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ORIGINAL ARTICLE

Targeted disruption of PI3K/Akt/mTOR signaling pathway, via PI3K inhibitors, promotes growth inhibitory effects in oral cancer cells

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Abstract

Purpose The phosphoinositide-3-kinase (PI3K) pathway is the frequently altered in human cancer. This has led to the development and study of novel PI3K inhibitors for targeted therapy and also to overcome resistance to radiotherapy.

Method The anti-tumour effects of PI3K inhibitors (PI-828, PI-103 and PX-866) in terms of cell proliferation, colony formation, induction of apoptosis, cell cycle arrest, invasion, autophagy, and pNF-κB/p65 translocation in SCC-4, SCC-9 and SCC-25 cells were studied by performing MTT, clonogenic, DAPI staining, propidium iodide staining, annexin-V binding, matrigel invasion, acridine orange staining and immuno-fluorescence assay. Western blot assay was performed to assess the alteration in the expression of various proteins.

Result PI-828 and PI-103 treatment exhibited dose-dependent inhibition of growth and proliferation of OSCC cells with a concomitant induction of apoptosis, altered cell cycle regulation and decreased invasiveness ($p < 0.01$). PX-866 induced apoptosis, cell cycle arrest, autophagy and a significant decrease in the invasiveness of oral cancer cells as compared to untreated cells ($p < 0.01$). These compounds significantly reduced expression of COX-2, cyclin-D1 and VEGF in the treated cells besides cytoplasmic accumulation of pNF-κB/p65 protein. In addition to PI3Kα, inactivation of downstream components, i.e. Akt and mTOR was seen.

Conclusion PI3K inhibitors such as PI-103, PI-828 and PX-866 may be developed as potential therapeutic agents for effective treatment of oral squamous cell carcinoma (OSCC) patients, associated with activated PI3K/Akt pathway.

Keywords Oral cancer · PI3K signaling · PI3K inhibitors · PI-103 · PI-828 · PX-866 · Apoptosis · NF-κB · Autophagy

Sadhna Aggarwal and Sarah John: equal contribution as first author.

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Introduction

Oral cancer is the third most common cancer in India with 75,000–80,000 new cases being reported every year [1]. There is a significant difference in the incidence rates of oral cancer around the world, with the age-adjusted rates varying from over 20 per 100,000 in India, to 10 per 100,000 in the USA and less than 2 per 100,000 in the Middle East [2]. The death rate associated with oral cancer has always remained very high due to the lack of early stage detection and poor prognosis leading to disease recurrence. The 5-year survival rate (75.68%) of oral cancer patients has not changed in the past few decades. It has been reported to be the most common and fatal cancer in the Indian males [3].

Balanced regulation and appropriate coordination of the signaling pathways are required for normal cellular physiology. Any turbulence in these important signalling pathways may cause uncontrolled growth of cells leding to cancer. PI3K/Akt pathway is one of the signaling pathways that are

found to be frequently disturbed in human cancers [4]. This pathway results in the complete activation of the Akt protein (protein kinase B) which further modulates the wide range of cellular functions of numerous substrates involved, like transcription, survival, angiogenesis and apoptosis [5, 6]. PI3K pathway is the most frequently (11–30%) mutated pathway in HNSCC patients. In fact, PIK3CA or PIK3R1 was shown to be the only mutated gene in a group of HPV + tumours [7]. Many germline (H1047R) or somatic (E542K, E545K and Y343C) mutations have been reported in the exon 7 of this gene [4, 8]. Mutations in Exon 9 (E545K) and Exon 20 (H1047Q, H1047Y and H1048Q) have also been reported in south Indian population [9]. It appears that the mutational analysis of PI3K pathway can serve as the predictive biomarker for the personalized therapies for OSCC patients [7, 8, 10, 11]. Also, it is the proof of the fact that targeting PI3K pathway via its inhibitors may offer highly effective strategy to treat substantial group of OSCC patients that has hyper active PI3K pathway. The PI3K inhibitors, therefore, may impede aberrant alterations in this pathway, leading to the arrest of cellular growth and cell cycle progression in oral cancer cells.

