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ORIGINAL ARTICLE

Erufosine, an alkylphosphocholine, with differential toxicity to human cancer cells and bone marrow cells

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Abstract
Purpose To investigate the activity and myeloprotective properties of erufosine, a novel alkylphosphocholine (APC), on human malignant cells and normal bone marrow cells.
Methods Human or mouse bone marrow cells were exposed to erufosine, miltefosine, perifosine, or edelfosine in CFU-GM assays. Human MDA-MB-231 breast carcinoma, Panc-1 pancreatic carcinoma, and RPMI8226 multiple myeloma cells were exposed to erufosine in colony formation assays. Colony formation of Panc-1 tumor cells and mouse bone marrow cells ex vivo were quantified following intravenous administration of erufosine to tumor-bearing mice. Western blotting methods were applied to human U87 glioblastoma cells exposed to erufosine to investigate Akt inhibition.
Results Erufosine was less toxic to human and mouse bone marrow cells than perifosine, miltefosine, and edelfosine and was equally toxic to human and mouse CFU-GM. The human cancer cells MDA-MB-231 breast, Panc-1 pancreatic, and RPMI8226 MM cells were more sensitive to erufosine in a colony formation assay than were human bone marrow cells generating an approximately tenfold differential in IC_{90} values. Erufosine injected intravenously significantly reduced Panc-1 tumor cell colony formation ex vivo but not mouse bone marrow CFU-GM. Erufosine inhibited Akt phosphorylation in human U87 glioblastoma cells.
Conclusions Erufosine offers potential as a novel therapeutic for cancer with a reduced toxicity profile to bone marrow cells compared with other agents in this class. Human cancer cells were more sensitive to erufosine than human or mouse bone marrow cells indicating a favorable therapeutic window for erufosine.

Keywords Erufosine · Alkylphosphocholine · Lipid · Bone marrow · Cancer · Toxicity

Abbreviations

APL	Alkylphospholipid
APC	Alkylphosphocholine
CFU-GM	Colony-forming unit–granulocyte–macrophage
IC	Inhibitory concentration
FBS	Fetal bovine serum
rm	Recombinant murine
rh	Recombinant human
GBM	Glioblastoma
IV	Intravenous
CML	Chronic myeloid leukemia
AML	Acute myeloid leukemia
MM	Multiple myeloma

Introduction

Alkylphosholipids (APLs) are a class of synthetic lipids that are characterized by two aliphatic side chains linked to glycerol phosphocholine by either an ether or thio-ether bond. APLs target the cell membrane resulting in inhibition of proliferation followed by apoptosis thereby offering potential as novel anti-cancer drugs. One of the first APLs

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evaluated for cancer was the alkyl-lysophospholipid edelfosine, 1-O-methyl-*rac*-glycero-3-phosphocholine, that selectively induced apoptosis in human leukemic and multiple myeloma (MM) cells but not in normal bone marrow cells [1, 2]. Although edelfosine showed some benefit in clinical trials in non-small cell lung cancer, the treatment was not an improvement over conventional therapies and therefore was not pursued as a cancer therapeutic [3, 4].

The rapid metabolism of APLs was a limiting characteristic; therefore, the next generation of molecules in this class, alkylphosphocholines (APCs), was synthesized. APCs are derived from APLs by the removal of the glycerol group that enhances the stability of the compounds to integrate into the lipid bilayer of the cell membrane via lipid rafts [5–7]. Miltefosine (hexadecylphosphocholine) was one of the first APC developed, is approved as an oral treatment for Indian visceral leishmaniasis, and has antifungal activity [8–10]. Miltefosine showed some efficacy when applied topically to cutaneous breast cancer metastasis [11] and cutaneous lymphomas [12]. Intravenous miltefosine administration resulted in hemolysis thus preventing its use as a systemic agent [13–16].

Due to the side effects experienced by cancer patients treated with miltefosine, perifosine (octadecyl-(1,1-dimethyl-4-piperidinio-4-yl)-phosphate) emerged as an improved APC analogue with oral bioavailability for anticancer therapy. Similar to edelfosine, perifosine induced selective apoptosis in vitro in human multiple myeloma cells [1, 17]. Clinical trials with perifosine in advanced or metastatic breast cancer, head and neck cancer, soft tissue sarcomas, metastatic melanoma, and other advanced solid tumors yielded disappointing results and/or gastrointestinal toxicities similar to those that were observed in the clinic with miltefosine [18–23]. However, perifosine has had clinical success in combination regimens including in combination with capecitabine in metastatic colorectal cancer and in combination with bortezomib in multiple myeloma [24–26]. Perifosine has been given Fast Track status by the FDA in these indications and is currently in Phase III clinical trials.

The structure of the newest APC erufosine (erucylphosphohomocholine or erucylphospho-N,N,N-trimethylpropylammonium) improves upon previous derivatives with a longer (22-carbon) chain containing a *cis*-13,14 double bond. This modification resulted in reduced hemolytic toxicity [27, 28] thereby enabling systemic delivery that was not feasible with edelfosine, miltefosine, or perifosine. Erufosine induced apoptosis in human astrocytoma and glioblastoma cell lines in vitro and influenced rat C6 glioma tumor growth in vivo [29–31]. Erufosine synergized with gemcitabine to increase cytotoxicity in leukemia cells while lowering myelotoxicity [32]. Erufosine was cytotoxic to acute myelogenous leukemia (AML) cells via JNK- and PP2A-dependent signaling pathways and showed additivity with etoposide and cytarabine [33, 34]. Erufosine induced apoptosis in human chronic lymphocytic leukemia (CLL) cells and in multiple myeloma and urinary bladder carcinoma cell lines [35–37]. The anti-cancer therapeutic potential of erufosine warranted evaluation in a Phase I clinical trial [38].

