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ORIGINAL ARTICLE

Erucylphospho-*N*,*N*,*N*-trimethylpropylammonium (erufosine) is a potential antimyeloma drug devoid of myelotoxicity

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Abstract
Purpose Erufosine is an i.v. injectable alkylphosphocholine which is active against various haematological malignancies in vitro. In the present study, its effects on multiple myeloma (MM) cell lines and on murine and human hematopoietic progenitor cells (HPCs) were investigated.
Methods The following MM cell lines were used: RPMI-8226, U-266 and OPM-2. The cytotoxicity of erufosine against these cell lines was determined by the MTT-dye reduction assay. Bcl-2, Bcl-X_L and pAkt expression levels, activation of caspases, as well as cleavage of PARP, were studied by Western blotting. Migration was evaluated by a modified Boyden-chamber assay. The haematologic toxicity of erufosine was assessed using clonogenicity assays with normal HPCs of murine or human origin.
Results Significant cytotoxic activity of erufosine against the MM cell lines was found. Comparison of the characteristics of erufosine-induced cell death in the three cell lines revealed a complex mode of action with apoptotic mechanisms prevailing in OPM-2 cells and non-apoptotic mechanisms prevailing in U-266 cells. The sensitivity of the MM cell lines to erufosine-induced apoptosis correlated inversely with the Bcl-X_L expression level. Erufosine participated in synergistic interactions with various drugs. Furthermore, it showed potent migration-inhibiting activity in RPMI-8226 cells. Erufosine was not toxic to normal HPCs of murine or human origin and even stimulated progenitors from human umbilical cord blood to form granulocyte/macrophage colonies. Moreover, erufosine ameliorated the toxicity of bendamustine to murine HPCs.
Conclusions Overall, the data presented reveal that erufosine could have potential as an antimyeloma drug and deserves further development.

Keywords Erufosine · Alkylphosphocholines · Multiple myeloma · Cytotoxicity · Haematopoietic progenitors · Antimigratory activity

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Introduction

Multiple myeloma (MM) is a relatively frequent haematological malignancy accounting for 15% of all cases of lymphoproliferative diseases [1]. The disease is characterized by malignant plasma cells that secrete monoclonal immunoglobulins and infiltrate the bone marrow. MM cells interact extensively with the bone marrow microenvironment via adhesion molecules and are stimulated by

cytokines including interleukin (IL)-6, vascular endothelial growth factor (VEGF), macrophage inflammatory protein (MIP)-1α, MIP-1β and stromal cell-derived factor (SDF)-1 [2]. These influences intensify the signalling of three important pathways [phosphoinositide-3 kinase (PI3 K)/Akt, Ras/Raf/mitogen-activated protein kinase (MAPK) and Janus kinase (JAK)] and as a result promote MM cell growth and survival, as well as confer drug resistance.

With traditional induction regimens, about 50% of patients with MM show an initial response but ultimately develop drug resistance [3, 4]. Newer lenalidomide- and bortezomib-based regimens achieve an initial response in up to 90% of patients [3], albeit at the expense of significant side effects, especially neurotoxicity (bortezomib) and myelosuppression (lenalidomide and bortezomib) [5]. Despite these advances, MM remains incurable and there is an enormous need for new therapeutic options which are more effective and less toxic than the currently used drugs. Because MM cells are long surviving rather than fast growing, drugs that cause apoptosis are of particular interest [6].

Alkylphosphocholines are a class of antineoplastic compounds that were originally derived from alkylglycerophosphocholines but they lack the glycerol backbone [7]. They exhibit significant cytotoxic and pro-apoptotic activity towards a vast number of malignantly transformed haematopoietic cell lines [8]. Alkylphosphocholines enhance the cytotoxicity of various conventional cytostatics [9–11] and sensitize tumour cells to radiation-induced cell death [12]. Interestingly, alkylphosphocholines do not target DNA but primarily interact with cell membranes, thus differing from classical chemotherapeutic drugs [13]. This is based on their structure, which precludes intensive metabolism and leads to accumulation within membranes [14, 15]. There is evidence that edelfosine and the alkylphosphocholine perifosine promote clustering of cell surface lipid rafts in human T-lymphoid Jurkat and Peer leukaemic cells, as well as in multiple myeloma MM144 cells [16]. This is accompanied by recruitment of Fas/CD95 and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors-1/2 (TRAIL-R1/R2), together with corresponding downstream signalling molecules [6, 16]. Studies on various malignantly transformed haematopoietic cell lines showed that alkylphosphocholines reduce the level of phosphorylated Akt [11, 17] and phosphorylated Rb [18], up-regulate Rb [11, 18] and down-regulate β-catenin and survivin [19].

Different from perifosine and other alkylphosphocholines, erucylphospho-*N*,*N*,*N*-trimethylpropylammonium (ErPC3, erucylphosphohomocholine, erufosine) does not cause haemolysis upon intravenous application and its cholinomimetic properties are less pronounced when compared to perifosine [20]. Preliminary results from a phase I trial in patients with chronic lymphocytic leukaemia (CLL) show that plasma concentrations of more than 60 μM erufosine can be achieved in humans without significant toxicity [21]. It was recently demonstrated by us that erufosine is cytotoxic in three human MM cell lines [22]. The IC_{50} values at 72 h were in the range between 3.2 and 16.2 μM and distinguished two of the cell lines (RPMI-8226 and OPM-2) as more sensitive than the third (U-266).

In the current study, we investigated the characteristics of erufosine-induced cell death in the three MM cell lines, the influence of erufosine on MM cell migration, the combined cytotoxic effects of erufosine with other drugs and the influence of erufosine on normal haematopoiesis.

Materials and methods

Drugs, chemicals and antibodies

Erufosine was synthesized by H. Eibl (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), dissolved in ethanol/PBS (1:1, v/v) and stored at 4°C. The chemical structure of erufosine was published elsewhere [9]. Bendamustine was obtained from Astellas Pharma GmbH, Munich, Germany. Melphalan and curcumin were purchased from Sigma-Aldrich. Bendamustine and melphalan were used as fresh stock solutions, prepared in ethanol or methanol, respectively. Curcumin was dissolved in ethanol and stored at −20°C. Bortezomib (Velcade®) of clinical grade was dissolved in dimethylsulphoxide (DMSO) and stored at −20°C. DAPI (4′,6-diamidino-2-phenylindole) was purchased from Roche Diagnostics (Mannheim, Germany), dissolved in phosphate-buffered saline (PBS) and stored at −20°C. All the antibodies used for Western blot were obtained from Santa Cruz Biotechnology (Heidelberg, Germany): goat polyclonal antibodies against caspase-8 and β-actin (sc-6136 and sc-1615, respectively), mouse monoclonal antibodies against caspase-3, caspase-9, poly (ADP)-ribose polymerase (PARP) and Bcl-2 (sc-56053, sc-17784, sc-8007 and sc-509, respectively), rabbit polyclonal antibodies against Bcl-$X_{S/L}$, AKT1/2/3 and phosphorylated Akt1/2/3 (sc-104, sc-8312 and sc-7985-R, respectively) and corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. All other chemicals were supplied by Sigma-Aldrich if not otherwise specified.