The presently used anticancer drugs aim non-selective cytotoxicity by inducing apoptosis of cancer cells. The chemotherapeutic drugs that are most often used for oral cavity and oropharynx cancers are cisplatin, carboplatin, 5-fluorouracil (5-FU), paclitaxel (Taxol®), docetaxel (Taxotere®), hydroxyurea and other drugs are used less often (methotrexate, bleomycin and capecitabine). These may be used either alone or in combination, depending on the stage and location of the tumour [12]. These drugs show generalized toxicity to almost all dividing cells, hence show enhanced toxicity to normal cells as well. Targeted therapy for oral cancer with epidermal growth factor receptor (EGFR) monoclonal antibodies, EGFR tyrosine kinase inhibitors, VEGFR inhibitors and various other immune-checkpoint inhibitors; is still at phase II or III clinical trials, represents relatively newer and costlier concept, that needs further research to confirm its clinical efficacy [13, 14].

Lately, it has been observed that the cancer cells develop the resistance to apoptosis due to their clonogenic survival signals being provided by the PI3K/Akt pathway. Therefore, PI3K/Akt pathway has become an attractive target for drug development. PI3K inhibitors are of great interest, as they can be used as combinatorial therapy to effectively inhibit all the abnormal downstream signaling which is required for cancer cell survival and growth. The PI3K targeted (pan PI3K, PI3Kα/β/γ or PI3K/mTOR) therapies like LY3023414, GSK2636771, Alpelisib (BYL719), Buparlisib (BKM120), PX-866, Copanlisib and INCB050465 are already under phase I/II/III clinical trials in combination with other chemo, radio or immuno therapies for effective treatment of oral cancer patients [15–17].

The present literature favors the fact that oral cancers are an exciting setting for PI3K pharmacologic intervention. Hence, the rationale of our study was to observe the anti-tumour effects of PI3K inhibitors (PI-103, PI-828 and PX-866) on three different oral cancer cell lines. PI-103 is a cell permeable inhibitor of PI3K isoforms that has been reported to block PI3K signaling in glioma cells via its ability to inhibit both PI3Kα and mTOR [18]. PX-866 is a ring-opened analogue of wortmannin that potently and irreversibly inhibits PI3K. It targets p110α, p110δ and p110γ with single-digit nanomolar IC50 values [19]. PI-828, an efficient variation of LY294002, can be immobilized on a solid phase and is a highly potential PI3K inhibitor.

Materials and methods

Cell culture and maintenance

Human oral squamous cell carcinoma (OSCC) cell lines, SCC-4 (American Type Culture Collection; ATCC, Manassas, VA, USA), SCC-9 and SCC-25 (DKFZ, Germany; authenticated by DSMZ, Braunschweig, Germany) were used for the experiments [20]. The cells were maintained in DMEM supplemented with 10% (v/v) FBS, and antibiotics (100 units/ml Penicillin, 100 mg/ml Streptomycin, 50 units/ml Gentamycin) in 25 or 75 cm^2 tissue culture flasks at pH 7.4, 37 °C, 5% CO_2 and 95% relative humidity.

Tumour cell growth inhibition by MTT assay

PI-828, PI-103 and PX-866 were dissolved in dimethylsulphoxide (DMSO), which were further diluted in media as needed. MTT [3,4–(4,5-dimethylthiazol-2-yl)-2, 5-di-phenyl tetrazolium bromide] assay was performed to determine IC50 value of the PI3K inhibitors at 24, 48 and 72 h of treatment in all the cell lines. Briefly, 5000 cells/well (SCC-4, SCC-9 and SCC-25) were plated in 96-well plates and incubated overnight at 37 °C in the CO_2 incubator. The cancer cells with similar percentage of DMSO in the media were devised as the control well for each cell line. The following day, tumour cells were treated with various concentrations of PI-828, PX-866, PI-103 and cisplatin (2 µg/ml) in triplicate for 24, 48 and 72 h. After incubation, 0.5 mg/ml MTT solution was added and the cells were re-incubated for 3 h in the dark at 37 °C. The purple-colored formazan crystals thus formed were solubilized in DMSO and the absorbance was measured at 570 nm using spectrophotometer. % Viability was calculated as mentioned below:

$$\% \text{ Viability} = \left(1 - \text{OD}_{\text{treated}}/\text{OD}_{\text{untreated}}\right) \times 100.$$

All subsequent experiments were performed using respective IC50 doses.