We have further investigated the advantages that erufosine may offer for oncology over other APCs. Erufosine was directly compared against edelfosine, miltefosine, and perifosine in colony-forming unit (CFU) assays with murine and human bone marrow granulocyte–macrophage progenitor cells and found that erufosine was less cytotoxic than the other compounds. Erufosine was evaluated in several human cancer cell lines in CFU assays, and the data generated indicated a tenfold increased sensitivity of cancer cells to erufosine compared with human bone marrow cells. Erufosine was administered intravenously to Panc-1 tumor-bearing mice resulting in a reduction in tumor cell colony formation while sparing the bone marrow. Erufosine inhibited the phosphorylation of Akt, a signaling molecule that is involved in cell survival and apoptosis. Erufosine is a novel synthetic compound with lower bone marrow toxicity than other compounds in this class allowing intravenous infusion at higher doses and therefore holds promise as a monotherapy or in combination regimens.

Materials and methods

Materials

Edelfosine (Bachem Bioscience, King of Prussia, PA), miltefosine (Cayman Chemical, Ann Arbor, MI), perifosine (Cayman Chemical), and erufosine (Genzyme Pharmaceuticals, Liestal, Switzerland) were prepared as white powders and dissolved at a concentration of 20 mM in 0.9% saline that was preheated to 60°C. Compounds were vortex mixed until completely dissolved. Stock solutions were freshly prepared on the day of the experiment.

Mouse bone marrow

Naïve, male Balb/c mice (Charles River Laboratories, Wilmington, MA) were euthanized by CO_2 inhalation at 7–10 weeks of age for bone marrow collection. A skin incision was made throughout the whole hind limb from mid-point of inguinal ligament to the medial side of the foot. The muscle and other connective tissues were removed, and both tibias and the femurs were extracted. A blunt syringe needle (27G) was inserted into the

medullary cavity of the tibia or femur, and the cavity was flushed with 2 ml of sterile RPMI1640/5% FBS. The resulting cellular suspension was collected into a 50-ml conical bottom tube and kept on wet ice. Approximately 25 million cells were obtained from each mouse. All procedures involving animals described here and later were carried out according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with the Federal Animal Welfare Act (9 CFR, 1992) and were conducted in an AAALAC accredited facility.

Bone marrow CFU-GM assays

For mouse CFU-GM assays, freshly isolated mouse bone marrow cells were cultured in MethoCult (#M3534) containing the cytokines rmSCF, rmIL-3, and rhIL-6 (StemCell Technologies, Vancouver, BC). For human CFU-GM assays, viable human bone marrow cells (purchased as frozen vials from All Cells, LLC via StemCell Technologies, #ABM009) were prepared with DNase treatment (#07900, StemCell Technologies), washed in a protocol provided by the vendor, and cultured. The culture medium used was MethoCult GF (#H4534), containing the cytokines hSCF, hGM-CSF, and hIL-3 (StemCell Technologies).

For experiments, 150 µl of 20× concentration of the compounds, along with 150 µl of a suspension of mouse or human bone marrow cells, were added to the 3 ml tube of thawed MethoCult medium and vigorously mixed by vortex. Final cell concentrations were 4×10^4 cells/plate for mouse cells and 8×10^4 cells/plate for human cells. Tubes were then allowed to sit undisturbed for 10–15 min until the air bubbles dissipated. A blunt needle attached to a 3-ml syringe was used to collect 2.5 ml of the cell suspension, the air was voided from the syringe, and 1 ml was placed in the center of duplicate 100 cm^2 culture dishes. Dishes were gently rotated evenly covering of the bottom surface of each dish and then placed in a 37°C incubator for colony formation for 13 days for mouse bone marrow and 15 days for human bone marrow. Colonies were defined as clusters containing 30 or more cells.

Cancer cell colony formation assays

Human MDA-MB-231 breast carcinoma cells, Panc-1 pancreatic cells, and RPMI8226 multiple myeloma cells were purchased from ATCC (Manassas, VA) and cultured in RPMI1640/5% FBS (Invitrogen, Carlsbad, CA). MDA-MB-231 (500 cells/well) or PANC-1 (1,000 cells/well) and compounds were added to each well of a 6-well plate in a final volume of 3 mls; RPMI8226 multiple myeloma cells and compounds were added to 3 mls Methocult medium (AllCells); RPMI8226/Methocult mixture was vortex mixed and 1.1 mls was dispensed into 35 mm dishes in duplicate (1,000 RPMI8226 cells per dish). Cultures were incubated at 37°C, 5% CO_2 for 7 days. Colonies grown on plastic were fixed in methanol, stained with crystal violet, and counted.