Cell lines and culture conditions

The following human cell lines were used: multiple myeloma RPMI-8226, OPM-2 and U-266 cells and T-cell leukaemia KE-37 (former SKW-3) cells. All of them were

obtained from DSMZ, Braunschweig, Germany. All cells were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) and 2 mM L-glutamine (all from GIBCO/Invitrogen, Karlsruhe, Germany) under standard conditions (37°C in an incubator with humidified atmosphere containing 5% CO_2). Cells were passaged two or three times a week to maintain them in log phase.

Nuclear staining with DAPI

After treatment, cells were washed in PBS, centrifuged onto glass slides using a cytospin centrifuge (Shandon, Pittsburgh, PA, USA) and stained with DAPI solution (1 μg/ml in PBS). The slides were kept in the dark at 4°C until analysis using a fluorescence microscope.

Detection of apoptotic oligonucleosomal DNA fragmentation

About 5×10^6 treated or control cells were washed in PBS and lysed in 0.75-ml buffer [1 mM KH_2PO_4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), 1 mM Na_3VO_4, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% glycerol, 0.3% Triton X-100; pH 7.4]. After incubation for 10 min on ice, the lysates were centrifuged at 13,000×*g* for 20 min. Supernatants were harvested and processed by adding 0.187 ml 6 M NaCl solution and 0.937 ml 2-propanol, while gently mixing. Samples were incubated at −20°C overnight, thereafter centrifuged at 13,000×*g* for 20 min and the DNA pellets were washed with 70% ethanol, air-dried, re-dissolved in distilled water and analysed by electrophoresis in an 0.8% agarose gel. DNA was visualized by ethidium bromide staining and photographed using an UV transilluminator equipped with a digital camera (Herolab E.A.S.Y., Wiesloch, Germany).

A previously described experimental procedure for the detection of eventual oligonucleosomal DNA fragmentation in nuclei of untreated cells incubated with cytosolic extracts from apoptotic cells was used [9]. Briefly, treated or untreated cells were lysed in 0.75-ml buffer (see earlier) on ice for 10 min. Thereafter, lysates were spun for 10 min at 10,600 × *g*. Supernatants of treated samples were used as cytosolic fraction. Nuclear pellets of untreated cells were additionally washed in the same buffer, but without Triton X-100. Incubation with the cytosolic fraction was started immediately under continuous shaking at 37°C. At the time points indicated later, corresponding samples were centrifuged for 10 min at 10,600×*g*. Nuclei were lysed, and DNA was extracted using spin columns (QIAmp DNA Blood mini, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated DNA was analysed for oligonucleosomal apoptotic laddering by electrophoresis as described elsewhere.

Western blot

Cell pellets from control or treated cells ($4\text{-}5 \times 10^6$ cells) were re-suspended in buffer containing 0.1 M NaCl, 10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA). Thereafter, an equal quantity of lysis buffer (100 mM Tris-HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol) was added. Both buffers were supplemented before use with Na_3VO_4 to a final concentration of 1 mM, as well as with Complete™ protease inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany) as recommended by the manufacturer. The lysis buffer was additionally supplemented with dithiotreitol (DTT) to a final concentration of 200 mM. Lysates were simultaneously vortexed and boiled for 10 min at 100°C and thereafter centrifuged at 10,600×*g* for 10 min at 4°C. The protein concentration in a portion of each sample, lysed without DTT, was determined using the Pierce BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol. Volumes containing 50 μg of protein were taken from each sample, mixed with loading buffer (4×) and then loaded on NuPAGE® 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA). The electrophoresis was carried out using MES buffer (50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 50 mM Tris, 3.5 mM SDS, 1 mM EDTA; pH 7.3). The proteins were transferred to a polyvinylidene difluoride membrane which was thereafter blocked (5% skimmed milk and 0.1% Tween 20 in Tris-buffered saline (TBS)) and incubated with specific primary antibodies in a solution containing TBS, 0.5% milk and 0.1% Tween 20. The blots were washed, exposed to corresponding HRP-conjugated secondary antibodies, washed again and visualized using the Western Lightning™ system (PerkinElmer Life Sciences, Boston, MA, USA). Densitometric analysis was performed using the Scion Image for Windows program (Scion Corporation, Frederick, MD, USA).

Migration assay

Cell migration was evaluated by a modified Boyden chamber assay, using Millicell™ hanging inserts with polyethylene terephthalate membrane of 8 μm pore size (Millipore, Bedford, MA, USA) and 24-well Cellstar™ plates (Greiner, Frickenhausen, Germany). Initially, myeloma cells were starved in serum-free Opti-MEM® medium for 24 h. For the final 5 h of this period, they were grown in the presence or absence of erufosine. Thereafter, the cells were centrifuged, re-suspended in fresh medium and added to the upper compartment. The attractant

medium in the lower chamber contained 0.8% methylcellulose and 10% FCS in RPMI-1640 medium. Twelve hours later, cells that had migrated into the lower compartment were counted under an inverted microscope.

MTT assay for cell survival and proliferation

Logarithmically growing cells were seeded into 96-well microplates (100 µl/well at a density of 2×10^5 cells/ml) and exposed to various concentrations of the test compounds for 72 h. The cell survival fraction was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide)-dye reduction assay as described by Mosmann [23], with some modifications. Briefly, after incubation with the test compound, MTT solution (10 mg/ml in PBS) was added (10 µl/well). Plates were further incubated for 3 h at 37°C, and the formazan crystals formed were dissolved by addition of 110-µl solvent (5% formic acid in 2-propanol) per well and mixing. Absorption was measured by an automated microtiter plate spectrophotometer (Labexim LMR-1, Lengau, Austria) at 550 nm. For each concentration, at least eight wells were used. Complete medium (100 µl), MTT solution (10 µl) and 5% formic acid in 2-propanol (110 µl) were used as blank solution.

