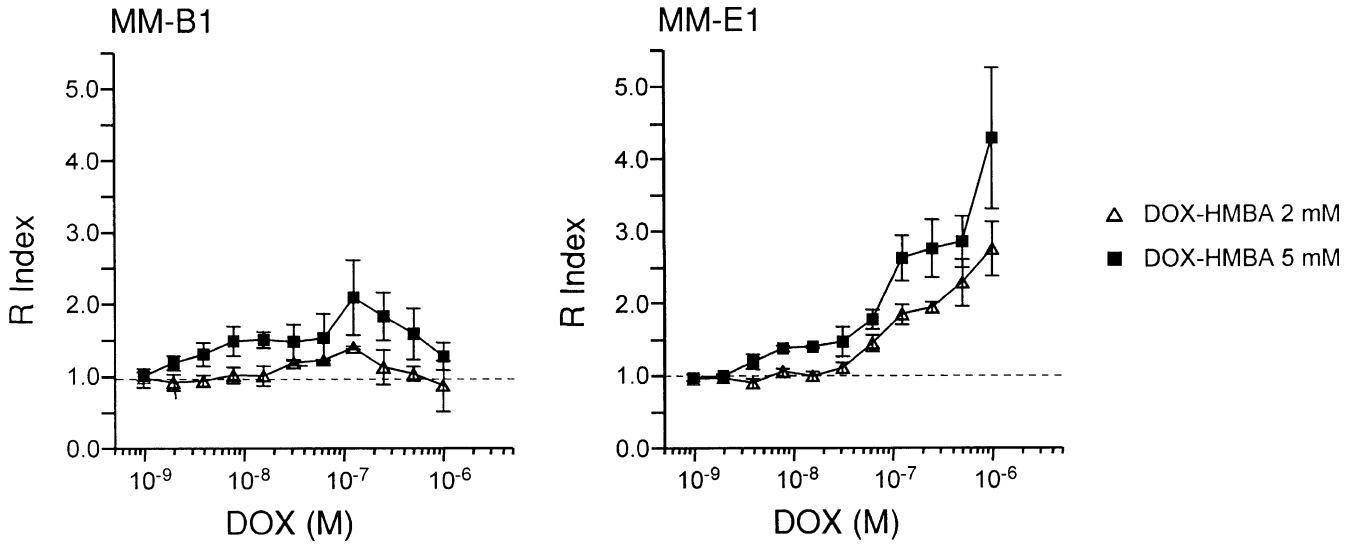

Fig. 3 Drug interaction analysis: R indexes for DOX–HMBA 2 m*M* and DOX–HMBA 5 m *M* combined treatments. An R index >1 indicates a synergistic interaction, an R index $=1$ indicates additivity, an R index <1 indicates that the combination exerts a less-than-additive effect

responding to the specific IC_{50} of each cell line. As shown in Fig. 4, the cultures treated with DOX had an increase of both necrotic and apoptotic cells, whereas the cultures treated with 5 m*M* HMBA did not show a significant increase over their spontaneous death rates, as stated above. Nonetheless, the rates of necrosis and apoptosis of DOX–HMBA-treated cultures were greater than those of the cultures treated with DOX alone, with an increase of about 50% in both cell lines according to trypan blue staining, and an increase of about 60% and 150% in MM-B1 and MM-E1 cell cultures, respectively, according to the TUNEL assay. The combination of HMBA and DOX thus resulted in synergistic cell killing.

