Erfosine simultaneously induces apoptosis and autophagy by modulating the Akt–mTOR signaling pathway in oral squamous cell carcinoma

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1. Introduction

Oral squamous cell carcinoma (OSCC), which falls in the head and neck cancer category, represents one of the six most common cancers in the world [1]. World wide, there are 400,000 new cases each year and most of these cases have been reported from Asian countries [1]. In India, it is the leading cancer among males and the third most common malignancy in females [2]. In spite of many advances in treatment modalities, the five-year survival rate after diagnosis remains approximately 50%, which is considerably lower than that for other cancers, such as colorectal, cervix and breast origin [3]. The limited survival is because early tumors are asymptomatic and remain unnoticed until they spread to the regional lymph nodes in the later stages. In addition, there is a lack of early detection markers and many patients show drug-resistance to the available chemotherapy [4]. Therefore, there is a need for novel molecular targeted therapies that impair aberrantly activated signaling pathways, which can influence the proliferation, survival and drug-resistance of OSCC. One such pathway is the mammalian target of rapamycin (mTOR) signaling network, which has been found deregulated in head and neck cancers [5,6].

mTOR is a serine–threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K) related family. It exists in two functional complexes, mTORC1 and mTORC2, and regulates cell growth and proliferation by integrating signals arising from growth factors, nutrients and energy status. mTORC1 regulates the activity of the translation machinery by modulating eukaryotic translation initiation factor 4E (eIF4E) activity and S6 kinase (p70S6K) through direct phosphorylation. S6K monophosphorylates 4EBP1 and eIF4E is released and the activity of the translation machinery is increased [7]. The ribosomal protein S6, which is a subunit of S6K is involved in the translation of proteins that are regulators of protein synthesis and can act as proto-oncogenes, hence, mTOR signaling activity is associated with cancer cell growth and survival [8,9].

Recently, inhibitors of proteins that are involved in the mTOR signaling pathway are under active clinical or preclinical investigation for cancer therapy. Although most of them appear as potential therapeutic agents, not much success has been obtained so far. Thus, there is a need to look for more potent inhibitors with minimum side effects. Erfosine (erucylphospho-N,N,N-trimethylpropylammonium, ErPC3) is a novel chemotherapeutic agent belonging to a group of substances referred to as alkylphosphocholines (APCs). These ether–lipid-derived synthetic compounds [10] exhibit strong pro-apoptotic activity on a variety of malignant cell lines [11,12] and primary tumor cells [13]. It is noteworthy that APCs are not myelotoxic and even stimulate normal haematopoiesis in the bone marrow, in contrast to conventional anticancer drugs [14,15]. However, their exact mechanism of action is not fully understood besides they modulate different signal transduction pathways.
through interaction with membrane components [12,14,16–21]. For example, they are known to inhibit the phosphoinositide pathway [14,16,21] and lead to de-phosphorylation of Akt and Rb [12]. In the present study, we for the first time investigated the anti-tumor potential of erufosine in oral squamous cell carcinoma cells. Its possible synergies with the commonly used drugs for OSCC like cisplatin and 5-flourouracil were also investigated. We report here the induction of autophagy and apoptosis, as well as of cell cycle and proliferation inhibition by erufosine via down-regulation of the mTOR signaling cascade.

2. Materials and methods

2.1. Cell lines and reagents

Four human oral squamous cell carcinoma cell lines Ca127, FaDu, SCC9 and SCC25 were obtained as a kind gift from Dr. Gustavo Acuna Sanhueza in Prof. Angel’s lab, DKFZ, Germany. Ca27 and FaDu cells were maintained in MEM medium (Gibco, Germany) and SCC9 and SCC25 were maintained in DMEM medium, all containing 10% fetal calf serum (Biochrom, Germany) and 2 mM l-glutamine (Invitrogen, Germany), at 37 °C under a humidified atmosphere with 5% CO2. All lines were recently tested and authenticated by the DSMZ (Dr. W. Dirks, DSMZ, Braunschweig, Germany) using a short tandem repeat profiling in accordance with AACR best practices. Cells were passaged two or three times per week to keep them in log phase. Erufosine (erufosine-NN,N-trimethylpropanolamine, ErPC3) was kindly provided by Prof. H. Ebi, Max Plank-Institute, Goettingen, Germany. It was dissolved in normal saline and stored at 4 °C. Rapamycin (Cyanam Chemical, USA) was dissolved in dimethylsulfoxide and added to concentrations at 25, 50 and 100 nM. Cisplatin (Medac, Germany) and 5-flourouracil (Sigma, Germany) are used for chemotherapy of oral squamous cell carcinoma, were employed to check their effect on cell proliferation in combination with erufosine.