Clonogenic cell survival assay

The clonogenic survival assay was performed to determine the ability of oral cancer cells to grow indefinitely and independently, thereby forming a large colony from a single cell as described earlier [20]. The data were represented as percentage of colony-forming unit (CFU).

$$\%\ \text{CFU} = (\text{Number of colonies formed after drug treatment} / \text{Number of colonies formed in untreated well}) \times 100.$$

Cell cycle analysis by propidium iodide (PI) labeling and flow cytometry

To study the effects of PI3K inhibitors on cell cycle regulation in oral cancer cells, the % cell in different phases of cell cycle was measured by labeling the cells with nucleic acid specific propidium iodide (Sigma-Aldrich, USA) followed by flow cytometric analysis as described before [21]. The distribution of cells in different phases of the cell cycle (G_0/G_1, S and G_2/M) was determined using ModFit cell cycle analysis software (Becton Dickinson, San Jose, CA, USA).

Assessment of apoptosis by Annexin-V/PI staining assay

Cells (10^6 cells/per well) were seeded in 6-well plate and incubated for 18 h, 37 °C in a 5% CO_2 incubator. Then, cells were treated with IC50 value of PI-828, PI-103 PX-866 and cisplatin for 48 h and processed as described earlier [21]. The data were analyzed using BD FACSDiva™ software (BD Biosciences, CA, USA).

Characterization of apoptosis by DAP-I staining

The effect of PI3K inhibitors on apoptosis of oral cancer cells was also characterized using a 4-6-diamidino-2-phenylindole (DAP-I) staining assay. Briefly, cells were treated with IC50 doses of drugs and incubated for 48 h, 37 °C in a 5% CO_2 incubator. The cells were washed twice with cold 1XPBS and fixed with 2% para-formaldehyde (PFA) for 10 min. Further, the cells were stained with DAP-I (300 nM) for 30 min at room temperature in dark, washed and mounted. Slides were then observed under fluorescence microscope (Nikon, Japan) at 40 × magnification.

Assessment of the invasive ability of cells by BD BIOCOAT™ matrigel cell invasion assay

100 µl of the diluted matrigel (1:5 ratios in serum free-cold cell culture DMEM) was added onto 8 µM PET membrane insert that was placed in 24-well plate. The plates were incubated at 37 °C for 4–5 h at 37 °C. The drug-treated cells were harvested from cell culture flasks, washed with DMEM and seeded (5×10^4 cells/100 µL) onto the matrigel. Lower chamber was filled with 500 µL of complete media (with 10% fetal bovine serum) as a chemo attractant for the cells. Plates were then incubated at 37 °C for 22–24 h and the non-invading cells were removed from the upper surface using a cotton swab. The insert was fixed with methanol: acetic acid (3:1) mixture and then stained with 0.5% crystal violet. The invaded cells were counted under inverted microscope and percentage invasion was calculated as below:

$$\%\ \text{Invasion} = (\text{No. of cells invaded in drug treated well} / \text{No. of cells invaded in untreated well}) \times 100.$$

Immunofluorescence assay

The tumour cells were treated with drugs (IC50) for 48 h, 37 °C in 5% CO_2 incubator. An indirect immunofluorescence assay was performed to localize pNF-κB/p65 (nuclear factor kappa-light-chain-enhancer of activated B cells) as described earlier, using anti-pNF-κB/p65-rabbit (Cell Signalling Technology, Boston, MA, USA) and anti-rabbit-I_gG(H + L)-Alexa Fluor®488 (Cell Signalling Technology, Boston, MA, USA) antibodies [21].