The effects of the cytostatic HMBA at 5 m*M* on the expression of the antiapoptotic Bcl-2 and Bcl-X_L and the proapoptotic Bax proteins were then investigated in time-course experiments. The cell lines used in the present study expressed Bcl-X_L and Bax at similar levels, whereas much lower Bcl-2 levels were detected in MM-B1 cells than in MM-E1 cells (Fig. 5a). Interestingly, the anti-Bax antibody recognized two bands in the protein extracts of MM cells, compatible with the full-length 21-kDa Bax (p21) and the recently described 18-kDa Bax form (p18). The latter is generally detected upon apoptotic induction [29], whereas it was expressed in the absence of exogenous apoptotic stimuli in both the MM-B1 and MM-E1 cell lines. This baseline p18 Bax expression could be either intrinsic to our MM cells or related to their spontaneous death rates. HMBA caused an early downregulation of Bcl-X_L in both cell lines and this effect, with a transient and partial interruption on the third day of treatment, persisted throughout the 96 h of exposure to the drug. Bcl-2 was also progressively downregulated in MM-E1 cells, whereas both p21 and p18 Bax underwent an early and sustained upregulation, with the p21/p18 ratios unmodified. In contrast, no major changes in Bcl-2 or Bax expression were detected in HMBA-treated MM-B1 cells. Figure 5b illustrates the effects of the treatment on the ratio between the antiapoptotic Bcl-2 and Bcl-X_L and the proapoptotic Bax proteins, as quantified via densitometric analysis of

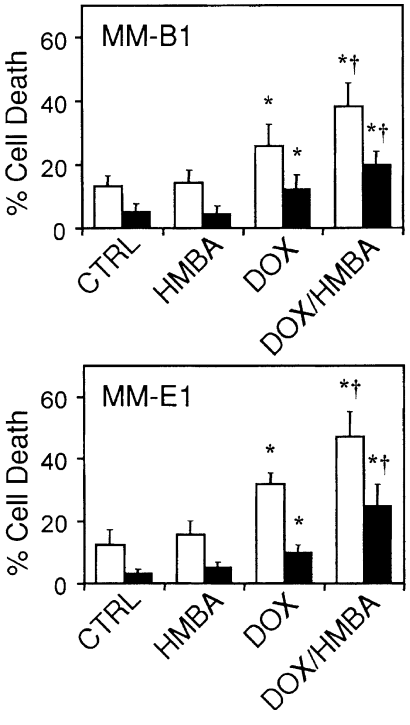

Fig. 4 Cell death rates of MM cultures after 96 h of growth without treatment (*CTRL*) or under treatment with HMBA, DOX and DOX–HMBA, as evaluated by trypan blue staining (*white bars*) and TUNEL (*black bars*). HMBA was used at 5 m*M*, whereas DOX was used at a dose corresponding to the specific IC_{50} of each cell line. $^{*}P<0.05$ vs untreated cells, $^{\dagger}P<0.05$ vs cells treated with DOX alone