2.2. Cell survival by MTT-dye reduction assay and determination of ATP levels

To analyze the cytotoxic effect of ErPC3 on the oral cancer cells, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay was performed as previously described [22], with some modifications. Briefly, 7 × 10^4 cells/100 μl media were seeded in 96-well plates 24 h before the experiment. Cells were then incubated with 10 different concentrations of ErPC3 for 24, 48 and 72 h as indicated. 10 μl/well MTT-solution (10 mg/ml in PBS) was then added and plates were further incubated for 1 h at 37 °C. The formazan crystals were dissolved by adding 110 μl/well of 0.4 N HCl in 2-propanol. Absorbance was measured by a microculture plate reader (Anthos 2001, Anthos Labtec, Wals, Austria) at 540 nm, reference filter 690 nm. 100 μl medium with 10 μl MTT stock-solution and 110 μl 0.4 N HCl in 2-propanol was used as blank solution to correct for background absorbance. Data obtained were presented as percent of untreated control in the best-fit (linear or sigmoidal) dose response curves. IC50 values at 95% confidence intervals were calculated for all cell lines. For each concentration, at least eight wells were used. In addition, cellular ATP concentrations were determined based on the luciferin–luciferase reaction using the CellTitertek Luminescence reader (Anthos Labtec, Austria). Levels of ATP were quantified using ATP assay kit (Promega, Germany), at 37 °C using luciferase as per manufacturer’s instructions. The total protein lysates (30–50 μg) were subjected to electrophoresis on 8% or 10% polyacrylamide SDS gels. Proteins were transferred onto PVDF membranes (Immobilon, Millipore, Germany), at 37 °C and then incubated with 5% dry milk at room temperature for 1 h. The membranes were washed two times with Tris buffered saline with Tween-20 (TBS-T). The membranes were incubated with primary antibodies (Cell Signaling Technologies, USA) as per manufacturer’s instructions. After washing for 3 times, the membranes were incubated with secondary antibody for 1 h. Proteins were visualized by chemiluminescence using the Auto-Imager (Alpha Innotech Corp, San Leandro, CA). The bands were quantified by densitometry and normalized to β-actin and quantified for protein expression using the ImageJ software. The percentage of apoptotic cells was determined by the sub-G1 peak in the DNA histogram using the ModFit LT software. The percentage of apoptotic cells was determined by the sub-G1 peak in the DNA histogram using the ModFit LT software. The significance of differences between two variables was analyzed using student’s t-test. A P-value <0.05 was considered to be statistically significant.

2.3. Assay for cytotoxic activity combined with other anti-tumor agents

As described above, all four cell lines were treated for 48 h with different combinations of erufosine, combined with cisplatin, 5-flourouracil and rapamycin. The MTT assay was used to determine their combination effects. The evaluation of the combination effects was performed using the method published by Ludwig and Berg [23]. Expected theoretical values were calculated according to the equation, c = a × b / 100, in which a and b are cell survival values after single agent treatment, given as percent of untreated control. For each combination, expected values were calculated and compared with the result obtained. intersection of cross curve was scored by an inverted microscope after 7–10 days. At least, five Petri dishes per treatment protocol were used. Data are represented as percentage of colony forming units.

2.4. Clonogenic survival assay

10^3 cells/ml from each sample (after treatment with ErPC3) were transferred into 5 ml of semi-solid medium containing 0.8% methylcellulose and 30% FCS. Cells were plated into Petri dishes (1.2 ml/Petri dish). Normal cell culture conditions (37 °C, 5% CO2 in humidified air) were applied. Colony formation (clusters of 20 or more cells) was scored by an inverted microscope after 7–10 days. At least, three Petri dishes per treatment protocol were used. Data are represented as percentage of colony forming units.

