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Effect of Erufosine on MDA-MB 231 Breast Cancer Cells

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Abstract

We investigated the anticancer activity of erufosine on the triple negative breast cancer cell line MDA-MB 231 in terms of cytotoxicity, induction of apoptosis and inhibition of cell migration. The antiproliferative effect was determined by MTT test. The induced cell death was revealed by using acridine orange/ethidium bromide (AO/EtBr) live cell staining. The effect on cell migration was evaluated by wound-healing assay. We show that IC50 dose for EPC3 treatment of MDA-MB231 cells was obtained-20μM, 13μM and 8μM for 24, 48 and 72 h, respectively. Our data pointed out that the treatment with erufosine caused inhibition of cell proliferation, an increase of early and late stage apoptotic cells and polyploid cells, which are sign for mitotic catastrophe. The prolonged treatment with erufosine did cells more sensitive and the apoptosis was visible at lower drug concentration. In the same time the anti-tumor agent caused slower closure of "wound" comparing to the untreated control cells.

Taken together, our results strongly suggest that erufosine have dose- and time-dependent inhibitory effects on a cell death and migration and may be is effective as anti-tumor agent against breast cancer.

Keywords: Erufosine; Breast cancer cells; Cytotoxicity; Apoptosis; Migration

Abbreviations

EPC3: Erufosine; ALPs: Alkylphosphocholines; ATL: Anti-tumor lipids; AO: Acridine Orange; EtBr: Etidium Bromide; EDTA: Ethylene Diaminetetraacetic Acid; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

Introduction

Erufosine (erucylphospho-N,N,N,-trimethylpropylammonium, EPC3) is an antitumor lipid that belongs to the group of synthetic alkylphosphocholines (APCs). Unlike classical antitumor agents, APCs target the cell membrane instead of DNA. Erufosine has a polar head and an unsaturated hydrocarbon chain that locates in the membrane raft domains, altering their fluidity and functions [1]. It is the first injected agent that loses its hemolytic properties and shows increased therapeutic features *in vivo*. Unlike the other anti- tumor lipids erufosine cross the blood-brain barrier and can accumulate in the brain [2]. EPC3 affects various signal transduction pathways starting from the cell membrane. Therefore, EPC3 modulates tumor proliferation and migration. It has high levels of cytotoxity that influences different types of tumors: gastrointestinal, cervical, brain, breast etc. The agent has favorable IC50 (the half-maximal inhibitory concentration) values for different tumor cell lines. As tumor becomes more malignant, erufosine's anti-tumor effect increases, IC50 decreases, respectively. The suppressing effect on high metastatic cancer cells is combined with stimulating effect on hematopoiesis [3]. It was noticed that erofusine manifests antitumor activity in wide range cancer cell lines such as MDA-MB 435 and MDA-MB 231 during preclinical trials [4]. Suppression of tumor proliferation and migration is due to the destruction of focal adhesion contacts containing β1 integrins and subsequent inhibition of Protein Kinase B (PKB) [5,6]. In addition, the anti-proliferative effect of erufosine was found to be due to inhibition of the PI3K/Akt/mTor transduction pathway [6], which subsequently leads to retention in phase G2/M of the cell cycle [7]. Because of the cell cycle arrest is obtained a formation of giant cells, which afterwards are subjected to apoptosis.

In the present study, we show for the first time that the cytotoxic effect of erufosine on breast

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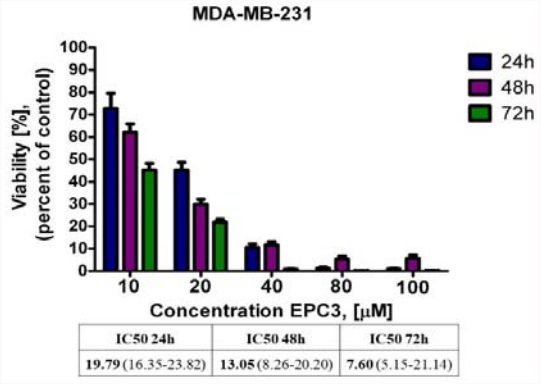

IC50 24h	IC50 48h	IC50 72h
19.79 (16.35-23.82)	13.05 (8.26-20.20)	7.60 (5.15-21.14)

Figure 1: Cell viability of MDA-MB 231 treated with erufosine for 24h., 48h. and 72h. Statistical analysis is done by ANOVA one-way test and Tukey post test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

cancer cell line MDA-MB-231 and induction of apoptosis is time-dependent. The sensitivity of MDA-MB-231 cells to erufosine increases with the treatment time, and IC50 decreases over time. An early stage of apoptosis was also found at a lower erufosine concentration as the treatment time progressed. The disturbance of cell proliferation and the induction of apoptosis led to inhibition of cell migration after erufosine treatment.