Detection of autophagy by acridine orange staining

The seeded cells were allowed to adhere on a 12-well plate overnight. On the following day, cells were treated with IC50 dose of PI-103 and cisplatin for 48 h and then stained with 1 µg/ml acridine orange for 15 min. PBS washed cells were then fixed with 4% PFA, mounted and examined under a fluorescence microscope (ZEISS Axio Imager 2, Zeiss, Germany). The autophagic cells were characterized by bright green and dim red fluorescence in the cytoplasm and nucleus, respectively, and ultra-bright red fluorescence in the acidic vacuoles.

Western blotting and enhanced chemiluminescence

Alteration in expression of few proteins, post PI-103 treatment, was studied in SCC-4 cells using western blot analysis as described earlier [22]. The primary antibodies Cyclin D1 and VEGFa were obtained from Abcam, Cambridge, UK, while rest of the antibodies were obtained from Cell Signaling Technology, Boston, MA, USA [21–23].

Statistical analysis

The statistical significance of the data was determined by Student's *t*-test using GraphPad PRISM version 6.0 (Qiagen)

and the results were expressed as the mean ± SD, unless indicated otherwise.

Results

PI3K inhibitors induced dose-dependent cytotoxicity in oral cancer cells

MTT assay revealed the dose-dependent cytotoxicity in all the three oral cancer cell lines after PI-103 and PI-828 drugs treatment (Fig. 1). IC50 dose for PI-103 was found to be 1.8 µg/ml or 5.17 µM in SCC-4, 1.9 µg/ml or 5.54 µM in SCC-9 and 1.2 µg/ml or 3.44 µM in SCC-25 cells. Similarly, for PI-828 calculated IC50 dose was 5.625 µg/ml or 17.44 µM in SCC-4, 7.5 µg/ml or 23.26 µM in SCC-9 and 10 µg/ml or 31.02 µM in SCC-25 cells at 48 h. We have used previously determined IC50 values (0.4 µM and 0.8 µM) of PX-866 drug in all subsequent experiments [24].

The cytotoxic effects of these drugs were also evaluated on peripheral blood mononuclear cells (PBMCs) from healthy volunteers. It induced negligible cytotoxicity (< 5%) in treated cells as compared to untreated control. The morphological changes indicating cell growth inhibition were also observed microscopically, i.e. smaller size, irregular shape, detachment from base of culture flask and membrane blebbing (data not shown).

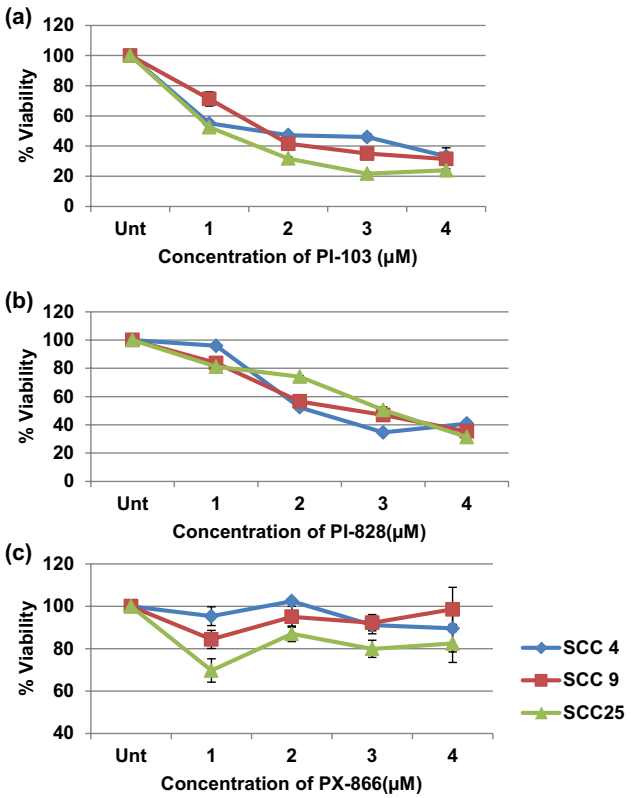

Fig. 1 Representative line graph showing effects of **a** PI-103, **b** PI-828 and **c** PX-866 on oral cancer cell proliferation by MTT assay. PI-103 and PI-828 treatment showed dose dependent inhibition of tumour cells, whereas PX-866 treatment failed to show dose-dependent cytotoxicity

PI3K inhibitors abrogated the clonogenic potential of oral cancer cells

The anti-proliferative activity of PI3K inhibitors (using the IC50 derived by MTT assay) on tumour cell growth was determined by in vitro clonogenic assay. A significant reduction in colony forming units (CFU) was observed in SCC-4, SCC-9 and SCC-25 cells after treatment with PI-828, PI-103 and PX-866 (Supplementary Fig. 1).