2.5. Immunoblotting analysis for protein expression

The modulation of mTOR signaling pathway components by ErPC3 treatment was tested by immunoblot analysis. All four cell lines were treated in 25 cm² culture flasks with the indicated concentrations of ErPC3 for 17, 24 and 48 h. After treatment, cells were harvested, transferred to 1.5 ml microcentrifuge tubes (Eppendorf, Germany), washed in PBS and centrifuged for 5 min at 2000 rpm in an Eppendorf microcentrifuge. Pellets (2 × 10⁶ cells) were lysed with buffer containing 100 mM Tris-HCl with pH 8.0 (Sigma), 4% SDS (Sigma), 20% Glyceral (Sigma), 200 mM DTT (dithiothreitol, Sigma) supplemented with complete protease inhibitor cocktail tablets (Roche). After vigorous vortexing, lysates were boiled for 10 min and then centrifuged at 13,000 rpm for 10 min at 4 °C. Aliquots of 10 μl were taken from the lysates before adding DTT, diluted five times in distilled water and quantified for protein concentration using the Pierce Protein Assay (Pierce, USA). The total protein lysates (30–50 μg) were subjected to electrophoresis on 8% or 10% polyacrylamide SDS gels. Proteins were transferred onto PVDF membranes (Sigma–Aldrich) and the membranes were probed for different proteins: (Ser/Thr phosphorylated (p)-Akt, Thr/Ser phosphorylated (p)-Akt, p-PRAS40, p-Raptor, mTOR, Ser2448/mTOR, p-p70S6K, p-4E-BP1, LC3B, cleaved PARP, cyclic D1) using specific antibodies (Cell Signaling Technologies, USA) as per manufacturer’s instructions. Immunoblots were developed using a HRP-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling Technologies, USA) and ECL System (Amersham Pharmaacia Biotech, Germany). Levels of β-Actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autorigraphic images performed using the Photoshop CS3 Program.

2.6. Caspase activity assay

Caspase 3/7 activity, after treatment with different concentrations of erufosine for different cell lines was analyzed in a 96-well microplate format using the Apo-Glo™ assay (Promega, Germany) according to the manufacturer’s recommendations. The activity was measured in a luminometer (Berthold Technologies, Germany) and expressed as percentage of untreated control.

2.7. Detection of autophagy with acridine orange staining

Acidic vesicular organelles were imaged by staining of the cells with 1 μg/ml acridine orange solution for 20 min. Samples were then examined under a Zeiss Cell Observer Microscope (Carl Zeiss Inc., Germany) using the excitation filter 488 nm and emission filter 505–530 nm and >650 nm [25].

2.8. Cell cycle analysis and determination of hypodiploid (apoptotic) cells by flow cytometry

The effect of erufosine on the cell cycle was checked in all cell lines by propidium iodide staining and flow cytometry. Briefly, 1 × 10⁶ cells were washed in PBS, centrifuged and fixed in 4 ml ice cold 70% ethanol by incubation at 4 °C for 1 h. Cells were then centrifuged and re-suspended in phosphate buffered saline. RNA was digested by incubating the samples with 1 mg/ml RNase A (Invitrogen, Germany) for 30 min at 37 °C. Propidium iodide (50 μg/ml) was then added and the samples were acquired in a FACS Canto (BD Biosciences) after 10 min incubation. The distribution of cells in G0/G1, S and G2/M phases of cell cycle was estimated using the ModFit LT software. The percentage of apoptotic cells was determined by the sub-G1 peak in the DNA histogram using the FACS Diva software (BD Biosciences).

2.9. Transfection of siRNA

The cells were transfected using cationic liposomes (Lipofectamine™ reagent, Invitrogen) according to the manufacturer’s instructions. Briefly, cells at a concentration of 5 × 10⁵/ml cells were incubated for 24 h in six-well plates in culture medium. The cells were then transfected with 160 nM mTOR or non-sense control (NSO) siRNA in the presence of lipofectamine for 24 h. The cells were again transfected in the same way (double transfection) for another 24 h. To determine whether erufosine-induced cytotoxicity is affected by mTOR siRNA, OSCC cells transfected with mTOR or NSO siRNA were treated with erufosine, rapamycin and cisplatin for 48 h and MTT assay and Clonogenicity assay was performed as described above.