Materials and Methods

Materials

Erufosine was synthesized in the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, and was most graciously provided by Prof. M. Berger. It was dissolved in PBS (phosphate buffer saline, pH7.4) at 20mM stock concentration and was stored at +4°C.

MTT reagent (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is from Invitrogen, USA.

Acridine orange (AO) and ethidium bromide (EtBr) were from Sigma Aldrich, Germany. All chemicals were used as received without any further purification.

Cell line

MDA-MB 231 highly invasive breast cancer cell line was from American Type Culture Collection (ATCC, USA).

Methods

MTT assay: To observe the cytotoxicity of erufosine the cells were maintained as described previously [7]. The adherent cells were treated with erufosine in concentration range between 10µM and 100µM for 24, 48 and 72 hours. MTT test was performed as described by Mosmann [9] with some modifications. Briefly, after the incubation period the cell medium was changed with fresh medium (200µL/well). Then, 50µL of MTT solution (5mg/mL in PBS) was added. Plates were further incubated for 4h at 37°C, and the formed formazan crystals were dissolved by addition of 250µL solvent (5% formic acid in 2-propanol) per well and mixing. The absorbance was recorded at 570nm with the 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). Three independent experiments were performed in quadruplicate. Complete medium (200µL) and 5% formic acid in 2-propanol (250µL) were used as a blank solution. Cytotoxicity corresponding to cell survival after incubation with erufosine was presented as percentage to the cell viability of the positive control – non-treated cells.

Double AO/EtBr staining for detection of apoptosis

MDA-MB 231 cells, seeded onto round glass coverslips, were treated with 10, 20 and 30 µM erufosine and were incubated for 24 or 48 h. Untreated tumor cells were used as a control. Adherent cells were stained with solution (10µl) containing 10µg/ml AO and 10µg/ml EtBr. The cells were visualized under fluorescence microscope (Nu2, Carl Zeiss, Germany) using camera Canon EOS 5D Mark II.

In vitro wound healing (scratch) assay

Confluent MDA-MB 231 monolayers were scratched in a straight line using a 200µl sterile plastic pipette tip. The cells were treated with 10, 20 and 30 µM erufosine and were incubated for 72h. Untreated tumor cells were used as a control. Wound healing assay was repeated 3 times. Photographs were analyzed by Image J. Statistical analyses were performed using Graph Pad Prism 5.02.

Results and Discussion

Cell survival

To determine cell survival after treatment with erufosine, we used an MTT assay based on the conversion of tetrazolium salt from oxidoreductase enzymes to insoluble formazan (color compound) and measurement of the absorption intensity of staining. The data obtained was processed by non-linear regression to obtain IC50 of erufosine for each incubation time. The triple negative breast cancer cell line MDA-MB 231 (ER, PR and HER2 negative) showed significantly high sensitivity to treatment with erufosine that was rose to the time of treatment (Figure 1). The calculated IC50 for erufosine was 19.79µM for MDA-MB 231 treated for 24 hours and 13.05µM, and 7.60µM for 48 hours and 72 hours treatment, respectively.

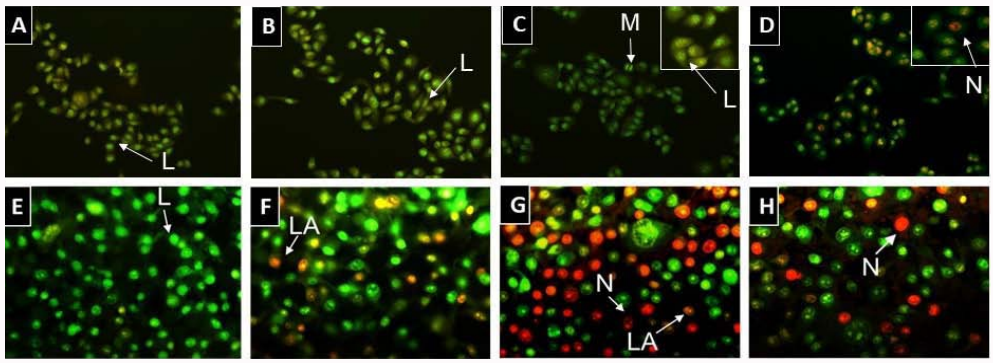

Figure 2: Morphology changes in MDA-MB 231 cells after treatment with erufosine. Cells were incubated 24h (A, B, C and D) and 48h (E, F, G and H) and stained for AO (green)/EtBr (red). Control cells (A, E), cells treated with 10 µM (B, F), 20 µM (C, G), 30 µM (D, H) erufosine, Objective magnifications 20X (24 h) and 40X (48 h). Double acridine AO/EtBr staining method was repeated 3 times.