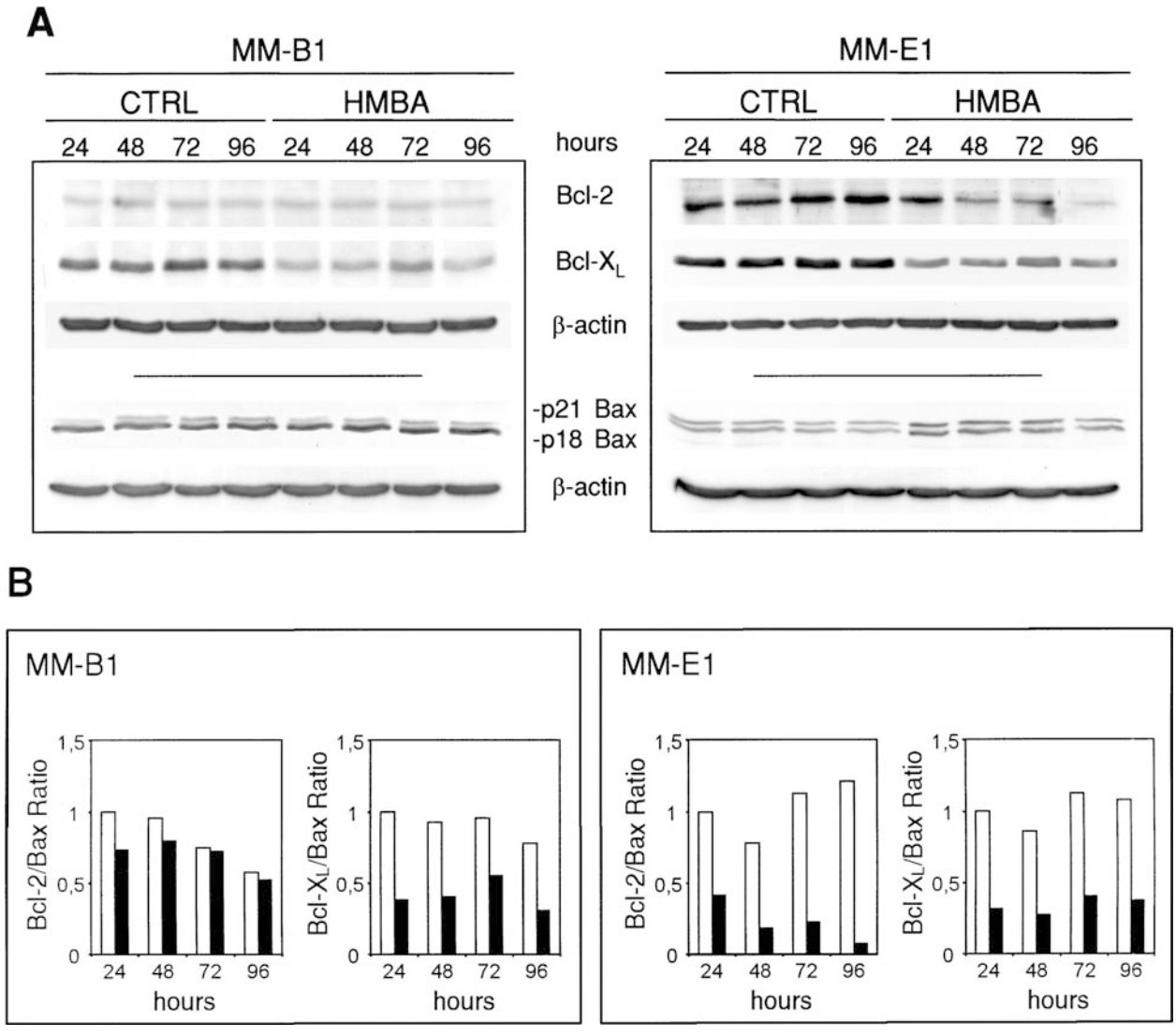

Fig. 5a, b Effect of HMBA on the expression of Bcl-2, Bcl-X_L, and Bax proteins. **a** Time-course of Bcl-2, Bcl-X_L, and Bax expression in cultures untreated (*CTRL*) or treated with 5 m*M* HMBA for 96 h, as determined by Western blot analysis. A representative experiment is shown. The same membrane was successively probed with anti-Bcl-2, anti-Bcl-X_L and anti-β-actin antibodies or, alternatively, with anti-Bax and anti-β-actin antibodies. **b** Bcl-2/Bax and Bcl-X_L/Bax ratios of untreated (*white bars*) and HMBA-treated cultures (*black bars*) as obtained from densitometric analysis of Western blot autoradiograms. The densitometric values of Bcl-2, Bcl-X_L, and Bax were normalized to those of β-actin, and the normalized values are expressed as fold variation vs the values from untreated cultures at 24 h that were arbitrarily set equal to 1. HMBA treatment reduced the Bcl-X_L/Bax ratio in MM-B1 cells and both the Bcl-2/Bax and Bcl-X_L/Bax ratios in MM-E1 cells

Western blot autoradiograms. The cytostatic HMBA at 5 m*M* lowered the threshold for death commitment by reducing the ratio between antiapoptotic and proapoptotic Bcl-2 family proteins.

Experiments were also performed to determine whether the modulation of Bcl-2 family members induced by HMBA occurred at the transcriptional or post-transcriptional level. Semiquantitative RT-PCR analysis showed that treatment with 5 m*M* HMBA induced a downregulation of bcl-2 and bcl-X_L mRNAs, with no major modifications of bax mRNA, in either cell line (Fig. 6). The results from Western blot and RT-PCR analyses taken together thus indicate that Bax protein levels are upregulated by HMBA though post-transcriptional mechanisms in MM-E1 cells, as previously reported for other cell types [7, 20].