2.10. Statistical analysis

Experiments were performed at minimum in triplicate and the results are expressed as means with corresponding standard deviations. The significance of differences between two variables was analyzed using student’s t-test. A P-value <0.05 was considered to be statistically significant.
3. Results

3.1. Erufosine induces cytotoxicity in human oral squamous cell carcinoma cell lines

Tumor cells were treated with 0–100 μM erufosine for 24, 48 and 72 h and the MTT assay was used to evaluate the resulting cytotoxic effects. Erufosine showed dose-dependent cytotoxicity in all the OSCC cell lines (Fig. 1). The IC50 for Cal27, FaDu, SCC9 and SCC25 were 33, 41, 50 and 46 μM, respectively, after 48 h treatment.

All the tumor cells showed morphological changes within 24 h treatment only (Suppl. Fig. 1). The prominent morphological changes in erufosine treated cells were cell shrinkage, detachment from the surface of the culture dish and membrane blebbing. While SCC9 and SCC25 cells showed appearance of characteristic vacuoles inside the cells after treatment with 50 μM erufosine for 24 h, these intracellular vacuoles were not very pronounced in the case of Cal27 and FaDu cells. In addition, a 5–10 fold decrease in ATP levels was also observed at a 100 μM dose of erufosine after 48 h in all cell lines (Suppl. Fig. 2).

3.2. Erufosine inhibits colony growth of human OSCC cells

The effects of erufosine on tumor cell growth were determined by clonogenic survival assay. The results are shown in Fig. 2. A significant dose-dependent reduction in CFU was observed in Cal27, FaDu and SCC25 cell lines after both 24 and 48 h treatment. While Cal27 cells showed an almost 30-fold and 20-fold reduction in CFU after 24 h and 48 h, respectively, in response to a 30 μM dose, FaDu cells showed recovery in CFU at 48 h treatment following both doses of erufosine (15 and 25 μM). SCC9 cells did not form colonies in semi-solid media.

3.3. Erufosine induces apoptosis in OSCC cells by activating caspase 3/7 and cleavage of PARP

To determine whether erufosine induces apoptosis in OSCC cells, cells were treated in the absence or presence of increasing concentrations of erufosine for 24 h, at which point the sub-G1 fraction of cells was analyzed (Fig. 3A). A dose-dependent increase in the sub-G1 peak, consistent with apoptosis, was observed in all four cell lines treated with increasing concentrations of erufosine. Treatment also increased caspase-3/-7 activity in a dose-dependent manner (Fig. 3B). As compared to the untreated control, 50 μM erufosine enhanced the caspase-3/-7 activity to approximately 6-fold in Cal27, 4-fold in SCC25, 3-fold in FaDu and 2-fold in SCC9 cells at the indicated time points. The results were further confirmed by Western blot analysis of cleavage of the caspase substrate PARP (Fig. 3C). Erufosine treatment resulted in the processing of full-length PARP (110 kDa) to its 89 kDa cleaved form in OSCC cells in a concentration and time dependent manner suggesting that it activates a caspase-dependent apoptotic pathway.

3.4. Erufosine induces autophagy in OSCC cells

We next examined if in addition to apoptosis erufosine induces autophagy in OSCC cells. Autophagy was analyzed using acridine orange staining for acidic vesicular organelles (AVOs) including autophagic vacuoles. In untreated cells, the cytoplasm and nucleus fluoresced bright green while erufosine treatment with the indicated concentrations for 24 h induced the development of AVOs in OSCC cells, as shown by the concentrated bright red fluorescence in acidic compartments (Fig. 4A). To confirm autophagy, we examined the expression of LC3B-I and its cleavage product LC3B-II associated with the autophagosome membrane by Western blot analysis. The results showed an increased ratio of LC3B-II to LC3B-I in all OSCC cells after treatment with erufosine as compared to untreated controls (Fig. 4B).

3.5. Erufosine induces G2 arrest and modulates cyclin D1

Cell cycle analysis and Western blot assay was performed to observe if erufosine induces cell cycle arrest in OSCC cell lines. The results have been shown in Figs. 5 and 6, respectively. All tumor cell lines showed a dose-dependent increase in tumor cells in G2 phase as compared to the untreated control. While Cal27 showed an increase in G2 phase from 4% to 17%, FaDu, SCC9 and SCC25 showed increases from 7% to 19%, 9–21% and 9–47%, respectively. Although cyclin D1 expression was downregulated at higher concentrations of erufosine in all the cell lines, an upregulation was seen at lower doses in FaDu and SCC9 cell lines.