Long-term bone marrow cell cultures (LTBMCCs) and colony-forming unit (CFU) assay

LTBMCCs were derived from murine femurs as previously described [8]. Briefly, female and male ICR mice (National Breeding Center, Slivnitza, Bulgaria) were used as donors. After killing the animals by cervical dislocation, femurs were removed and bone marrow was flushed out aseptically into LTBMCC medium [10% FCS (Biochrome, Germany), 10% horse serum (Invitrogen, USA), 1 µM hydrocortisone, 0.017 mg/ml transferrin, 0.007 µl/ml β-mercaptoethanol, 100 IU/ml penicillin (Invitrogen, USA), 100 µg/ml streptomycin (Invitrogen, USA) and 2.5 µg/ml amphotericin B (Applichem, Germany) in MEM-α (Invitrogen, USA)] using a 21-gauge needle fitted to a syringe. A single-cell suspension was prepared by repeated gentle aspiration of the marrow plug. The primary cells were plated into 25-cm^2 culture flasks (Greiner, Frickenhausen, Germany) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Every week half of the medium was exchanged. After an adherent layer had been established (in approx. 2 weeks), LTBMCCs were re-fed by adding fresh bone marrow. After two more weeks, when clusters of haematopoietic progenitor cells were visible, the cultures were treated with erufosine, bendamustine or combinations of both drugs. Exposure of cells to drugs was maintained for 24 h, and then the medium was changed. In combination experiments, this was followed by treatment with the second drug for further 24 h. After the treatment period, the cells were detached by trypsinisation and plated in semisolid medium (0.8% methylcellulose, 30% horse serum and 0.1 ng/ml recombinant mouse granulocyte/macrophage colony-stimulating factor in RPMI-1640). The 24-well plates (at least three wells per treatment group) were kept under standard cell culture conditions for 10–14 days. Colonies (clusters of 20 or more cells) were counted using an inverted microscope (TMS, Nikon, Japan).

CFU assay with mononuclear cells from human cord blood

Umbilical cord blood was obtained following the informed consent of three mothers. Each individual sample was subjected to density gradient centrifugation ($400 \times g$ for 30 min) on Ficoll-Paque® PLUS (Amersham Biosciences, Uppsala, Sweden). The mononuclear cell fractions were isolated and stored in liquid nitrogen in medium containing 10% DMSO and 90% FCS. Later on, all collected samples were pooled, and the cells were cultured under standard conditions in FCS-enriched RPMI-1640 medium for 24 h, in the presence or absence of erufosine. Thereafter, aliquots of 50,000 cells were taken and mixed with 1-ml MethoCult® GF H4434 medium (StemCell Technologies, Vancouver, BC, Canada), according to the recommendations of the manufacturer. Each sample was distributed into 3 wells of a 24-well culture plate (300 µl/well), and the plate was incubated under standard conditions. Ten days later, the colonies were scored under an inverted microscope (TMS, Nikon, Japan).

Statistics and evaluation of combination effects

The cell survival fractions were calculated as the ratio of MTT absorptions from treated and control cells. Expected additive effects were calculated according to the equation:

$$SF_{A+B(expected)} = SF_A \times SF_B,$$

where SF_A and SF_B are the cell survival fractions after single agent treatment with drug A or B, respectively. The resulting values were compared with the respective experimentally observed values ($SF_{A+B\ (measured)}$). If $0.7 \times SF_{A+B\ (expected)} < SF_{A+B\ (measured)} < 1.3 \times SF_{A+B\ (expected)}$, the combination effect was considered additive. If $SF_{A+B\ (measured)} \leq 0.7 \times SF_{A+B\ (expected)}$, synergistic drug interaction was assumed and if $SF_{A+B\ (measured)} \geq 1.3 \times SF_{A+B\ (expected)}$, the combination effect was considered antagonistic. All values were normalized to 100% and represented in graphical form, together with the corresponding standard deviations (SD). In addition, the

95- and 99%-confidence intervals for $SF_{A+B\ (measured)}$ and for $SF_{A+B\ (expected)}$ were determined from the respective standard errors of the mean and then used for estimation of significance, e.g. the differences were considered significant at the 5% level when the respective 95% confidence intervals did not overlap. Moreover, non-overlapping of 95% confidence intervals for the distribution of individual samples in the experimental populations (calculated from standard deviations) was used to demonstrate especially large differences between expected and observed mean values. Differences between individual results from experiments on migration and clonogenicity were assessed for significance by means of Student's *t*-test.

Results

Nuclear changes and oligonucleosomal DNA fragmentation after exposure of MM cells to erufosine

Treatment of OPM-2 cells with 15 μM erufosine for 24 h caused nuclear shrinkage, chromatin condensation and nuclear fragmentation (Fig. 1a). In contrast, nuclei of U-266 cells did not undergo any visible changes, even when the cells were treated with 40 μM erufosine for time periods as long as 72 h (data not shown). There was also no oligonucleosomal DNA fragmentation in these cells (data not shown). In order to verify the susceptibility of U-266 nuclei to undergo oligonucleosomal DNA fragmentation, a cell-free system was established. It consisted of nuclei from untreated U-266 cells and cytosolic extracts from KE-37 cells, pretreated with 20 μM erufosine for 24 h. In this system, marked oligonucleosomal DNA fragmentation was observed, even after incubation times as short as 15 min (Fig. 1b). The phenomenon of DNA laddering was also detected when longer incubation times (up to 60 min) were used. DNA isolated from control U-266 nuclei that were not exposed to cytosolic extracts from apoptotic KE-37 cells was not fragmented, thus ruling out the possibility of artefacts due to the isolation method.

Cleavage of caspases and PARP in response to treatment with erufosine

To further characterize the pro-apoptotic activity of erufosine, we searched for the cleavage of caspases and their substrate PARP in OPM-2 and in U-266 cells.[1] OPM-2 cells responded by concentration-dependent activation of the initiator caspases-8 and -9 and the effector caspase-3 (Fig. 2a). The DNA repair enzyme PARP was accordingly inactivated by cleavage. These alterations were detectable

