EFFECT OF ERUFOSINE ON THE REORGANIZATION OF CYTOSKELETON AND CELL DEATH IN ADHERENT TUMOR AND NON-TUMORIGENIC CELLS

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ABSTRACT
Cell adhesion plays a key role in tumor progression and its control could diminish the tumor metastases. The action of erufosine on cell survival, reorganization of cytoskeleton, and apoptosis was analyzed in breast cancer and mammary epithelial cells. Breast cancer cell lines MDA-MB-231 and MCF-7 as well as the non-tumorigenic line MCF-10A were treated with erufosine (5 μmol/L to 15 μmol/L). MTS test, FACS analysis, actin, tubulin and DAPI staining were performed. For MDA-MB-231 erufosine provoked apoptosis and actin reorganization, while MCF-7 and MCF-10A were less sensitive to the action of erufosine. The organization of microtubules was not affected by erufosine treatment. The cytotoxic action of erufosine on the investigated cells was cell-specific. The highly invasive MDA-MB-231 cell line was found to be most sensitive, since 15 μmol/L erufosine caused actin cytoskeleton reorganization and apoptosis.

Keywords: erufosine, breast cancer cells, cytoskeleton, apoptosis

Introduction
The alkylphosphocholines (APc) are a new group of antitumor agents, which show cytotoxic activity against different tumor cell lines in vitro and antineoplastic activity in vivo (1, 12). Unlike standard chemotherapy and irradiation, which work on the DNA level, APc are membrane-operating agents which inhibit protein kinase C and modulate the signal transduction pathways originating from the membrane (5). They induce apoptosis in many tumor models (7, 11) and selectively damage leukemic cells without affecting (by the same concentration) the normal cells of the bone marrow (10). Recently, a new alkylphosphocholine analog was obtained, called erucylphospho-N, N, N-trimethylpropylammonium (ErPC₃, erufosine). This is the first injected agent which loses its hemolytic properties and shows increased therapeutic ratio in vivo (2). The drug can even stimulate the production of hematopoietic progenitor cells (6, 15). Cell adhesion is a fundamental process, which, in non-tumorigenic cells, plays a crucial role in the cell growth and survival because it sustains the organization of tissues and organs. For tumorigenic cells the presence of adherence contacts is not a requirement for cell growth and survival (4). The changes in the cells’ adhesion behavior determine their modified morphology and migration behaviors which affect the cells’s invasive properties during all the stages of tumorigenesis. Thus, the manipulation of cell adhesiveness is an important prerequisite for the control of cancer cell growth and invasion. The cytoskeleton plays a crucial role for the manifestation of cell adhesiveness. Actin filaments, filopodia and lamelipodia ensure the cell adhesion, spreading...
we have described earlier (13) and were analyzed with an inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems GmbH, Germany) with objective HCX PL FLUOTAR 63x/1.25 oil.

**Tubulin staining**

After 24 h incubation with erufosine, the cells were washed with PBS and fixed with 3 % paraformaldehyde (PFA) for 15 min at room temperature. After washing with PBS, cells were permeabilized with 0.5 % Triton X-100 for 5 min. Saturation of the remaining free aldehyde was performed with 1 % BSA in PBS for 30 min. The slides were washed with PBS and incubated with primary monoclonal antibody against α-tubulin (Sigma, TG199) diluted 1:100 in PBS with 1 % BSA for 30 min at room temperature. After washing with PBS, cells were incubated with secondary rabbit anti-mouse IgG-Cy3-conjugated antibody (Sigma, C2181) at a dilution of 1:200 for 30 min. Finally, samples were washed with PBS and distilled water, mounted on objective slides and studied with an inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems GmbH, Germany) with objective HCX PL FLUOTAR 63x/1.25 oil.

**DAPI staining of the nucleus**

After erufosine treatment for 24 h, the cells were rinsed with PBS and stained with DAPI (1 μg/mL in PBS). The slides were conserved in the dark at 4 °C and were analyzed with an inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems GmbH, Germany) with objective HCX PL FLUOTAR 63x/1.25 oil.