Panc-1 tumor model

Human Panc-1 pancreatic cancer cells were cultured as described earlier. Female Balb/c nude (*nu/nu*) mice (Charles River Labs) were implanted subcutaneously with 5×10^6 Panc-1 cells in a 1:1 mixture of RPMI1640:Matrigel (BD Biosciences, Bedford, MA) in a 200 µl volume. When tumors reached approximately 7–10 mm in width and length, the mice received 0.9% saline or 120 mg/kg erufosine in 0.9% saline by intravenous injection via the tail vein in a volume of 10 µl per gram of body weight. Mice were euthanized 24 h later, and the bone marrow was collected for CFU-GM assay as described earlier. Subcutaneous Panc-1 tumors were excised, minced with scalpels, and a single cell suspension was prepared by dissociation in RPMI1640/1% FBS with 4 mg/ml collagenase (Roche Applied Science, Minneapolis, MN) and 0.4 mg/ml elastase (Roche) in a rotational incubator 37°C for 1 h. The resulting cell suspension was washed twice by centrifugation with RPMI1640/1% FBS. Panc-1 tumors cells were counted and plated for colony formation assay in monolayer as described earlier.

Western blot

Human U87 glioma cells (ATCC) in RPMI1640/5% FBS were exposed to 1–20 µm of erufosine or perifosine for 5–24 h. To prepare lysates, flasks were placed on ice and washed once with cold PBS. Cells were scraped in 1 ml cold PBS and centrifuged for 5 min at 2,000 RPM at 4°C. The supernatant was removed and lysis buffer containing 150 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP40 and proteinase inhibitor cocktail tablets in autoclaved ddH_2O were added to the cells (Roche Diagnostics, Inc., Indianapolis, IN). Cell lysates were incubated on ice for 30 min, centrifuged for 15 min at 14,000 RPM at 4°C. Protein concentration in the supernatant was measured using the Biorad protein determination assay. Fifty micrograms of protein in sample buffer containing 1 part NuPage reducing agent and 2.5 parts NuPage LDS sample buffer were loaded onto 4–12% Bis–Tris gradient gels and run in MOPS running buffer (Invitrogen). Samples were heated for 10 min at 70°C prior to loading onto the gel. The gel was transferred to nitrocellulose membranes for 2 h according to the manufacturer's instructions (Invitrogen). The membranes were blocked for 1 h at RT with Odyssey blocking buffer (Li-Cor, Lincoln, NE) and probed with antibodies against Akt and pAkt at a 1:1,000 dilution (Cell Signaling

Technology) and actin at 1:100,000 dilution (Sigma–Aldrich, St. Louis, MO) overnight at 4°C. The membranes were washed in 3 × 15 min washes in PBS/0.1% Tween 20 (Sigma). Secondary anti-rabbit antibodies conjugated to IR700 or IR800 dyes (Rockland Immunochemicals, Inc., Gilbertsville, PA) were added at a dilution of 1:1,000 in Odyssey blocking buffer and incubated a 1 h at RT. Washes were performed as described earlier. Detection of the signal was conducted using the Odyssey imaging system (LI-COR).

Statistics

Statistical significance was determined by student's *t*-test with GraphPad Prism 5.0 (GraphPad Prism Software, Inc., La Jolla, CA); $P < 0.05$ was considered statistically significant. EC values were determined using nonlinear regression analysis with GraphPad Prism software.

Results

Mouse and human bone marrow CFU-GM assays

The compounds edelfosine, miltefosine, perifosine, and erufosine are alkylphospholipids with the distinction that edelfosine is an alkyl-lysophospholipid and the other three molecules belong to a subclass of alkylphosphocholines (Fig. 1). The cytotoxicity of edelfosine, miltefosine, perifosine, and erufosine were directly compared in half-log increments from 3 to 300 μM concentrations in both mouse and human bone marrow in CFU-GM assays. The cells were exposed continuously to the agents for 14–16 days. The IC_{50} and IC_{90} values in the mouse CFU-GM assay were higher for erufosine (170.4 ± 11.5 μM; 288.3 ± 5.2 μM) than for edelfosine (29.4 ± 7.8 μM; 76.7 ± 17.7 μM), miltefosine (111.7 ± 19.3 μM; 194.5 ± 42.2 μM), and perifosine (120.8 ± 35.2 μM; 237.2 ± 34.3 μM) (Table 1; Fig. 2a). Erufosine was less toxic to murine bone marrow cells in particular at the 100 μM concentration. In the human CFU-GM assay, the IC_{50} and IC_{90} values for erufosine (216.0 ± 3.1 μM; 284.3 ± 3.0 μM) were also higher than for the miltefosine (31.7 ± 1.7 μM; 118.3 ± 20.9 μM) and perifosine (<3 μM; 83.3 ± 4.4 μM) (Table 1; Fig. 2b). The differences between erufosine and miltefosine and perifosine were noticeable across concentrations ranging from 10 to 100 μM. Although edelfosine was tested in the assay, the edelfosine altered the methylcellulose semisolid matrix medium for the human CFU-GM assay so that it was more liquid thereby preventing any countable colony formation.

In addition to the reduced cytotoxicity of erufosine on bone marrow cells compared with other APC, our results show that erufosine can enhance survival and colony formation. In repeated experiments, we observed an increase in colony formation by human or murine bone marrow cells compared with untreated cells (Fig. 2a, b). This effect also occurred upon exposure of the bone marrow cells to perifosine and miltefosine. However, the enhancement of the human CFU-GM by erufosine over the other agents was significantly more pronounced with human bone marrow cells. The optimal concentration of erufosine exposure was 30 μM resulting in a doubling in the CFU-GM compared with untreated controls (Fig. 2b).