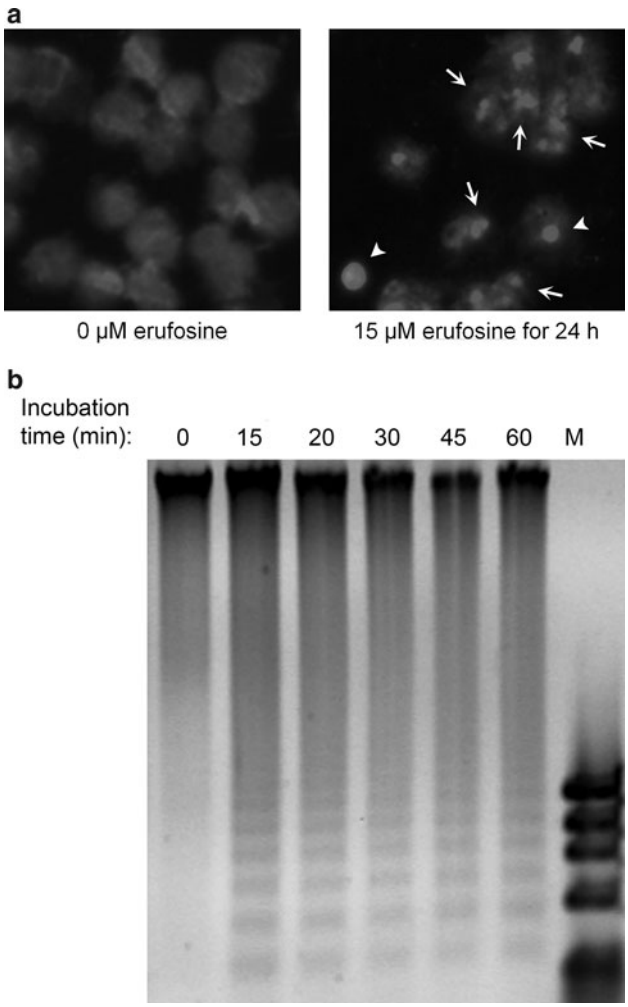

Fig. 1 **a** Effect of erufosine on the morphology of OPM-2 cell nuclei. After treatment with 0 (left image) or 15 (right image) μM erufosine for 24 h, the cells were mounted on slides and their nuclei were stained with DAPI. The *arrow-heads* indicate nuclear shrinkage and chromatin condensation. The *whole arrows* denote nuclear fragmentation. **b** Cell-free transfer of apoptotic stimuli from cytosolic extracts of KE-37 (SKW-3) cells, pretreated with 20 μM erufosine for 24 h, to nuclei of untreated U-266 cells. Nuclei of U-266 cells were incubated with the cytosolic extracts for 0, 15, 20, 30, 45 or 60 min, and then nuclear DNA was extracted and subjected to electrophoresis in an agarose gel. *Lane M*, size marker

following exposure to erufosine concentrations as low as 5 μM for 24 h. Neither caspase activation nor PARP cleavage was observed in U-266 cells even after 72 h treatment with 20 μM erufosine (Fig. 2b).

MM cell lines differ in their Bcl-2 and Bcl-X_L expression levels

When searching for a possible explanation for the differential susceptibility of the three MM cell lines to erufosine-induced apoptosis, we compared their expression levels of the antiapoptotic proteins Bcl-2 and Bcl-X_L. U-266 cells

[1] Activation of caspases and PARP cleavage in RPMI-8226 cells in response to erufosine has been published before [22].

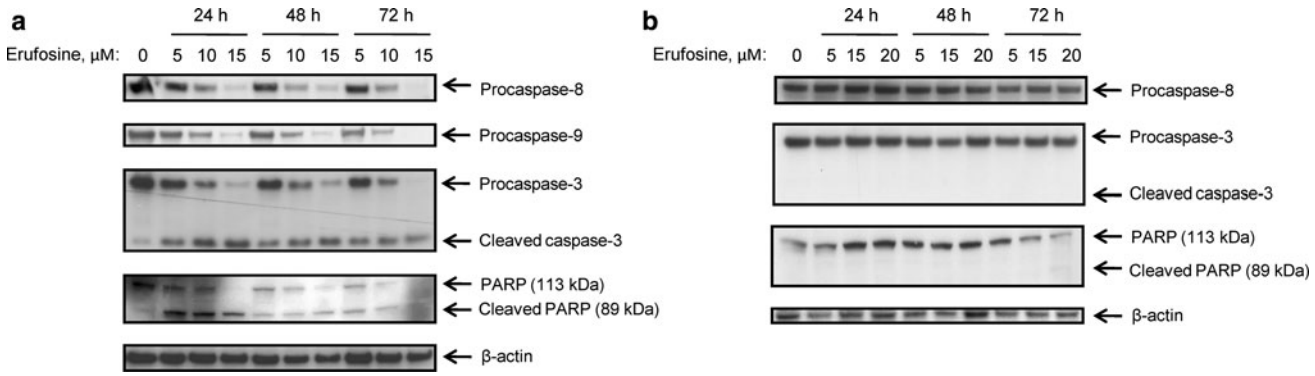

Fig. 2 **a** Concentration-dependent activation of apoptotic cascades after treatment of OPM-2 cells with up to 15 μM erufosine for 24–72 h. Both the extrinsic and intrinsic pathways of apoptosis were activated as certified by consumption of pro-caspases-8 and -9, respectively. The downstream effector caspase-3 was cleaved and activated, too. PARP was accordingly cleaved and inactivated. **b** In U-266 cells, no activation of the apoptotic cascade was observed after treatment with up to 20 μM erufosine for 24–72 h. *β*-actin controls for equal protein loading are also shown for both sets of Western blots (**a** and **b**)

had the highest amount of Bcl-2, followed by OPM-2 and RPMI-8226 cells (Fig. 3a). U-266 cells also had a very high content of Bcl-X_L, while the cell line most prone to apoptosis, OPM-2, barely had any (Fig. 3b).

Erufosine-induced apoptosis in OPM-2 cells involves Bcl-2 cleavage

We next tried to elucidate whether erufosine induces down-regulation or other changes of the above-mentioned antiapoptotic proteins. No change in the expression of Bcl-X_L was detected in RPMI-8226 or in OPM-2 cells (data not shown). The Bcl-2 level also remained constant in RPMI-8226 cells. In OPM-2 cells, however, erufosine led to concentration-dependent and persistent fragmentation of Bcl-2 (Fig. 3c).

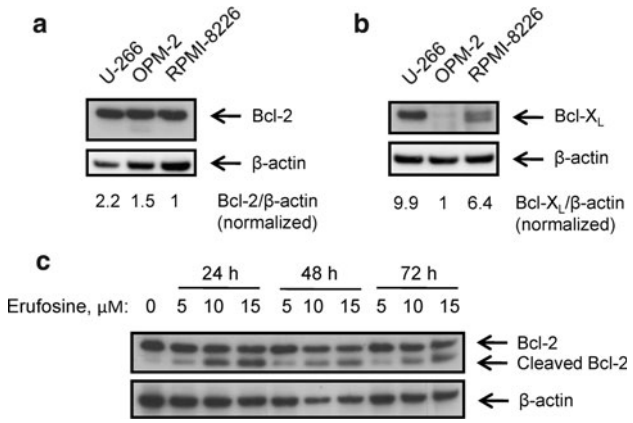

Fig. 3 Basal expression of the antiapoptotic proteins Bcl-2 (**a**) and Bcl-X_L (**b**) in three MM cell lines. The numbers beneath the Western blots represent the relative expression of Bcl-2 or Bcl-X_L in the three cell lines. The values were derived by dividing the densitometric output for each Bcl-2 or Bcl-X_L band by the densitometric output for the corresponding *β*-actin band and subsequent normalization. **c** Erufosine induces concentration-dependent and persistent fragmentation of Bcl-2 in OPM-2 cells as determined by immunoblotting

Erufosine induces dephosphorylation of Akt in OPM-2 cells

Erufosine reduced the level of phosphorylated Akt (pAkt) in OPM-2 cells (Fig. 4a). The reduction was about 75% in cells treated with 5 μM erufosine for 6 h and became more pronounced with higher concentrations and longer incubation time. Concentration-dependent cleavage of Akt was also observed, starting at 10 μM erufosine (Fig. 4a). The molecular weight of the detected fragment was about 44 kDa. No significant dephosphorylation or cleavage of Akt was observed in U-266 or in RPMI-8226 cells (Fig. 4b, c, respectively).