3.6. Erufosine down-regulates the mTOR signaling pathway in OSCC cells

Western blot analysis showed that the treatment of all four OSCC cell lines with erufosine resulted in decreased expression levels of Ser2448 p-mTOR, p-PRAS40 and p-Raptor in a dose-dependent manner (Fig. 7A–C). In fact, p-PRAS40 was reduced to undetectable levels at the highest concentration of erufosine. However, total mTOR levels were unaffected in Cal27, SCC9 and SCC25 cells, although a decrease was seen in FaDu cells after treatment with 50 μM erufosine (Fig. 8A). Ser473 p-Akt, which is phosphorylated by the mTORC2 complex and Thr308 p-Akt, the upstream kinase of the mTORC1 complex were also de-phosphorylated (Figs. 8A and 9). The downstream substrates of mTORC1 p70S6K and 4E-BP1 were also efficiently de-phosphorylated by erufosine treatment in all cell lines (Figs. 7 and 8A).

To further study the effects of mTOR down-regulation on the anticancer potential of erufosine, we performed transient transfection experiments using siRNA specific for mTOR. Treatment of FaDu cells with the mTOR specific siRNA resulted in a marked decrease of Ser2448 p-mTOR (Fig. 8B). These transfected cells were treated with the indicated concentrations of erufosine, rapamycin, cisplatin and then subjected to MTT and clonogenecity assays. As observed from the MTT assay, mTOR down-regulation enhanced the cytotoxic potential of erufosine, which was higher than that of rapamycin and comparable to cisplatin. While 20 μM erufosine alone showed 25% cytotoxicity, it induced 50% cytotoxicity in cells transfected with siRNA. The results were further confirmed by CFU assay.

3.7. Erufosine shows additive effects with cisplatin and 5-FU in human OSCC cells

The combination effects of erufosine with cisplatin and 5-FU are shown in Supplementary Table 1. All four cell lines responded in a similar way to the combination of erufosine with cisplatin or 5-FU. The cytotoxic effects of the combinations were additive as the observed effects were ≤30% higher than the expected effects.

4. Discussion

Erufosine is a novel ether lipid alkylphosphocholine that has displayed a high efficacy against tumor models in vivo and in vitro [11–13,24,26]. It has been shown to induce apoptosis in many leukemia and glioma cell lines [14,24,27,28]. Compared to other alkylphosphocholines like perifosine, which have poor gastrointestinal tolerability and for their hemolytic potential cannot.
Fig. 1. In vitro cytotoxicity induced by erufosine in oral cancer cells. (A) Cal27, (B) FaDu, (C) SCC9 and (D) SCC25 cells were treated with 0–100 μM erufosine. Dose response curves representing percentage of untreated control versus erufosine concentration were generated after 24, 48 and 72 h of treatment. Tables below curves show the respective IC50 concentration with 95% confidence limits.

Fig. 2. Clonogenic survival assay. Cal27, FaDu and SCC25 cells were treated with the indicated concentrations of erufosine for 24 and 48 h. At day 10, cultures were assessed for colony formation. The percent colony forming units are summarized in the table. *p < 0.05, students t-test.

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be administered intravenously [29], erufosine is not myelotoxic and even stimulates normal hematopoiesis [14,15,30]. These features are attributed to its chemical structure, which has a long alkyl chain of 22 carbon atoms with one cis-double bond. This is the basis for erufosine’s low hemolytic potential, which is prerequisite for its systemic application and in turn allows high plasma levels. Preliminary results from a phase I trial in patients with chronic lymphocytic leukemia (CLL) show that plasma concentrations of more than 60 μM erufosine can be achieved in humans without significant toxicity [13]. It has been observed that erufosine modulates different signal transduction pathways through interaction with membrane components, however, its exact mechanism of action is not fully understood [12,14,16–21,31]. Since our earlier studies have demonstrated that erufosine inhibits the phosphoinositide pathway [14,16,21], leads to de-phosphorylation of Akt and de-phosphorylation as well as induction of Rb [12], we examined its anti-tumor activities and effect on the mTOR signaling pathway in Cal27, FaDu, SCC9 and SCC25 oral squamous cell carcinoma cells.