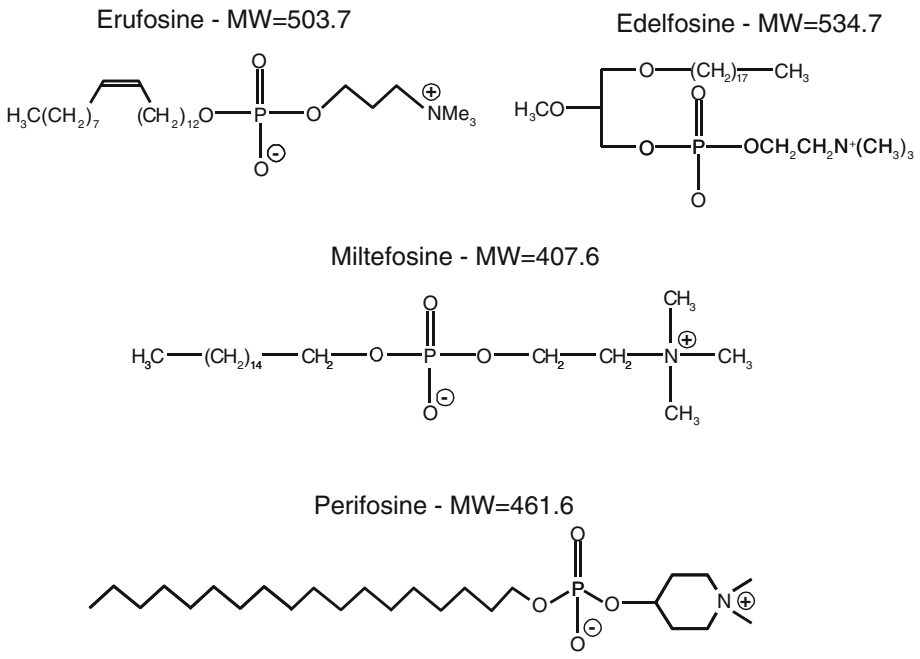

Fig. 1 Chemical structures. Erufosine is a 2nd generation alkylphosphocholine. Erufosine differs by a longer alkyl chain with 22 C-atoms, a cis double bond in the central position of the alkyl chain, and increased distance between P and N with 1 C-atom

Table 1 Four alkylphospholipids in mouse and human CFU-GM assays

Compound	Erufosine	Edelfosine	Miltefosine	Perifosine
Mouse CFU-GM				
IC_{50} (μM)	170.4 ± 11.5	29.4 ± 7.8*	111.7 ± 19.3*	120.8 ± 35.2
IC_{90} (μM)	288.3 ± 5.2	76.7 ± 17.7**	194.5 ± 42.2*	237.2 ± 34.3
Human CFU-GM				
IC_{50} (μM)	216.0 ± 3.1	NA	31.7 ± 1.7**	<3**
IC_{90} (μM)	284.3 ± 3.0	NA	118.3 ± 20.9**	83.3 ± 4.4*

IC_{50}s and IC_{90}s of four alkylphospholipids in a continuous exposure CFU-GM assay of mouse and human bone marrow cells. Data are expressed as the mean of 3 or more individual experiments ± SEM