Antimigratory effects of erufosine

Myeloma cells were exposed to erufosine (5 μM for OPM-2 and RPMI-8226 cells or 15 μM for U-266 cells) for 5 h, and their migration towards a compartment enriched with FCS was assessed. The migration rates of erufosine-treated OPM-2, U-266 and RPMI-8226 cells were 65, 49 and 38% of the respective control values for untreated cells (Fig. 5a). The differences were statistically significant for the U-266 and RPMI-8226 cells ($P < 0.05$). The viability of the cells that remained in the upper compartment was evaluated by the MTT-dye reduction assay, and no adverse effect in RPMI-8226 cells was observed (Fig. 5b). However, only 43% of OPM-2 cells and 34% of U-266 cells survived the treatment.

Effects of erufosine on mouse haematopoietic progenitor cells

No significant reduction in progenitor cell number (colony formation) was found after exposure of LTBMCCs to 30 μM erufosine for 24 h (Fig. 6a). However, 25 or 50 μM bendamustine reduced the number of colony-forming units

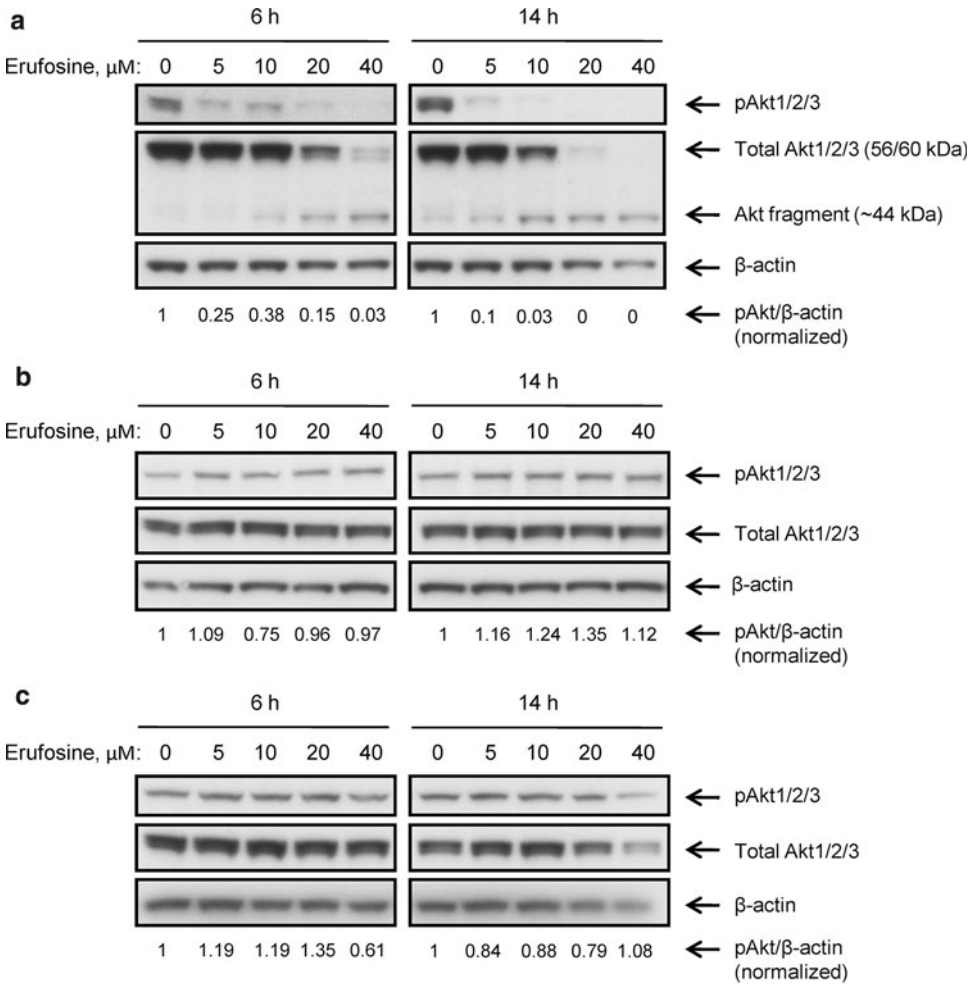

Fig. 4 Effect of erufosine on cellular pAkt levels. Erufosine induced significant dephosphorylation of pAkt in OPM-2 cells (**a**) but not in U-266 **(b)** or RPMI-8226 cells **(c)**. Additionally, cleavage of Akt was observed in OPM-2 cells **(a)**. The numbers beneath the Western blots represent the relative expression of pAkt when compared to the respective untreated control. The values were derived by dividing the densitometric output for each pAkt band by the densitometric output for the corresponding β-actin band and subsequent normalization

(CFUs) to 42 and 34% of the untreated control, respectively. The treatment of LTBMCCs with bendamustine at 25 or 50 μM as first agent and erufosine at 30 μM as second agent resulted in a clear reduction in bendamustine-induced progenitor suppression as evidenced by a colony formation of 98 or 86% of the untreated control, respectively. The opposite treatment schedule (erufosine as first agent and bendamustine as second agent) led to clonogenicity not much lower than that of the untreated control, too (colony formation of 86 or 72% of the untreated control for the combinations with 25 and 50 μM bendamustine, respectively).

Effects of erufosine on the clonogenicity of human cord blood cells (HCBCs)

Erufosine-treated HCBCs formed more colonies than the respective untreated control (Fig. 6b). This effect was dose dependent—18, 35 and 46% more colonies for erufosine concentrations of 5, 10 and 20 μM, respectively. The stimulation in the latter two cases was statistically significant ($P < 0.05$). Differential counting showed that the phenomenon of increased clonogenicity was restricted to the granulocyte/macrophage lineage and the formation of burst-forming units-erythroid (BFU-E) colonies was not stimulated.