In MTT assay erufosine appeared to be a potent inducer of cytotoxicity in all tumor cell lines with varying sensitivity. Cal27 was most sensitive to erufosine treatment followed by FaDu, SCC9 and SCC25 respectively. The treated tumor cells exhibited shrinkage in their size, detachment from the culture dish and membrane blebbing that are typical features of apoptotic cell death. While SCC9 and SCC25 displayed appearance of characteristic intracellular vacuoles, these were not very prominent in case of Cal27 and FaDu. Such vacuoles have been observed earlier in LN-18, LNT-229 and LN-308 human malignant glioma cells in response to treatment with alkylphosphocholines, though their origin is not clear [32]. Erufosine treatment for 48 h also led to five to ten-fold decreased ATP levels in tumor cells. Similar effects were observed earlier in human glioblastoma U87MG and U118MG cell lines [33] and were suggested to be due to the permeabilization and dissipation of mitochondrial membrane potential (ΔΨm) and may be related to the inhibition of the F0 subunit of the mitochondrial F0F1 ATP(synth)ase. Thus, it appears that erufosine exhibits anti-mitochondrial activity on human oral cancer cells and that this effect may contribute to abrogating growth and development of these tumor cells. Our results on colony formation in response to erufosine also support this finding. Erufosine significantly reduced colony formation in Cal27, FaDu and SCC25 cells, with the exception of SCC9 cells, which, as documented earlier, do not form colonies in semi-solid media [34]. It can thus be concluded from both assays that erufosine markedly reduces the surviving fraction of tumor cells.

We next examined if erufosine has any effect on tumor cell cycle using PI staining and flow cytometry. Treatment with erufosine
Fig. 4. Induction of autophagy by erufosine treatment. (A) Acridine orange staining was performed in cells grown on chamber slides with or without exposure to the indicated concentrations of erufosine for 24 h. White arrows indicate the presence of bright red fluorescing autophagic vacuoles in the cell cytoplasm. No such vacuoles can be seen in the untreated (0 μM) cells. (B) Western blot analysis for the expression of LC3B-I and LC3B-II in the lysates of Cal27, FaDu, SCC9 and SCC25 cells after treatment with erufosine at the indicated doses and time points. The numbers beneath the Western blots represent relative expression of the ratios of LC3BII: LC3B I when compared to the respective untreated control. The values were derived by dividing the densitometric output for each LC3BII band by that for the corresponding LC3BI band and subsequent normalization with β-actin.

Fig. 5. Effect of erufosine on cell cycle. Flow cytometry analysis of DNA content in different phases of the cell cycle after 24 h exposure to the indicated concentrations of erufosine. The percentage of cells in each phase is presented in the table, which shows a significant arrest in the G2 phase of the cell cycle in Cal27, FaDu, SCC9 and SCC25 cells. (For interpretation to colours in this figure, the reader is referred to the web version of this paper.)
resulted in a robust accumulation of tumor cells in the G2 phase of the cell cycle. In an earlier study, it was also shown to increase the G2/M fraction of THP1 cells and to decrease their G1 fraction of the cell cycle [35]. It further downregulated the expression of cyclin D1 at higher concentrations in all cell lines, although an upregulation was observed at lower (non-cytotoxic) concentrations in FaDu and SCC9 cells. It appears that in FaDu and SCC9, there is accumulation of cyclin D1 at lower concentrations while at higher concentration it is inhibited due to cell cycle arrest. Thus, it appears that erufo-sine causes cell cycle arrest by modulating cyclin D1 and possibly cyclin D1-dependent protein kinases.