* $P < 0.05$ versus erufosine, ** $P < 0.0001$ versus erufosine

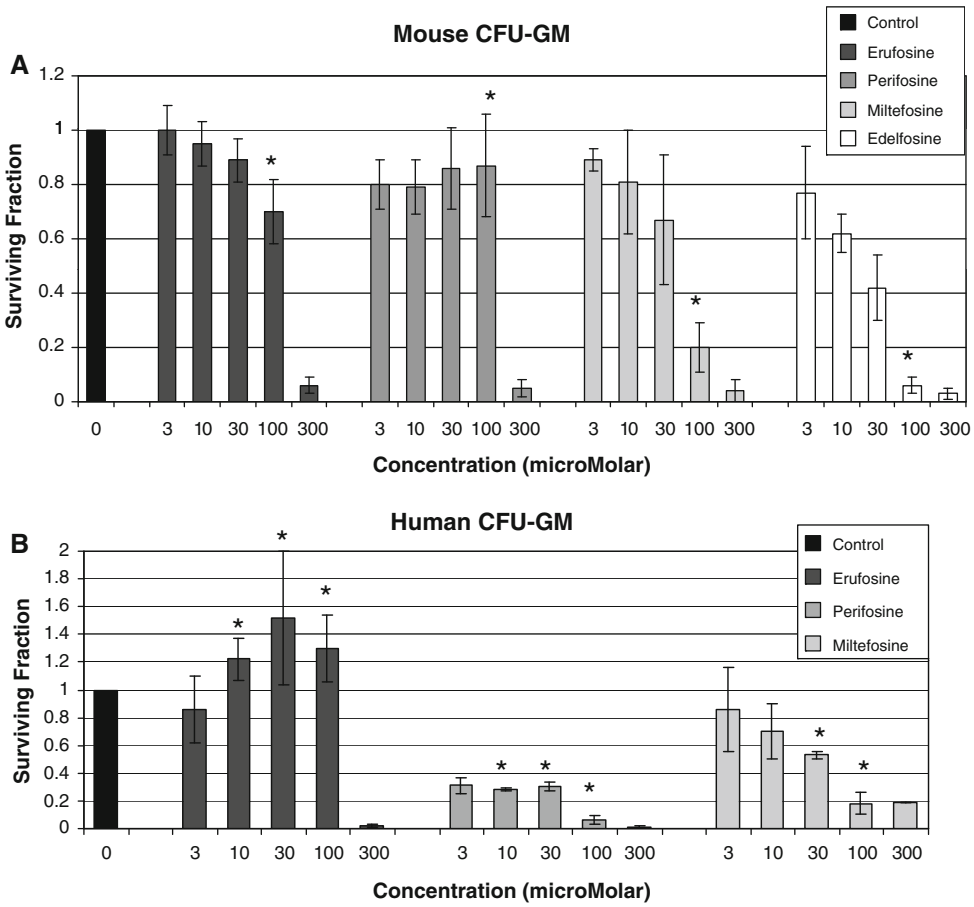

Fig. 2 CFU-GM assays. **a** Mouse bone marrow was exposed to increasing concentrations of erufosine, miltefosine, edelfosine, or perifosine. Erufosine and perifosine were significantly less toxic than miltefosine and edelfosine at a concentration of 100 μM ($P < 0.05$). Erufosine and perifosine were not significantly different from each other in the mouse CFU-GM assay. **b** Erufosine on human bone marrow in the CFU-GM assay was better tolerated than the other alkylphospholipids. Erufosine enhanced colony formation at 30 μM while perifosine and miltefosine did not. Erufosine was significantly less toxic than perifosine and miltefosine at 30 and 100 μM concentrations ($P < 0.05$). Erufosine was also significantly less toxic than perifosine at the 10 μM concentration, and miltefosine was significantly less toxic than perifosine at the 30 μM concentration ($P < 0.05$). The data are expressed as the mean of 2 or more independent experiments; *bars* are SEM

Human cancer colony formation assays

APCs such as edelfosine and perifosine have demonstrated selective cytotoxicity toward multiple myeloma and leukemia cells compared with bone marrow cells [1, 2, 17]. Previously, the cytotoxicity of erufosine toward cancer cells from solid tumors was demonstrated in several types of brain cancer cells [29, 30, 39]. In the current study, the effects of erufosine exposure on several human cancer cell lines: MDA-MB-231 breast carcinoma, Panc-1 pancreatic carcinoma, and RPMI8226 multiple myeloma cells were explored in a colony formation assay. The cancer cells were continuously exposed to erufosine for 1 week at subconfluent levels to promote colony formation.

RPMI8226 multiple myeloma colony formation assay yielded IC_{50} and IC_{90} values of 23.0 ± 6.4 μM and 41.7 ± 11.9 μM, respectively, upon exposure to erufosine (Fig. 3a). MDA-MB-231 breast carcinoma cells were more sensitive to erufosine than the RPMI8226 multiple myeloma cells and generated IC_{50} and IC_{90} values of

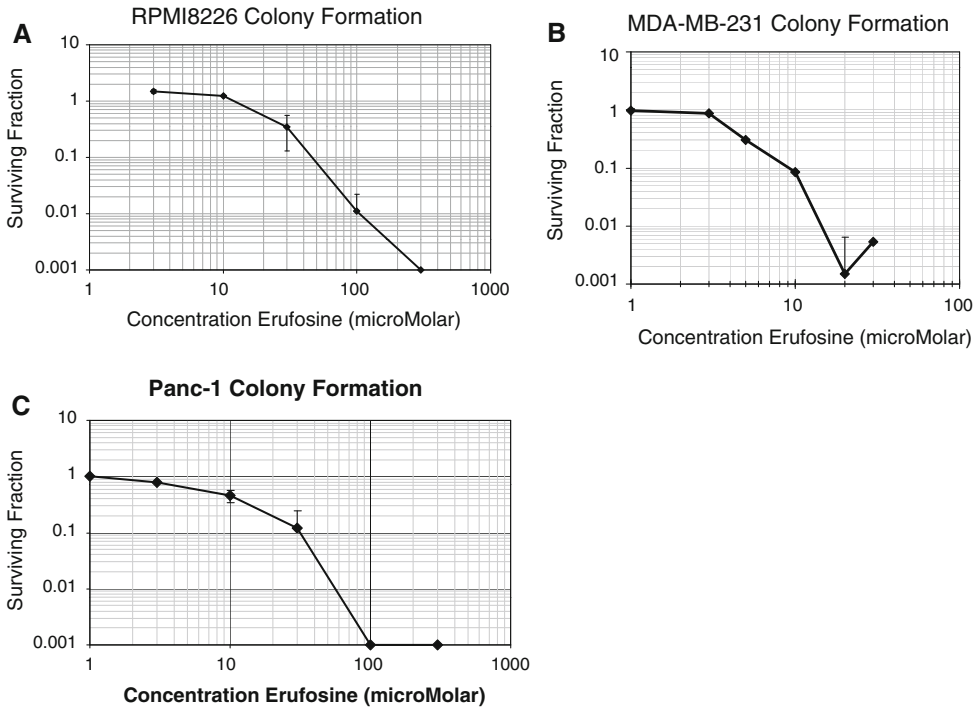

Fig. 3 Human cancer cell colony-formation assays. **a** RPMI8226 human multiple myeloma cells were exposed to erufosine. **b** MDA-MB-231 human breast cancer cells were more sensitive to erufosine than RPMI8226 cells. **c** Panc-1 human pancreatic cells were also sensitive to erufosine. The data are expressed as the mean of 2 independent experiments; *bars* are SEM

4.5 ± 0.6 μM and 9.8 ± 0.1 μM, respectively (Fig. 3b). The Panc-1 pancreatic carcinoma cells were sensitive to erufosine during colony formation and resulted in IC_{50} and IC_{90} values of 16.6 ± 13.1 μM and 42.7 ± 17.3 μM, respectively, in the colony formation assay (Fig. 3c).