Cytotoxic effects of erufosine in combination with other cytostatic drugs

The simultaneous treatment of U-266 cells with erufosine and alkylators (bendamustine or melphalan) led to cytotoxic effects that were significantly greater than expected (Fig. 7a, b). In the case of melphalan, the observed gain of efficacy was large enough (more than 30%) to consider the combination synergistic. For RPMI-8226 cells, the combinations of erufosine with bendamustine or bortezomib were synergistically cytotoxic, too (Fig. 7c, d). For OPM-2 cells, none of the combinations achieved a synergistic effect. All three cell lines responded in different ways to the combination of erufosine with curcumin. These two drugs antagonized their cytotoxic effects against OPM-2 cells and also against RPMI-8226 cells when the concentration of erufosine was sufficiently high (Fig. 8a, b,

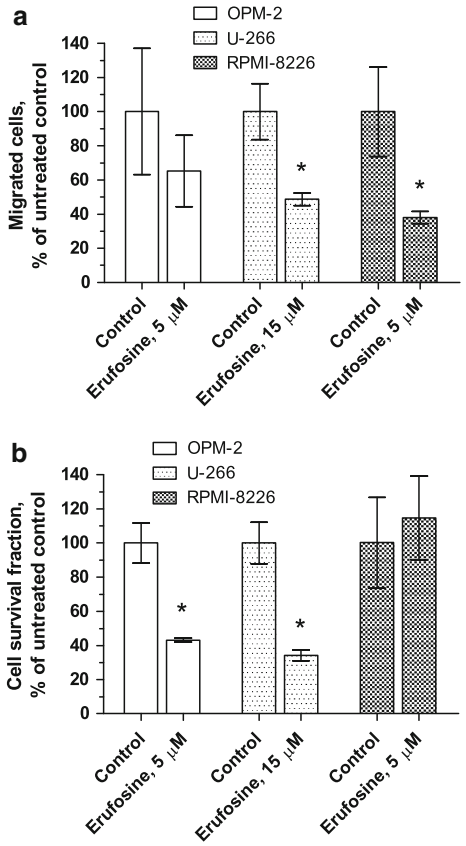

Fig. 5 Erufosine inhibits the migration of RPMI-8226 cells after short treatment that does not reduce their viability. Myeloma cells were starved in serum-free medium, treated with erufosine for 5 h and placed in fresh medium in the upper chambers of the transwell migration system. The attractant medium in the lower chambers was enriched with FCS. The cells were allowed to migrate for 12 h. **a** Migrated cells were counted and represented as a percentage of the untreated control. **b** Cells that had remained in the upper compartment were assessed for viability by the MTT-dye reduction assay. All data represent means ± SD of quadruplicate experiments. *asterisk*, $P < 0.05$

respectively). However, erufosine and curcumin were additive in their cytotoxic effects against U-266 cells, as well as against RPMI-8226 cells for low concentrations of erufosine, e.g. 2.5 μM (Fig. 8b, c, respectively).

Discussion

It has previously been reported that the alkylphosphocholine perifosine induces apoptosis in MM cells [24, 25]. In the present study, OPM-2 cells responded to erufosine treatment by nuclear shrinkage, chromatin condensation and nuclear fragmentation which are typical for apoptotic cell death. RPMI-8226 cells, however, despite being as sensitive to erufosine as OPM-2 cells, were previously found to undergo only nuclear shrinkage and chromatin condensation but no nuclear fragmentation [22]. This implies that even if erufosine induces apoptosis in RPMI-8226 cells, there could still be other mechanisms responsible for its cytotoxic action in these cells. Our current results also showed that the nuclei of U-266 cells were not affected at all by erufosine treatment, thus further confirming the observation that the effects of erufosine on the nuclear morphology of MM cells are cell line specific. Nevertheless, DNA laddering could be induced in a cell-free system consisting of U-266 nuclei and cytosolic extracts from erufosine-treated KE-37 cells. This showed that the oligonucleosomal DNA fragmentation blockade in U-266 cells is of extra-nuclear origin and could be explained by overexpression of antiapoptotic molecules and/or survival receptor activation.

The morphological changes in the OPM-2 cell line were corroborated by molecular proof for the activation of the apoptotic machinery. In these cells, erufosine activated both the extrinsic and intrinsic pathways of apoptosis. Some signalling blockade might be the reason for the lack of apoptosis induction in the U-266 cells, which have been reported to produce IL-6 and to use it as an autocrine stimulator [26]. In the two cell lines exhibiting apoptotic features, the concentrations needed to elicit apoptosis were higher in RPMI-8226 than in OPM-2 cells. This is remarkable because they were killed at comparable concentrations of erufosine [22]. A similar observation was made for perifosine [19]. For this agent, it was suggested that induction of stress response followed by apoptotic signalling cannot account, at least completely, for perifosine-induced MM cell cytotoxicity [19]. Moreover, in glioma cells, alkylphosphocholines induced a mixed type of cell death that involved features of apoptosis and necrosis and was not abrogated by the presence of multi-caspase inhibitors [27]. Two hypotheses for the mechanism of alkylphosphocholine-induced apoptosis have been proposed so far. According to the first, alkylphosphocholines can act by ligand-independent activation of Fas receptors [6, 14, 28]. The second hypothesis suggests that alkylphosphocholine-induced apoptosis occurs independently of death receptor signalling and the extrinsic pathway [29]. The main arguments in support of the latter are observations that erucylphosphocholine induces apoptosis in CD95- and TRAIL-resistant Jurkat cells, as well as insufficient caspase-3 activation in Jurkat cells expressing a dominant-negative caspase-9 or overexpressing Bcl-2 [30]. However, it was more recently shown that Bcl-2 overexpression does not rescue SKW6.4 leukaemic cells from alkylphosphocholine-induced apoptosis [14] and the presence of Fas/CD95 is essential for alkylphosphocholine-mediated apoptosis in MM cells [6]. These seemingly contradicting data could be explained by differences in the Fas-signalling pathways of the cell lines, e.g. SKW6.4 cells