**MTS test for cell survival and proliferation**

To analyze the cytotoxic effect of erufosine on the breast cancer and non-cancer cells, the MTS test (Promega) was performed as previously described (13). Briefly, the adherent cells were treated as described above and incubated additionally for 2 h, 24 h or 72 h. Then, 50 μL of MTS reagent was added directly to the adherent cells. They were incubated for 2 h at 37 °C and the absorbance at 490 nm was recorded with a 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). The survival of the cells treated with different concentrations of erufosine was presented as a relative number of adhered cells (O.D. at 490 nm). Three independent experiments were performed for each cell line.

**FACS analysis**

Cells were seeded at a density of 1×10⁶ cells/mL in 6-well plates and incubated for 24 h at 37 °C and 5 % CO₂. Then, erufosine was added at a concentration of 15 μmol/L and 50 μmol/L, and cells were additionally incubated for 24 h. Adhered cells were removed with 0.05 % trypsin/0.6 mmol/L ethylenediaminetetraacetic acid (EDTA) (Sigma), centrifuged (at 1×10³ RPM for 5 min) and resuspended to 1 mL PBS and centrifuged again. The pellet was resuspended in 0.3 mL of cold PBS and was added 0.7 mL of cold ethanol (70 %) drop by drop while gently vortexing. The samples were left for 1 day at 4 °C and after that they were washed and centrifuged twice. The pellets were resuspended in 0.25 mL of PBS, and 5 μL of 10 mg/mL Rnase A (Sigma, R-6513) was added. The samples were incubated for 1 h at 37 °C and 10 μL of 1 mg/mL PI (Sigma, P-4170) was added to each sample. The probes were analyzed by flow cytometry on a BD FACS Calibur flow cytometer at 585.

**Results and Discussion**

**Cell survival and cytotoxicity following treatment with erufosine**

The cell survival in the three adherent cell lines was not influenced during the two hours after the treatment with erufosine at a concentration of 5 μmol/L, 10 μmol/L, and 15 μmol/L (Fig. 1).

![Fig. 1. Cell survival and proliferation of MCF-10A, MCF-7 (ATCC) and MDA-MB-231 cells, after treatment with different concentrations of erufosine for 2 h, 24 h and 72 h. Statistical analysis was done by ANOVA one-way test and Tukey–Kramer post test (*P < 0.05; **P < 0.01; ***P < 0.001).](image-url)
The non-tumorigenic epithelial cell line MCF-10A was almost undisturbed by the effect of the drug, even after a longer period of treatment (24 h and 72 h) (Fig. 1). In MCF-7 cells a reduced proliferation activity could be seen after 72 h of treatment with 5 μmol/L erufosine. The invasive cell line MDA-MB-231 was shown to be most sensitive to the effect of erufosine. The proliferation activity was reduced after 24 h treatment with 5 μmol/L and higher concentrations of erufosine, and after 72 h the IC₅₀ (half maximal inhibitory concentration) level is reached at 5 μmol/L erufosine (Fig. 1).

**Cell death and cytoskeleton reorganization following treatment with erufosine**

The treatment of MDA-MB-231 cells with 15 μmol/L erufosine caused compression of the nucleus, chromatin condensation, and fragmentation of the nucleus (Fig. 2G). Unlike MDA-MB-231 cells, the nuclei of MCF-10A and MCF-7 cells did not appear to suffer any major changes from the treatment with the high erufosine concentration. By following the adhesive behaviour of MDA-MB-231 cells it was observed that the same concentration of erufusin (15 μmol/L), which caused apoptosis (Fig. 2G), provoked both reinforced adhesion and cell spreading and appearance of actin structures such as lamelipodia and philopodia on the cell surface (Fig. 2H). For the other two lines (MCF-10A and MCF-7) no change in their adhesive behaviour was observed after treatment with erufosine. Exposure of cells to 15 μmol/L erufosine did not lead to changes in the microtubule cytoskeletal network (Fig. 3). The microtubule network in MCF-10A cells was well presented: cables spread all over the control cells (Fig. 3A) and the erufosine-treated cells as well (Fig. 3D). In MCF-7 and MDA-MB-231 cells, although the tubulin network was more diffuse, it was not disturbed by the drug treatment (Fig. 3).