Differential toxicities to erufosine

The IC_{90} values generated from human bone marrow cells and from human cancer cells in colony formation assays indicated a substantial differential in the sensitivity of normal hematopoietic cells and malignant cancer cells to erufosine (Fig. 4). The MDA-MB-231, Panc-1, and RPMI8226 MM cells were more sensitive to erufosine than were human bone marrow CFU-GM generating IC_{90} values that range from approximately 10–40 μM. By comparison, the human and murine bone marrow cells had IC_{90} values of approximately 280 μM. The greater sensitivity of human cancer cells erufosine may be indicative of the possible potential of erufosine to be a cancer therapy with low bone marrow toxicity.

Panc-1 pancreatic carcinoma xenograft model

The differential sensitivities observed in vitro to erufosine between human cancer cells and bone marrow cells was further investigated in vivo in the Panc-1 pancreatic carcinoma xenograft model. Mice-bearing subcutaneous Panc-1 tumors received a single intravenous injection of erufosine (120 mg/kg) or saline control. Tumors and bone marrow were collected 24 h later and incorporated into colony formation and CFU-GM assays. Erufosine treatment produced a reduction in Panc-1 tumor cell colony formation resulting in a surviving fraction of 0.45 ($P < 0.05$) (Fig. 5). Thus, Panc-1 colony formation was reduced by approximately 55% compared with the saline control treatment. By comparison, mouse bone marrow CFU-GM was not significantly effected with a surviving fraction of 0.86 or a reduction of approximately 14% ($P > 0.05$). Erufosine (120 mg/kg) was well tolerated and there were no overt signs of toxicity. Thus, systemic delivery of a single-dose erufosine was cytotoxic toward the Panc-1 human pancreatic carcinoma in vivo as demonstrated by Panc-1 tumor colony formation while sparing the host bone marrow CFU-GM.

Akt signaling pathway

One mechanism of action of APCs such as perifosine is the inhibition of the Akt pathway [40]. To investigate whether erufosine could also affect the Akt signaling pathway, U87 human glioma cells were chosen as they are a cell line that constitutively phosphorylates Akt. In addition, erufosine inhibited Akt phosphorylation in other human glioblastoma cell lines [41]. U87 glioma cells were exposed to erufosine or perifosine as a positive control at concentrations ranging from 1 to 20 μM for 5 and 24 h. Western blot analysis revealed that erufosine inhibited Akt phosphorylation of U87 glioma cells at a concentration of 20 μM at 5 h and was more apparent at 24 h at 10–20 μM (Fig. 6a). In this

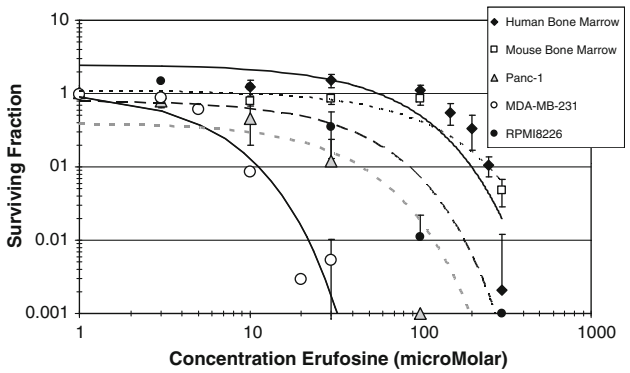

Fig. 4 Differential sensitivities. Human cancer cells are more sensitive to erufosine than either murine or human bone marrow cells when continuously exposed in colony or CFU-GM assays over a 1–2-week period. The data are expressed as the mean of 2 independent experiments; *bars* are SEM

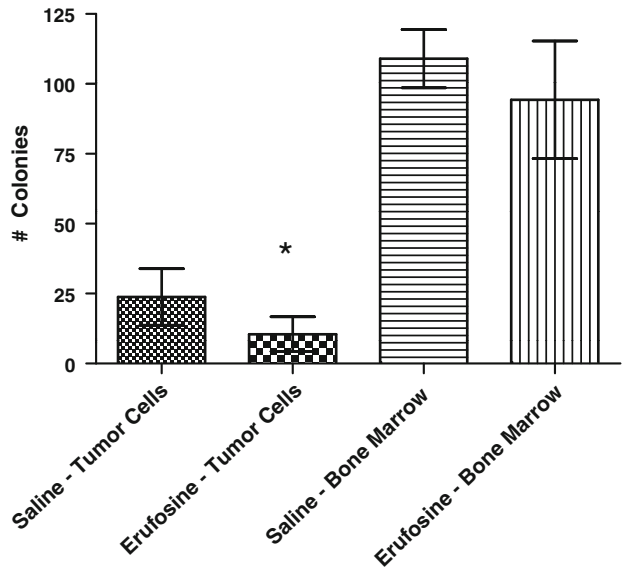

Fig. 5 Bone marrow–sparing effect and tumoricidal effect. Mice-bearing Panc-1 pancreatic xenograft tumors were treated with a single IV injection of 120 mg/kg erufosine or saline control. Twenty-four hours later, the bone marrow cells and tumor cells were collected and single cell suspensions were prepared for CFU-GM and colony-formation assays. Erufosine significantly reduced Panc-1 tumor cell formation ex vivo ($P < 0.05$) and did not significantly change bone marrow CFU-GM ($P > 0.05$). Data are expressed as the mean ± SD