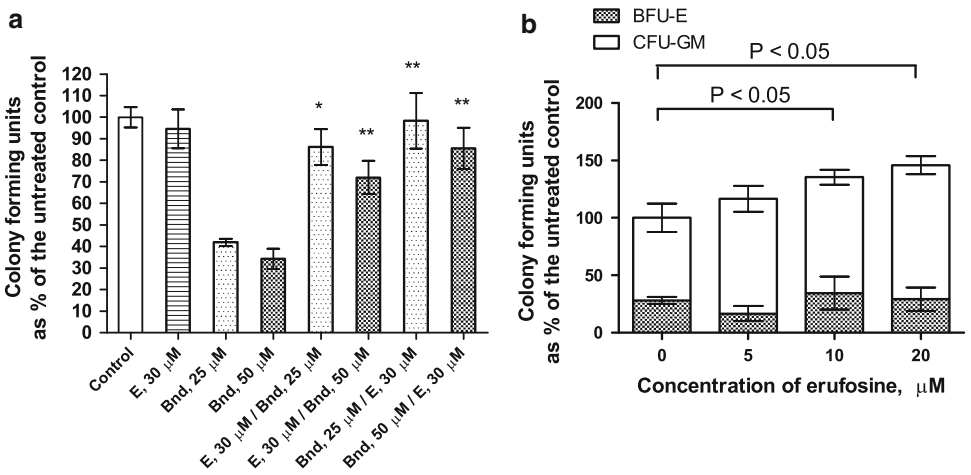

Fig. 6 **a** Erufosine protects LTBMCCs from bendamustine-induced clonal growth inhibition. LTBMCCs were treated with erufosine, bendamustine or combinations of both drugs, applied consecutively. Each drug was applied for 24 h. The number of colonies was determined after 10-day cultivation of the cells in a special semisolid medium. *Columns*, means of at least three wells; *error bars*, SD; *E*, erufosine; *Bnd*, bendamustine; *asterisk*, $P < 0.0005$; *two asterisks*, $P < 0.00005$. **b** Erufosine stimulates HCBCs to form colonies of granulocyte/macrophage type. HCBCs were subjected to treatment with several different concentrations of erufosine for 72 h. The number of colonies was determined after 10 days of further incubation in MethoCult® GF H4434 medium and is presented as a percentage of the untreated control. The relative shares of CFU-GM and BFU-E types of colonies are represented by the *white* or *cross-hatched* parts of the columns, respectively, together with the corresponding standard deviations ($n = 4$)

are type I and Jurkat cells are type II according to the classification of Scaffidi et al. [14, 30]. The modest level of caspase-8 activation in RPMI-8226 cells [22], together with the high expression level of Bcl-2 and Bcl-X_L proteins, hindering the function of the mitochondria amplification loop, suggests that these cells could be classified as type II. This hypothesis gains additional support by our data for the OPM-2 cell line. OPM-2 cells have negligible expression of Bcl-X_L, i.e. the amplification loop might possibly function in these cells. In agreement with this, we observed profound activation of both initiator caspases.

Numerous articles reported that Bcl-2 and Bcl-X_L confer drug resistance in MM [31, 32]. The increased expression of Bcl-2 provided resistance to interferon, dexamethasone, etoposide, doxorubicin and bortezomib [33–36], and overexpression of Bcl-X_L in MM cell lines increased resistance to doxorubicin and melphalan [37]. Furthermore, a gene expression study in 60 cancer cell lines has shown that Bcl-X_L plays a unique role in general resistance to cytotoxic agents [38]. In line with these findings, our results show that the sensitivity of the three MM cell lines to erufosine-induced apoptosis correlated inversely with their Bcl-X_L content. The very high content of Bcl-X_L in U-266 cells and their resistance to erufosine could possibly be explained by the presence of an IL-6 autocrine loop in these cells [25, 39].

Of the three MM cell lines studied, only OPM-2 cells responded to erufosine treatment with Bcl-2 cleavage, yielding a fragment of about 23 kDa. Such a fragment was described in several cell lines as a result of Bcl-2 cleavage at Asp34 by activated caspase-3 [40]. Importantly, this fragment was shown to further activate downstream caspases, thus amplifying apoptosis signalling [40] which could explain the sensitivity of the OPM-2 cell line to apoptosis induction in our experiments.

Studies on various malignantly transformed haematopoietic cell lines showed that alkylphosphocholines, including erufosine, reduce the level of phosphorylated Akt [11, 17, 24]. Perifosine is thought to reduce the phosphorylation of Akt not by inhibiting upstream kinases but by interfering directly with the pleckstrin homology domain of Akt, thus hindering its translocation to the plasma membrane, where Akt phosphorylation actually takes place [41]. In the present study, erufosine induced dephosphorylation of pAkt in OPM-2 cells but not in RPMI-8226 cells, although these two cell lines are equally sensitive to the cytotoxic action of erufosine [22]. The observed fragmentation of Akt in OPM-2 cells could be explained with cleavage by activated caspases [42]. The lack of significant pAkt dephosphorylation in RPMI-8226 and U-266 cells points to the importance of alternative molecular targets of alkylphosphocholines [43].

In the present work, the migration-inhibiting activity of erufosine is demonstrated for the first time. The migration of RPMI-8226 myeloma cells was strongly reduced (almost threefold) and this effect was not due to cytotoxicity. Similar findings are available for perifosine, which was shown to prevent migration of MM cells towards chemokines and cytokines that are present in the bone marrow, including SDF-1 and VEGF [25]. The short 5-h treatment