In this study, erufo-sine induced apoptosis in all the cell lines, as indicated by a time- and dose-dependent increase in the sub-G1 (hypodiploid) population and cleavage of PARP. At 30 μM concentration, an approximately 4-fold rise in the sub-G1 population was observed in Cal27 cells besides 3.5, 3 and 1.5-fold rises in SCC25, FaDu and SCC9 cells, respectively. This was mediated by the effector caspases 3 and 7. However, the pro-apoptotic effects of erufo-sine were least pronounced in SCC9 cells. Earlier studies have shown that erufo-sine induces apoptosis in a variety of tumor cell lines via the extrinsic or the intrinsic pathways [2,20,36]. Similarly, it has been reported that mitochondria as well as Apaf-1 and cas-

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**Fig. 6.** Western blot analysis for the expression of cyclin D1 in lysates of Cal27, FaDu, SCC9 and SCC25 cells after treatment with erufo-sine at the indicated doses and time points. β-actin was used as loading control. The numbers beneath the western blots represent relative expression of cyclin D1 when compared to the respective untreated control. The values were derived by dividing the densitometric output for each cyclin D1 band by that for the corresponding β-actin band and subsequent normalization.

**Fig. 7.** Effect of erufo-sine on mTOR signaling components. (A–C) Western blot analysis for expression of p-mTOR, mTOR, p-Akt, p-Raptor, p-PRAS40, p-4EBP1 and p-S6K in Cal27, SCC9 and SCC25 cells, respectively. The numbers beneath the Western blots represent relative expression of different mTOR signaling components when compared to their respective untreated control. The values were derived by dividing the densitometric output for each band by that for the corresponding β-actin band and subsequent normalization.

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pase 3 play a major role in erucylphosphocholine and erucylphosphohomocholine-induced apoptosis [28,37,38]. However, in addition to inducing apoptosis, erufosine also induced autophagy in all four cell lines as observed by acridine orange staining and expression of LC3BII. It may be mentioned here that autophagy is also an important component of tumorigenesis, making it a possible target for the development of potent anticancer agents. In this study, we show for the first time that autophagy is activated in response to erufosine treatment in oral cancer cell lines. This is an important observation as recently it has been shown that constitutively activated PI3K/Akt/mTOR pathway is linked to the ability of tumor cells to inhibit autophagy and promoting their growth and survival [39].

The Akt/mTOR pathway has been identified to be deregulated in head and neck squamous cell carcinoma and thus is a potential therapeutic target for chemoprevention and treatment of such cancers [6]. It has been shown that the inhibition of mTOR by chronic administration of rapamycin halts the malignant conversion of precancerous lesions and promotes the regression of advanced carcinogen-induced squamous cell carcinomas [40]. However, single inhibitors in this pathway like rapamycin, have limited efficacy. This is possibly due to the feedback signaling loops operating through p70S6K and PI3K [41]. Therefore, an inhibitor that targets this pathway at multiple levels may prevent pathway re-activation and development of drug resistance. Similar to our previous observations [11,42], erufosine induced efficient de-phosphorylation of Akt at the Ser473 residue (a marker of mTORC2/Akt activity) in all oral cancer cell lines. In addition, we show that it also reduced phosphorylation at the Thr308 residue of Akt that is required for activation of the mTORC1 complex. Alkylphosphocholines are 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 [43]. Nevertheless, PTEN phosphorylation was also examined (data not shown) and a dose dependent decrease was observed after erufosine treatment, although total PTEN levels remained unaffected. Thus, activation of PTEN could be another mechanism of erufosine mediated deactivation of the Akt–mTOR pathway. We have also shown earlier that in the multiple myeloma cell lines

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**Fig. 8.** Effect of silencing of mTOR in oral cancer cells. (A) Western blot analysis for expression of p-mTOR, mTOR, Ser473p-Akt, p-Raptor, p-PRAS40, p-4EBP1 and p-S6K in FaDu cells. (B) FaDu cells were transfected with NSO, non-sense control siRNA or mTOR specific siRNA and then subjected to treatment with erufosine, cisplatin or rapamycin. They were then analyzed for expression of p-mTOR by Western blot analysis, cytotoxicity by MTT assay, and percentage of cell survival by clonogenic survival (CFU) assay. wt., wild type or untransfected cells. The numbers beneath the Western blots represent relative expression of different mTOR signaling components when compared to their respective untreated control. The values were derived by dividing the densitometric output for each band by that for the corresponding β-actin band and subsequent normalization.