experiment, erufosine was slightly less potent than perifosine at the same concentrations on U87 GBM cells (Fig. 6b).

Summary

Erufosine is a novel APC with a chemical structure that potentially has an improved therapeutic window achieved through reduced cytotoxicity toward hematopoietic cells potentially lowering the risk of hemolysis and myelosuppression and allowing systemic delivery in the clinic. In granulocyte–macrophage colony-forming unit (CFU-GM) assays, erufosine was less cytotoxic toward murine and human bone marrow cells than other compounds in this class including edelfosine, miltefosine, and perifosine. Interestingly, in this cell culture assay, concentrations of erufosine between 10 and 100 micromolar appeared to stimulate colony formation indicating that exposure to these concentrations of erufosine was improving the survival of the granulocyte–macrophage progenitor cells compared with cells exposed to the same concentration of vehicle without erufosine. This effect of erufosine was not observed with the other APCs tested. The increase in colony formation with exposure to 30 micromolar increased the number of countable colonies to nearly 150% of the control (100%). There was an abrupt decrease in colony formation upon exposure of the hematopoietic progenitor cells to 300 micromolar erufosine. There was no indication of a similar effect when several human tumor cell lines were exposed to the same concentration range of erufosine, Thus, if similar concentrations can be reached in the bone marrow of patients, erufosine may provide a benefit by sparing the bone marrow potentially by stimulating the proliferation of bone marrow cell populations after treatment with erufosine in combination with cytotoxic chemotherapy. In vivo studies are needed to determine whether these cell culture observations may translate to tumor-bearing mice.

The mechanism of action of erufosine is continuing to be elucidated. APLs and APCs interact with lipid rafts at the cell membranes where they disrupt the natural balance and metabolism of phospholipids in turn leading to alterations in membrane-signaling pathways associated with apoptosis [1, 5–7, 38]. The data presented here demonstrate that erufosine inhibited the Akt pathway in human U87 GBM cells. Previously, erufosine was shown to cause a decrease in the chronic myeloid leukemia (CML) fusion protein p210 (BCR-ABL) while inducing Rb protein expression and increasing p27 levels in K-562 CML cells [42]. Furthermore, the sensitivity of CML cells to lower concentrations of erufosine proved to be dependent on the levels of retinoblastoma protein, a known tumor suppressor protein [43]. The mitochondrial F_0F_1-ATP(synth)ase has also been implicated in apoptosis induction by erufosine in human GBM cells [44]. In addition, erufosine exerted effects on rat liver mitochondria leading to a decrease in ATP synthesis [45]. It remains to be determined whether erufosine inhibits protein kinase C in the same manner as the APC miltefosine [8].

A phase I trial with erufosine has been completed [38]. Preclinical data generated with erufosine may serve as a guide to identify patient populations that may respond to