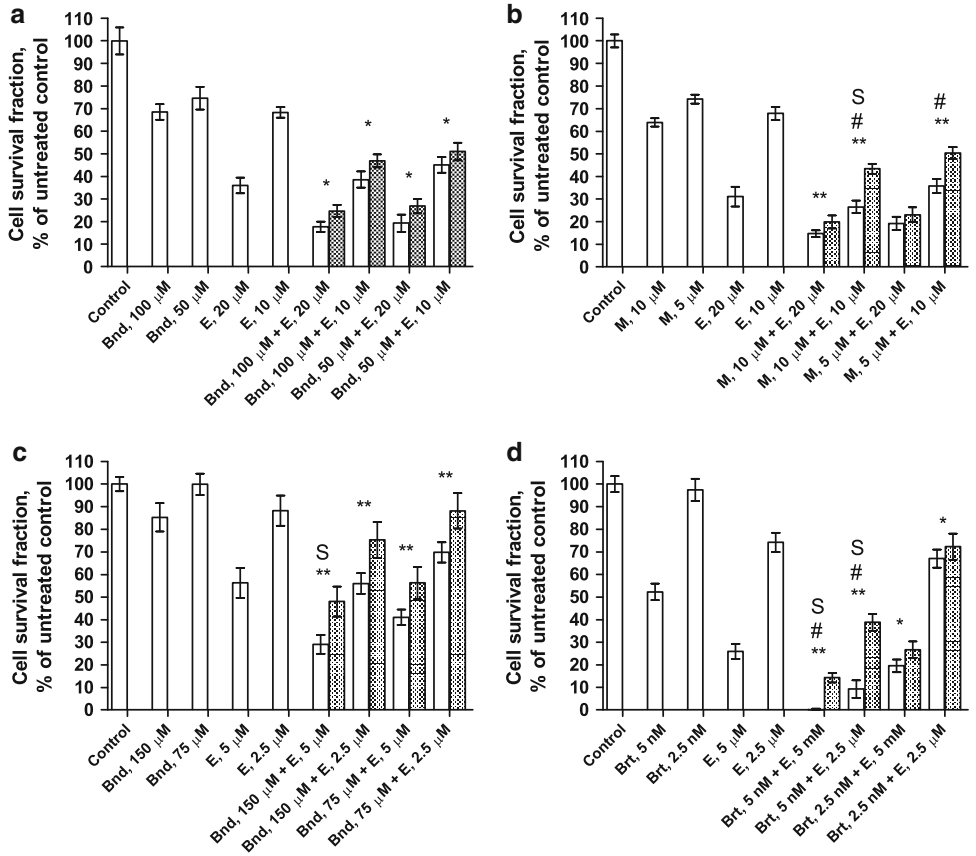

Fig. 7 Erufosine participates in synergistic drug interactions with other antimyeloma drugs like bendamustine (**a** and **c**), melphalan (**b**) and bortezomib (**d**). U-266 (**a** and **b**) and RPMI-8226 (**c** and **d**) cells were treated simultaneously with combinations of two drugs as shown in the graphs. Untreated controls and controls treated with one drug only were set up, too. Cytotoxicity was measured 72 h later using the MTT-dye reduction assay. *White columns*, means of observed data performed in tenfold; *cross-hatched columns*, cell survival fractions expected for additive drug interaction ($SF_{A+B\ (expected)}$); *error bars*, SD; *E*, erufosine; *Bnd*, bendamustine; *M*, melphalan; *Brt*, bortezomib; *S*, synergistic drug interaction ($SF_{A+B\ (measured)} \leq 0.7 \times SF_{A+B\ (expected)}$); *asterisk*, significant difference between true means at the 5% level; *two asterisks*, significant difference between true means at the 1% level; *crosshatch sign*, significant difference between population means at the 5% level

of OPM-2 and U-266 cells with erufosine was enough to cause cytotoxic effects that alone might explain the decrease in migration of these cells. The ability of erufosine in some cases to inhibit the migration of MM cells below its cytotoxic concentration could offer additional benefit, as MM is a disease involving migration and homing of malignant plasmocytes into the bone marrow.

It is known that alkylphosphocholines have low myelotoxicity [8, 44, 45], specifically they spare normal B and T cells, as well as vascular endothelial cells [6] and even stimulate normal haematopoiesis [8, 46]. To the best of our knowledge, the present article is the first to describe the stimulating effects of an alkylphosphocholine on granulocyte/macrophage progenitors from human umbilical cord blood. This opens a new perspective for using erufosine not only as an antineoplastic drug, but also in transplantation of haematopoietic stem cells. The present study also demonstrates that erufosine is able to ameliorate the myelotoxicity of conventional cytostatic drugs, such as bendamustine.

Our data also constitute the first evidence that erufosine could improve synergistically the antimyeloma effects of conventional cytostatics like melphalan and bendamustine, as well as the effect of the proteasome inhibitor bortezomib. There are similar data for perifosine in combination with dexamethasone, doxorubicin, melphalan or bortezomib [24]. However, curcumin antagonized the antineoplastic effects of erufosine in the MM cell lines in which the alkylphosphocholine induced apoptotic changes. Some of these interactions might occur at the level of JNK, a key mediator of alkylphosphocholine-induced apoptosis [16, 28, 47]. Bortezomib, for instance, inhibits the proteasome, thus preventing the degradation of JNK [46]. Curcumin, on the other hand, inhibits JNK activation by various agonists [48, 49].

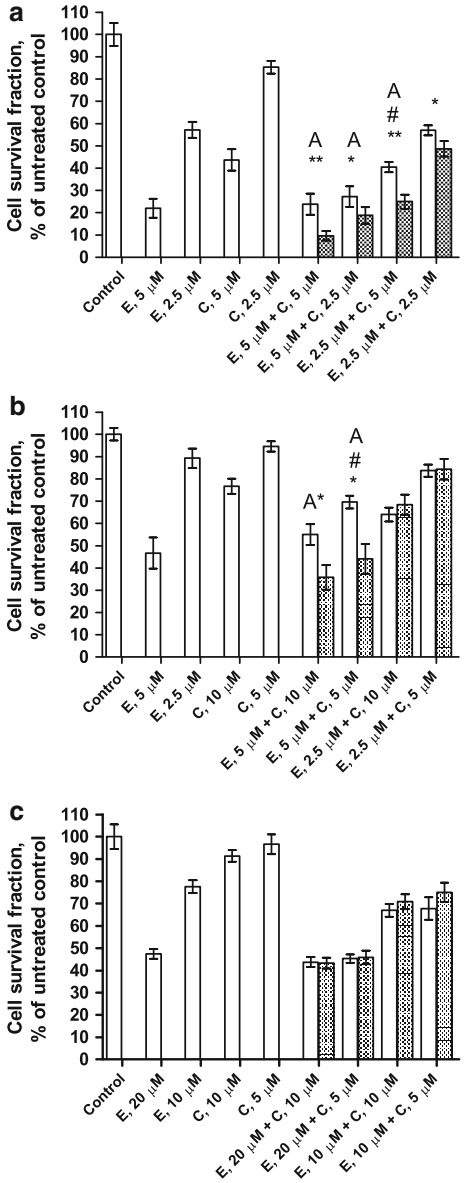

Fig. 8 Curcumin antagonizes the antimyeloma effects of erufosine in the MM cell lines in which the alkylphosphocholine induces apoptosis—OPM-2 (**a**), as well as in RPMI-8226 (**b**) when the concentration of erufosine is above the IC_{50}. In contrast, the combination of erufosine and curcumin is additively cytotoxic against U-266 cells (**c**), as well as against RPMI-8226 cells (**b**) for concentrations of erufosine below the IC_{50}. The MM cells were treated simultaneously with combinations of the two drugs as shown in the graphs. Untreated controls and controls treated with one drug only were set up, too. Cytotoxicity was measured 72 h later using the MTT-dye reduction assay. *White columns*, means of observed data performed in tenfold; *cross-hatched columns*, cell survival fractions expected for additive drug interaction ($SF_{A+B\ (expected)}$); *error bars*, SD; *E*, erufosine; *C*, curcumin; *A*, antagonistic drug interaction ($SF_{A+B\ (measured)} \geq 1.3 \times SF_{A+B\ (expected)}$); *asterisk*, significant difference between true population means at the 5% level; *two asterisks*, significant difference between true population means at the 1% level; *crosshatch sign*, significant difference between population means at the 5% level

In conclusion, erufosine is a promising antineoplastic drug that shows significant potential for treating human plasmocytoma, especially as a partner in combination chemotherapy regimens. In vitro it resulted in enhanced MM cell killing and ameliorated myelosuppressive effects of other commonly used drugs. Although only three cell lines were studied, it is inferred that erufosine has a complex mode of inducing cell death in MM, involving apoptotic and non-apoptotic mechanisms with the one or the other prevailing in different cell lines. Antimigratory properties of erufosine were demonstrated, too.

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