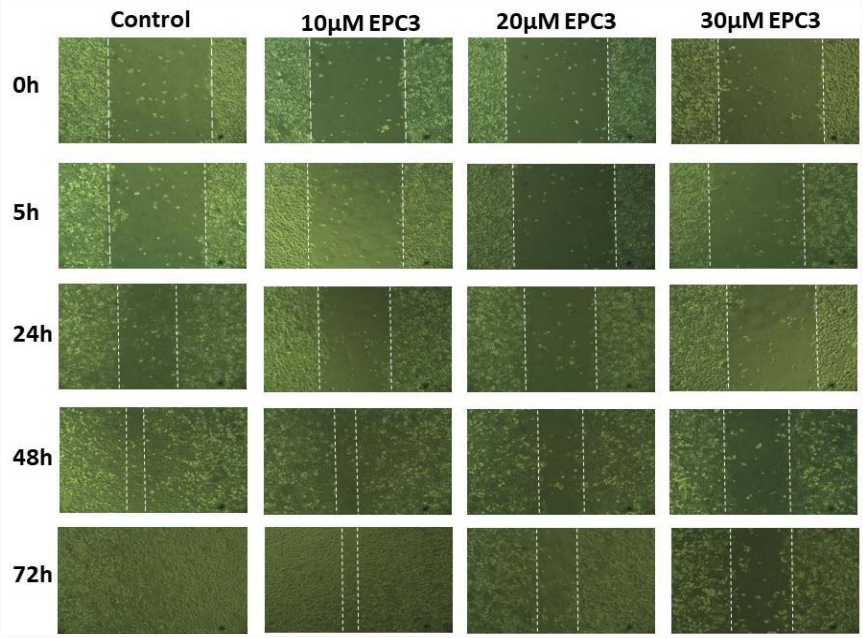

Figure 3: Effect of EPC3 on MDA-MB 231 cell migration. The cells were treated with 10, 20 and 30 μM erufosine for 72 h. Scratch zones were photographed with a microscope Leica DMI300 B, objective magnification 10X.

AO/EtBr double staining for detection of apoptotic and necrotic cells

To determine the predominant type of cell death, AO/EtBr staining was performed for MDA-MB 231 cells exposed to erufosine and untreated control cells.

Green live (L) and mitotic (M) MDA-MB 231 cells with a normal morphology were observed in the control (Figure 2A and Figure 2E). The cells treated with 10 and 20 μM erufosine for 24h were viable without indication of apoptosis or necrosis (Figure 2B and 2C). However, treatment with 20 μM erufosine tends to round out the cells, indicating that cytoskeletal alterations occur (Figure 2C). On treatment with a 30μM (IC75) concentration of erufosine (Figure 2D), were observed early-apoptotic, late-apoptotic and necrotic cells. The nuclei of the early apoptotic cells (L) were stained in green, similar to the control cell nuclei, but unlike them, the process of condensation of the nuclear chromatin has begun in the treated ones. Late-stage apoptotic cells (LA), with concentrated and asymmetrically localized orange nuclear EtBr staining, and necrotic cells (N) were also detected after treatment with 30μM erufosine (Figure 2D). Necrotic cells increased in cell population and showed uneven orange-red fluorescence at their periphery. Those cells were in a process of disintegration.

After 48h treatment cells showed higher sensitivity even at the lower concentrations of erufosine. The most of the MDA-MB 231 cells treated with 10μM erufosine for 48h (Figure 2F) are in early- and late- apoptotic stage and had changes in cell morphology. Kapoor and colleagues [Kapoor V, 2012] found that the anti-tumor agent induces cell cycle retention in phase G2 afterwards treatment of squamous cell carcinoma of the oral cavity resulting in multi-core cell formations. In Figure 2G, such a polyploid cell is observed. Its huge size is probably owing to erufosine-induced mitotic catastrophe.

Results from AO/EtBr live staining showed that with increasing the EPC3 concentration and the treatment time, the number of late-stage apoptotic cells is increased. Apoptosis is generally the predominant cell death after treatment with erufosine.

Wound healing assay in MDA-MB 231 cells

Wound healing assays have been used to study the effect of erufosine on MDA-MB 231 cell migration.

There were no significant changes in the scratch area after five hours treatment with erufosine. MDA-MB 231 treated with the lowest concentration of erufosine (10μM) had the same migration potential as the control cells. Migration potential of the cells decreased after treatment with 20 and 30 μM erufosine after 24h. While untreated cells had almost completely closed the wound within 72 hours, cells treated with 30 μM erufosine covered only about 50% of the total area during the same period of time (Figure 3).

We showed that the anti-tumor agent caused slower closure of "wound" comparing to the untreated control cells. In our previous study we showed that the cytotoxic effect of erufosine on MDA-MB 231 cells was accompanied by induction of adherent cell phenotype and apoptosis [8] which could be the reason for the decreased cell migration after erufosine treatment.

Conclusion

In the present investigation, we provide evidence that the cytotoxic effect of erufosine was dose- and time- dependent. We show that the increased erufosine concentration provoked apoptosis and inhibit migration potential in MDA-MB 231 cell line. In light of our findings, erufosine appears to be a promising chemotherapeutic agent in breast cancer.

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