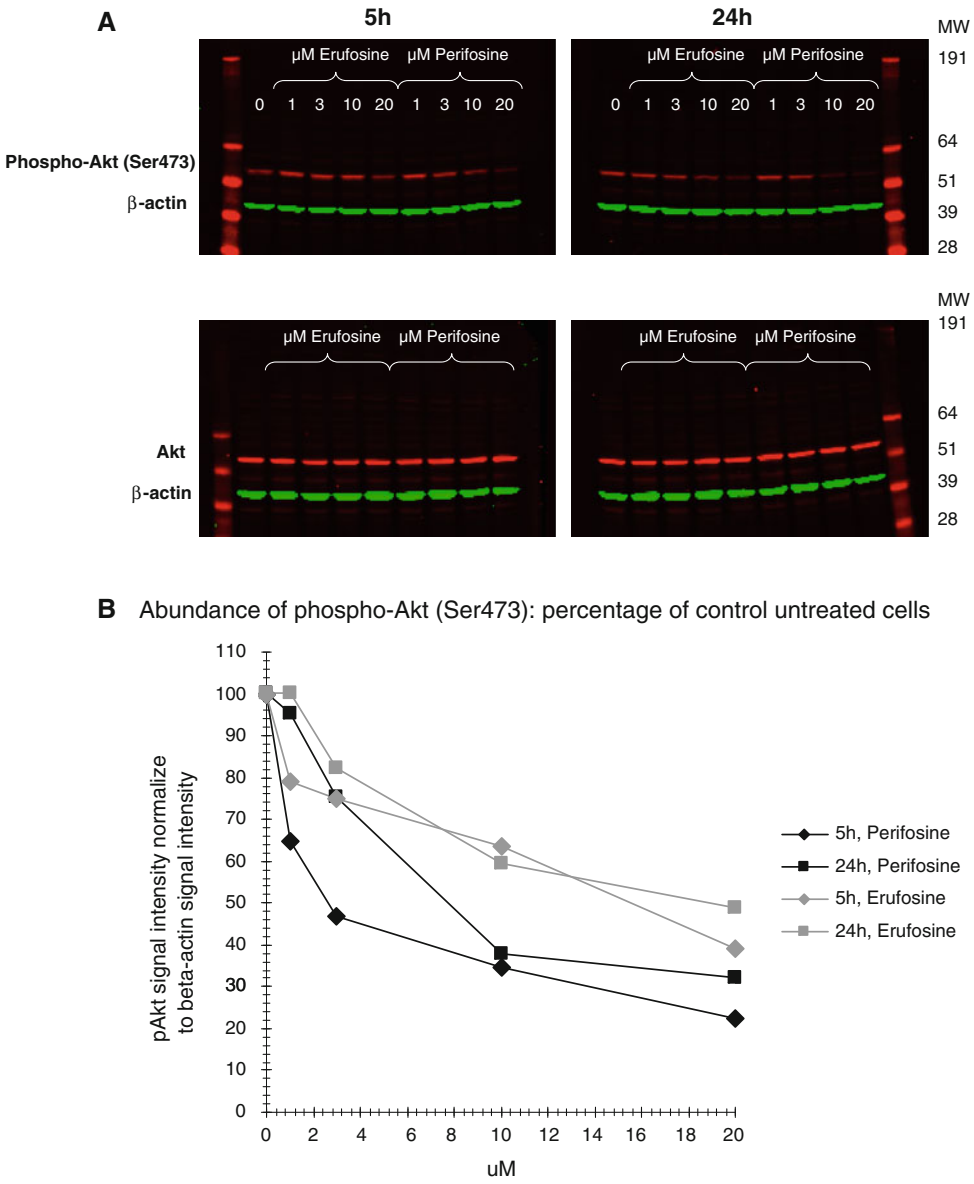

Fig. 6 Inhibition of Akt phosphorylation. U87 human glioma cells were exposed to erufosine or perifosine for 5–24 h. **a** Western blotting shows that erufosine inhibits phosphorylation of Akt. **b** Quantitative analysis indicates that perifosine at the same concentrations had a slightly greater effect in this cell line

erufosine therapy as a single agent or in combination regimens. The cell-based results presented here suggest that pancreatic and breast cancers as well as multiple myeloma are indications where erufosine may offer some clinical benefit. Multiple preclinical studies with erufosine have been conducted with human bladder, GBM, MM, and AML and CML cells demonstrated cytotoxic effects and induction of apoptosis by erufosine [29–31, 35–37, 44]. In addition, erufosine accumulated in subcutaneous rat C6 GBM tumors and in brain tissue in rats in vivo indicating that erufosine may inhibit the progression of malignant brain tumors [29, 46]. In a Phase II clinical trial, oral perifosine monotherapy was well tolerated and had encouraging activity with an overall response rate of 35% in patients with relapsed and/or refractory Waldenstrom Macroglobulinemia (WM), indicating WM may be another disease against which erufosine could potentially be effective [47]. Preclinical results also suggest that neuroblastomas are responsive to APCs [48].

Several clinical trials have demonstrated the limitations of perifosine as an oral single agent for treatment of solid tumors [40]. Perifosine has had positive clinical trials in combination regimens and is now undergoing Phase III clinical trial. Similarly, the potential of erufosine is not limited to its utility as a monotherapy. Preclinical studies have shown the radiosensitizing properties of erufosine particularly in GBM cells [39, 41, 49]. Using human CML cells K-562 and BV-173, the combination of erufosine with the EGFR inhibitor imatinib mesylate produced at least additive cytotoxicity [41]. Furthermore, erufosine proved to possess myeloprotective properties in combination with bendamustine and synergy with the drugs such as

bendamustine, melphalan, and bortezomib [37]. Synergistic cytotoxicity was observed with erufosine in combination with etoposide or cytarabine on AML cells as determined by isobologram analysis and combination index methodology [34].

Erufosine is a novel experimental therapeutic that may provide an advantage over other agents in this class due to the reduced hematopoietic toxicity and reduced risk of hemolysis; thus, erufosine can be administered by intravenous infusion in addition to other routes. An understanding of the mechanism of action of erufosine is continuing to evolve. Current preclinical data support the notion that the cytotoxic and apoptotic effects of erufosine in a wide variety of human cancer primary cells and cell lines are related to erufosine effects on the plasma membrane and direct or indirect disruption of signaling through the intracellular kinase Akt. The potential of erufosine is not limited to a single-agent therapy. Erufosine has been shown to be a radiation sensitizer and to increase the cytotoxicity of a variety of chemotherapeutic agents in simultaneous combination in cell culture. In vivo as a monotherapy, erufosine was more toxic to Panc-1 pancreatic carcinoma xenografts than to mouse bone marrow CFU-GM providing support for the notion that erufosine may be able to be used in combination with chemotherapeutic agents that cause dose-limiting bone marrow toxicity without additional bone marrow depletion. Erufosine may have the potential to enhance regrowth of the hematopoietic stem cell population in the bone marrow. The results presented here support further development of erufosine as a next generation APC for cancer therapy.

Conflict of interest None.

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