Finally, experiments were undertaken to determine whether the synergistic cell killing induced by the co-administration of DOX and HMBA could be associated with an enhanced modulation of Bcl-2 family members. In particular, we focused on Bcl-X_L, since this was the only protein we investigated that was affected by HMBA treatment in both MM-B1 and MM-E1 cell lines. Western blot analysis of cell lysates obtained from cultures grown either untreated or treated separately with HMBA 5 m*M*, DOX at the specific IC_{50} of each cell line, or with their combination, demonstrated that the co-administration of the two drugs resulted in a greater downregulation of this antiapoptotic protein than that obtained with either drug administered alone (Fig. 7).

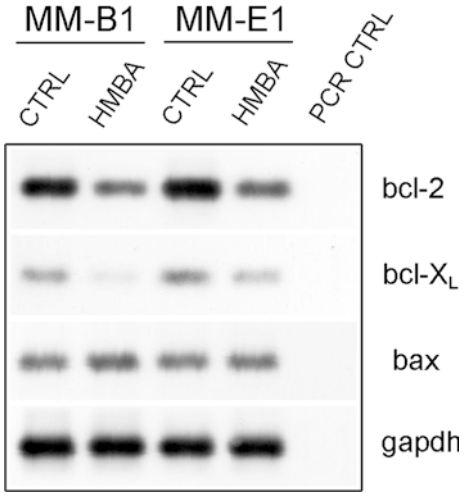

Fig. 6 Semiquantitative RT-PCR analysis of bcl-2, bcl-X_L, and bax expression in MM-B1 and MM-E1 cells grown either untreated (*CTRL*) or treated with 5 m*M* HMBA for 48 h. A negative control, in which cDNA was omitted, was used in every PCR assay (*PCR CTRL*). Amplification of GAPDH (*gapdh*) was used as a control for cDNA quality and loading

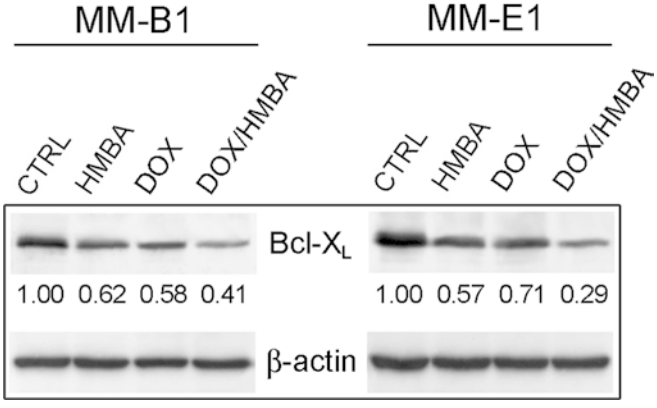

Fig. 7 Comparison of the effects of HMBA, DOX, and DOX–HMBA on Bcl-X_L protein levels, as determined by Western blot analysis. Cell lysates were obtained from MM cultures grown untreated (*CTRL*) or treated with HMBA 5 m*M*, DOX at the specific IC_{50} of each cell line, or with their combination for 48 h. A representative experiment is shown. The same membrane was successively probed with anti-Bcl-X_L and anti-β-actin antibodies. The densitometric values of Bcl-X_L were normalized to those of β-actin, and the normalized Bcl-X_L values are expressed as fold variation vs the values from untreated cultures. The combination of the two drugs resulted in a greater downregulation of Bcl-X_L than that obtained with either drug administered alone

Discussion

MM is poorly responsive to conventional therapeutic approaches. The present investigation demonstrated that MM cells in culture are sensitive to the cytotoxic effects of HMBA, a differentiating agent with cytostatic properties, whose cell death-inducing effects are receiving growing attention [26, 30]. In agreement with results obtained in different cell types [12, 28], HMBA concentrations of about 5 m*M* exerted a cytostatic effect on MM cells. On the other hand, MM cultures treated with HMBA at ≥10 m*M* showed a steep increase in both necrotic and apoptotic cells. Thus the sensitivity of MM cells to HMBA death-inducing effects is in the range of those reported for leukemic and colon carcinoma cells [26].