PI-103 inhibited colony growth from 100 to 4.4%, 5.6% and 2% in SCC-4, SCC-9 and SCC-25 cells, respectively. PI-828 inhibited colony growth from 100 to 25.2%, 6.8% and 22.2% in SCC-4, SCC-9 and SCC-25 respectively. PX-866 (0.4 µM) inhibited colony growth from 100 to 53.2%, 37.2% and 49% in SCC-4, SCC-9 and SCC-25 respectively. Similarly, PX-866 (0.8 µM) inhibited colony growth from 100 to 54%, 22.6% and 40% in SCC-4, SCC-9 and SCC-25, respectively. PI-103 induced the maximum inhibition of the colony formation by OSCC cells followed by PI-828 and PX-866, respectively.

PI3K inhibitors induced cell cycle arrest in oral cancer cells

PI-103 treatment resulted in enhanced accumulation of cells in the S phase, i.e. from 9.23 to 42.67%, 30.54–49.75% and 25–27.64% in SCC-4, SCC-9 and SCC-25 cells, respectively. PI-828 induced G0/G1 phase arrest in SCC-4 (75.4–86.23%) and SCC-9 (58.81–64.13%) cells. However, PX-866 showed G2/M phase arrest in SCC-4 (15.37–74.26%) and S phase arrest in SCC-9 (25–36.69%) and SCC-25 (25–31.02%) cell lines (Fig. 2).

PI3K inhibitors induced apoptotic cell death in oral cancer cells

The effects of these PI3K inhibitors were assessed on tumour cells by annexin-V binding assay as shown in Fig. 3. PI-103 treatment induced early-phase apoptosis from 1.9 to 13.3%, 7.6–33.6% in SCC-4, SCC-9 and SCC-25 cells, respectively, and in the late apoptotic phase from 0.8 to 4.1%, 3.3–56.7% and 5.4–13.4% in SCC-4, SCC-9 and SCC-25, respectively. Similarly, PI-828 treatment also induced early (10.6–43.3%, 7.5–27.1% and 42.4–44.3% in SCC-4, SCC-9 and SCC-25, respectively) and late (from 8.1 to 23.3%, 2.8–4.4% and 32.1–44.4% in SCC-4, SCC-9 and SCC-25, respectively) phase apoptosis. However, PX-866 treatment induced early