**Fig. 9.** Effect of erufosine on Thr308p-Akt levels in different oral cancer cell lines. The numbers beneath the Western blots represent relative expression of Thr308p-Akt when compared to their respective untreated control. The values were derived by dividing the densitometric output for each band by that for the corresponding β-actin band and subsequent normalization.
RPMI-8226 and U-266, Akt was not de-phosphorylated although they were sensitive to the cytotoxic activity of erufsine [42]. Similarly, another potent inhibitor of Akt, a phosphotyldinositol ether lipid analog has been shown to differentially regulate genes involved in apoptosis, wound response and angiogenesis (upregulation) and those involved in DNA replication, repair and mitosis (downregulation) in non-small cell lung carcinoma cells [44]. These observations point to the importance of other molecular targets of alkylphosphocholines.

In this study, we observed that p-mTOR was also de-phosphorylated at Ser2448, which is one of the most critical residues for catalytic activity of mTOR. However, total mTOR levels were unaffected in Cal27, SCC9 and SCC25 cells, although a decrease was seen in FaDu cells after treatment with 50 μM erufsine for the reason which remains unknown. In addition to p-mTOR, erfosine de-phosphorylated other important components of the mTORC1 complex such as p-PRAS40 and p-Raptor in a dose-dependent manner. This resulted in the efficient de-phosphorylation of the downstream substrates of mTORC1 such as p-4EBP1 and p70S6K.

To explore the possible benefits of combination therapy, we compared erfosine with cisplatin and 5-FU and demonstrated an additive effect of the combination. Erufosine therefore might be combined with DNA-targeting drugs to enhance their anticancer effects and overcome resistances to standard therapeutics in patients with multiple prior therapy or progressive disease. Taken together, our findings strongly suggest that erfosine acts on multiple targets in oral cancer cells such as apoptosis, autophagy and cell cycle regulators, Akt, mTOR, PRAS40, and Raptor. Thus, it appears to target the downstream substrates of mTORC1, p70S6K and p-4EBP1.

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Disclosure of potential conflicts of interest: No potential conflicts of interest are disclosed.

Appendix A. Supplementary material


References

[12] M.R. Berger, I. Tsoneva, S.M. Konstantinov, H. Eibl, Induction of apoptosis by erufsine-\[42\]. Similarly, another potent inhibitor of Akt, a phosphotyldinositol ether lipid analog has been shown to differentially regulate genes involved in apoptosis, wound response and angiogenesis (upregulation) and those involved in DNA replication, repair and mitosis (downregulation) in non-small cell lung carcinoma cells [44]. These observations point to the importance of other molecular targets of alkylphosphocholines.

In this study, we observed that p-mTOR was also de-phosphorylated at Ser2448, which is one of the most critical residues for catalytic activity of mTOR. However, total mTOR levels were unaffected in Cal27, SCC9 and SCC25 cells, although a decrease was seen in FaDu cells after treatment with 50 μM erufsine for the reason which remains unknown. In addition to p-mTOR, erfosine de-phosphorylated other important components of the mTORC1 complex such as p-PRAS40 and p-Raptor in a dose-dependent manner. This resulted in the efficient de-phosphorylation of the downstream substrates of mTORC1, p70S6K and p-4EBP1.

To explore the possible benefits of combination therapy, we compared erfosine with cisplatin and 5-FU and demonstrated an additive effect of the combination. Erufosine therefore might be combined with DNA-targeting drugs to enhance their anticancer effects and overcome resistances to standard therapeutics in patients with multiple prior therapy or progressive disease. Taken together, our findings strongly suggest that erfosine acts on multiple targets in oral cancer cells such as apoptosis, autophagy and cell cycle regulators, Akt, mTOR, PRAS40, and Raptor. Thus, it appears to target the signal pathways, which are often disturbed in tumor cells and this is combined with other advantageous properties like lack of myelosuppression. Thus, it appears to target the signal pathways, which are often disturbed in tumor cells and this is combined with other advantageous properties like lack of myelosuppression. Thus, it appears to target the signal pathways, which are often disturbed in tumor cells and this is combined with other advantageous properties like lack of myelosuppression. Thus, it appears to target the signal pathways, which are often disturbed in tumor cells and this is combined with other advantageous properties like lack of myelosuppression. Thus, it appears to target the signal pathways, which are often disturbed in tumor cells and this is combined with other advantageous properties like lack of myelosuppression.

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Appendix A. Supplementary material


References