Other authors have reported that some hybrid polar compounds can enhance cellular responses to antineoplastic treatments through mechanisms as yet undefined [4, 16, 37]. In the experiments reported here we found that HMBA inhibited MM cell survival synergistically in combination with the classical antineoplastic drug DOX. It is known that HMBA and DOX can affect both cell cycle progression and death [9, 18, 26, 30]. Their synergistic interaction could thus rely on multiple pathways and molecular determinants. In this context, we demonstrated that the supraadditive toxicity exerted by the two drugs in MM cells could, at least in part, rely on a synergistic cell killing. Furthermore, we found that HMBA, at a concentration not sufficient to induce cell death per se, reduced the ratio between antiapoptotic and proapoptotic Bcl-2 family members, thereby lowering the threshold for cell death commitment and sensitizing MM cells to the death inputs provided by DOX. The net balance between antiapoptotic and proapoptotic proteins of the Bcl-2 family is indeed considered critical for the determination of the fate and susceptibility of cells to death-inducing stimuli. This balance regulates the release of mitochondrial apoptogenic factors that, in turn, activate downstream effectors of apoptotic cell death [11]. Moreover, there is emerging evidence that Bcl-2 family members can influence necrotic as well as apoptotic cell death [13, 29]. It is worthy of note that the antiapoptotic Bcl-X_L was similarly downregulated by HMBA in both our MM cell lines and that the synergistic cell killing induced by the coadministration of DOX and HMBA was associated with an enhanced downregulation of this protein that has been addressed as a fundamental regulator of MM cell survival and a potential target in MM therapy [31].

Remarkably, the simultaneous administration of HMBA counteracted the chemoresistance induced by DOX in MM-E1 cells. Furthermore, the highest levels of synergy between HMBA and DOX were observed in the MM-E1 cell line using DOX doses in the range of resistance induction. These observations strongly suggest that a modulation of drug resistance mechanisms by HMBA may contribute to the synergistic interaction between the two drugs in this cell line. The mechanisms responsible for DOX-induced resistance in MM-E1 cells still have to be elucidated. As a more general matter, the molecular determinants of MM refractoriness to chemotherapy have not been clearly defined yet. MM cells show a high expression of genes involved in DNA repair, protection from oxidative stress, drug transport and detoxification, but the actual role of individual molecules in MM chemoresistance remains controversial [24, 32]. The multidrug resistance-associated P-glycoprotein has been found to be induced in MM cells treated with

DOX or other anticancer drugs in vitro, but further studies, performed both in vitro and in vivo, have demonstrated the absence of a functional correlation between the expression of this molecule and MM sensitivity to chemotherapy [14, 17, 21, 32].

HMBA has shown clinical activity both in leukemic patients and, to a lesser extent, in patients with solid tumors [1, 38]. However, the plasma concentrations that can be obtained and maintained through systemic delivery are not in excess of 2 m*M*, which limits the therapeutic effectiveness of the compound [6, 36, 38]. Remarkably, in the case of MM, this limitation could be overcome through intrapleural delivery of HMBA. In fact, intrapleural chemotherapy is currently performed in MM patients, with the double advantage of increasing local drug concentration and decreasing systemic toxicity [39]. Other tumors arising or metastasizing in the intracavitary environment may also be amenable to local treatment with HMBA. The effects of intrapleural HMBA have not been addressed, however, at least to our knowledge, either in clinical trials or in animal studies. The recently isolated second-generation hybrid polar compounds, which share some biological properties with HMBA but display much greater potencies, could also have therapeutic potential in MM [23].

In conclusion, we showed that HMBA exerts cytotoxic effects on MM cells in vitro, both as a single agent and through potentiation of DOX toxicity, and suggest that its therapeutic potential could be exploited, through intrapleural delivery, for the treatment of this locally aggressive tumor. Our findings indicate that further investigations on the feasibility of intracavitary therapy with HMBA are warranted.

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