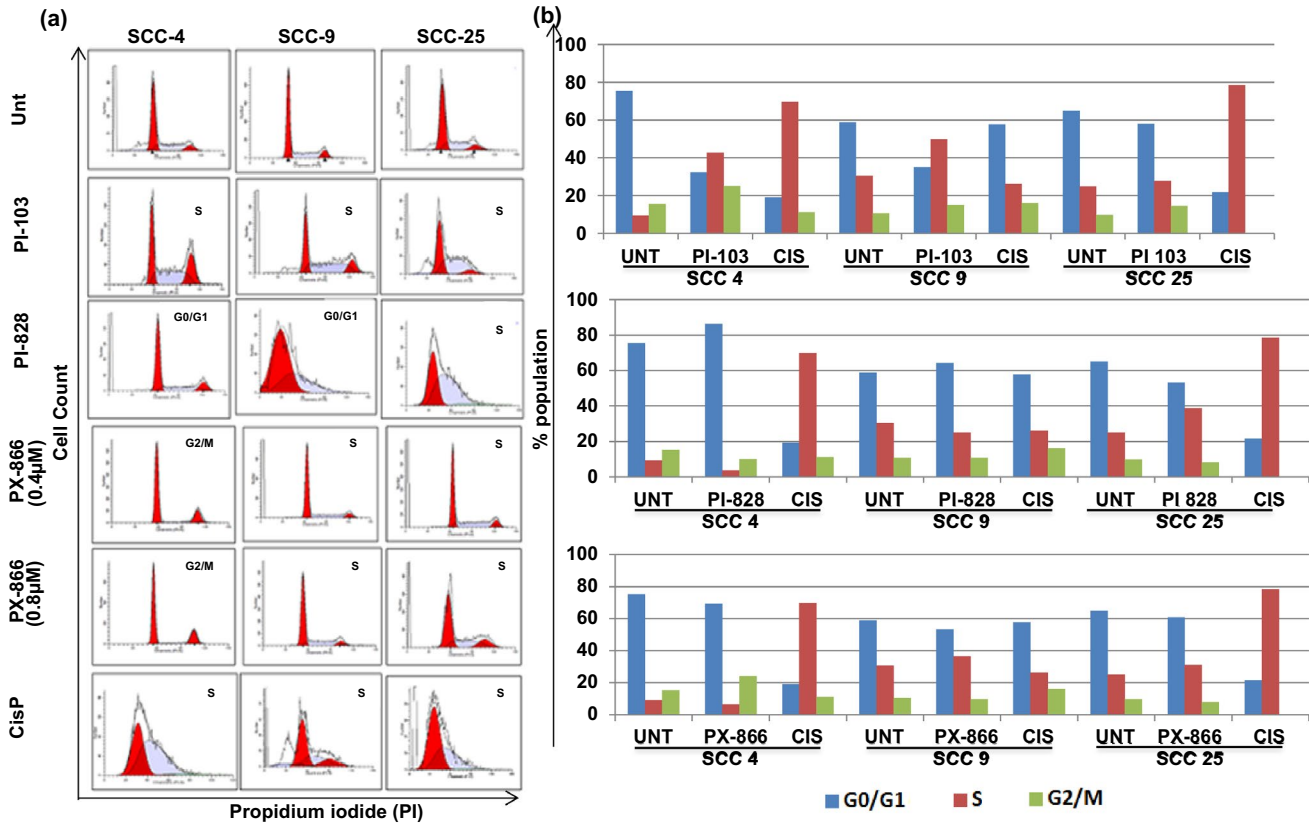

Fig. 2 Effects of PI-103, PI-828 and PX-866 treatment on cell cycle regulation of oral cancer cell lines. **a** Representative histograms showing alteration in percentages of cells in different phases of cell cycle after treatment with PI-103, PI-828 and PX-866. **b** Bar graphs showing the percentage (mean ± SD) of cells in each phase indicating an evident S-phase in all oral cancer cell lines. The IC50 doses of PI-103 (5.17 μM in SCC-4, 5.54 μM in SCC-9 and 3.44 μM in SCC-25 cells), PI-828 (17.44 μM in SCC-4, 23.26 μM in SCC-9 and 31.02 μM in SCC-25 cells) and PX-866 (0.4 μM and 0.8 μM) were used for the experiment

phase apoptosis only in SCC-4 (1.9–27.1%) cells and the late apoptotic phase apoptosis was induced in all the three OSCC cells, i.e. SCC-4 (0.8–4.1%), SCC-9 (3.3–4.2%) and SCC-25 (5.4–8.5%) cells.

SCC-4 cells exhibited the characteristic features of apoptotic nucleus after DAP-I staining on treatment with PI-103, PI-828 and PX-866, i.e. Smaller in size and more fragmented as compared to the nucleus of untreated cells (Supplementary Fig. 2).

PI3K inhibitors restricted cell migration and invasion of oral cancer cells

The effects of PI-103, PI-828 and PX-866 treatment on invasiveness and metastatic ability of SCC-4, SCC-9 and SCC-25 cells were studied by matrigel assay (Fig. 4). The migration rate of SCC-4 cells through matrigel decreased to 12.12%, 47% and 27% after PI-103, PI-828 and PX-866 treatment, respectively. Similarly, the migration rates of SCC-9 cells were declined to 16%, 22% and 38% after PI-103, PX-866 and PI-828 treatment, respectively. Also, the migration rate of SCC-25 cells was observed to have dropped 15%, 33% and 18% after PI-103, PI-828 and PX-866 treatment, respectively.