**FACS analysis**

To determine the quantity of apoptotic cells in the three cell lines in response to erufosine, flow cytometry analysis (FACS) was performed for cells exposed to erufosine and untreated control cells. The analysis of MCF-10 cells which had been treated with 50 μmol/L erufosine showed only a slight increase (about 10 %) in the population of apoptotic cells (data not shown). Exposure of MCF-7 cells to 15 μmol/L erufosine resulted in a 35 % increase in the number of apoptotic cells, but the higher drug concentration (50 μmol/L) increased this

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**Fig. 2.** Effect of erufosine on the cell nucleus and the actin cytoskeleton in MCF-10A (A, B, C), MCF-7 (D, E, F) and MDA-MB-231 (G, H, I) cells. Cells were treated with 15 μmol/L erufosine and sustained with DAPI (upper panel) and BODIPY/Phaloidin (middle panel). Lower panel: sustaining for actin of non-treated cells. Bar = 50 μm.
percentage only slightly more (to 38%) as compared to the lower concentration (Fig. 4A,C). Compared to MCF-7 cells, MDA-MB-231 cells were more sensitive to the lower drug concentration (15 µmol/L) as shown by 45% apoptotic cells. Interestingly, the higher drug concentration (50 µmol/L) considerably increased this percentage to over 75% in MDA-MB-231 cells (Fig. 4B,C). These data confirmed our microscopic observations and indicated that erufosine indeed could be applied as an effective and selective anti-tumor agent with little effect on normal and noninvasive tissue.

**Final remarks**

This study was part of experiments characterizing the activity of erufosine in preclinical breast cancer models. Remarkably, the experiments described here as well as those of a recently published paper (3) collectively suggest that erufosine could successfully be used in breast cancer patients. This suggestion is based on our finding that erufosine showed high antiproliferative activity in breast cancer cell lines at concentrations which did not exert toxicity in adherent non-tumorigenic breast tissue cells. Remarkably, the triple negative breast cancer cell line MDA-MB-231 responded even better to erufosine than the hormone-dependent cell line MCF-7. The high antineoplastic activity in breast cancer cell lines, together with the lack of toxicity in non-tumorigenic MCF-10 cells, corresponds to the experience with miltefosine (14). Miltefosine was the first alkylphosphocholine which was successfully used for treating cutaneous breast cancer metastases and its respectable effect after topical administration was linked with a good tolerance towards cells of the thoracic skin (14). In addition, erufosine was not only found to be devoid of toxicity towards normal hematopoietic cells, but even stimulated the colony forming potential of human bone marrow cells (15).

The observed reorganization of the actin cytoskeleton in MDA-MB-231 cells is interesting because it parallels the induction of apoptosis in response to erufosine and could be interpreted as a cellular response. A similar tendency towards formation of an adherent cell phenotype in response to erufosine was observed in LAMA-84 chronic myeloid cells, too (9). However, unlike the cells used in this study, LAMA-84 cells showed relatively high resistance to erufosine ($IC_{50} = 21 \mu$mol/L to 91 µmol/L). It is possible that the highly-invasive MDA-MB-231 cells are hit twofold by erufosine treatment. On the one hand, they suffer from erufosine’s interference with specific signaling pathways, such as those involving the ras and raf oncogenes (3), which are often mutated in tumor cells. On the other hand, the influence of erufosine on actin reorganization will impact tumor cell migration because the actin connecting proteins of the Rho family of the small GTPases will also be affected (8). In contrast, the lack of changes in tubulin networks supports the suggestion that microtubules do not participate in the trafficking of erufosine in cells, nor are they a target for erufosine’s action.

A complete analysis of the specific signaling pathways influenced by erufosine will be an object of future experiments.

**Conclusions**

The results from this study showed that the cytotoxic activity of erufosine towards adherent tumor cells is cell-specific. Most sensitive was the highly-metastatic cell line MDA-MB-231; and the non-tumorigenic cell line MCF-10A was non-sensitive. Certain erufosine concentrations (15 µmol/L) were observed to cause cell death and provoke the adherent cell phenotype of the highly-metastatic cell line MDA-MB-231. It was found that the tubulin network is not a target for erufosine’s action.

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**Fig. 3.** Effect of erufosine on tubulin organization in MCF-10A (A), MCF-7 (B) and MDA-MB-231 (C) cells treated with 15 µmol/L erufosine and stained for α-tubulin. Lower panel: non-treated MCF-10A (D), MCF-7 (E) and MDA-MB-21 (F). Bar = 50 µm.
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REFERENCES