PI3K inhibitors induced cytoplasmic translocation of pNF-κB/p65 in oral cancer cells

The intracellular immunofluorescence staining revealed altered expression of pNF-κB/p65 in nucleus and cytoplasm in PI-103-, PI-828- and PX-866-treated OSCC cells as compared to untreated controls. The results showed the higher expression of pNF-κB/p65 in the nucleus of untreated cells; however, the drug treatment-induced cytoplasmic accumulation of pNF-κB/p65 in oral cancer cells revealing inhibition of protein translocation and its subsequent degradation (Fig. 5a).

It was further observed that PI3K inhibitor (PI-103) also induced autophagy in SCC-4 cells (Fig. 5b).

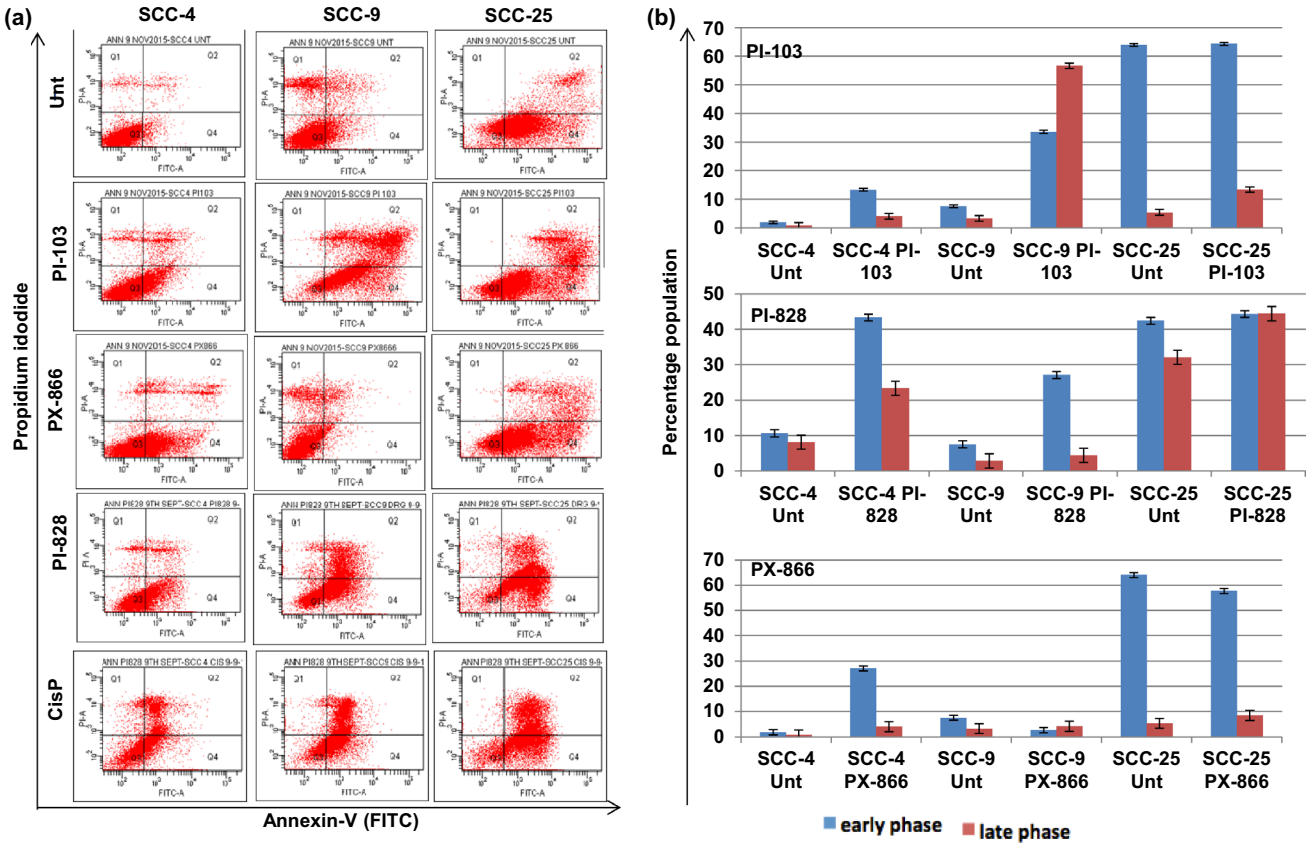

Fig. 3 Annexin-V/PI staining assay showing apoptotic effects of PI-103, PI-828 and PX-866 treatment on SCC-4, SCC-9 and SCC-25 oral cancer cells: **a** the percentage distribution of cells in Quad I (top left): necrotic (annexin-V FITC −/PI +); Quad II (top right): late apoptotic cells (annexin-V FITC +/PI +); Quad III (bottom left): live cells (annexin-V FITC +/PI −); Quad (bottom right): early apoptotic cells (annexin-V FITC +/PI −); **b** increase in the early, late and total apoptotic cells (mean ± SD) after treatment. The % cell population in early and late phases of apoptosis has been shown in blue and red bars respectively

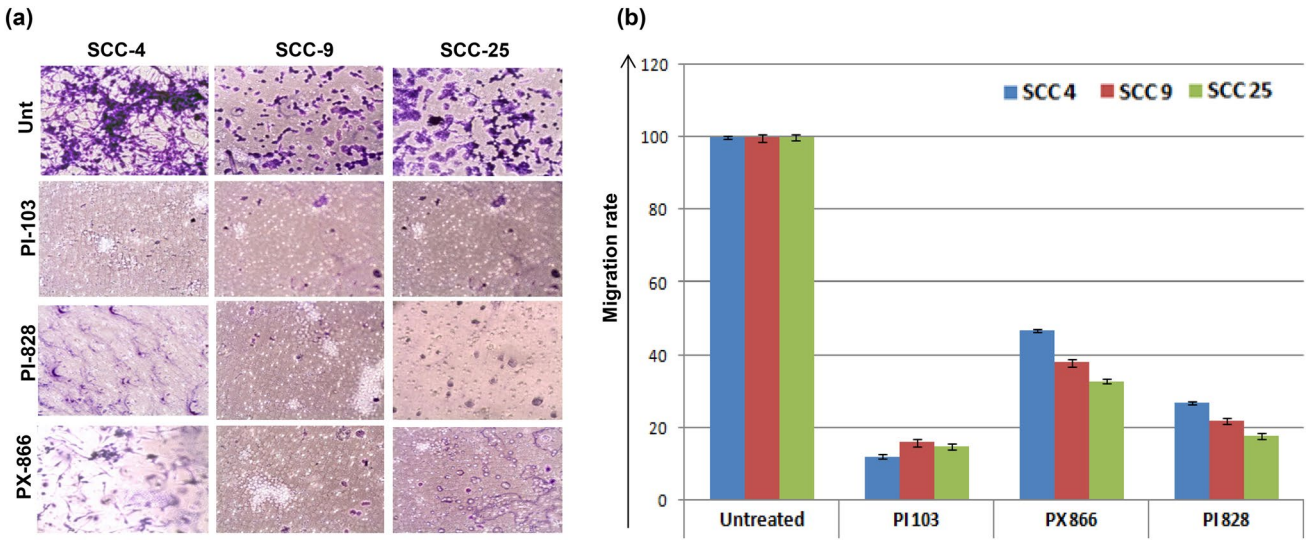

Fig. 4 Matrigel assay showing the effects of PI-103, PI-828 and PX-866 treatment on invasion and migration ability of oral cancer cells: **a** representative picture of migrated tumour cells after crystal violet staining. The experiment was performed in duplicates and three fields were counted for each chamber. **b** Bar graph showing decrease in percentage invasion (mean ± SD) after PI-103, PI828 and PX866 treatment in SCC-4, SCC-9 and SCC-25 cells

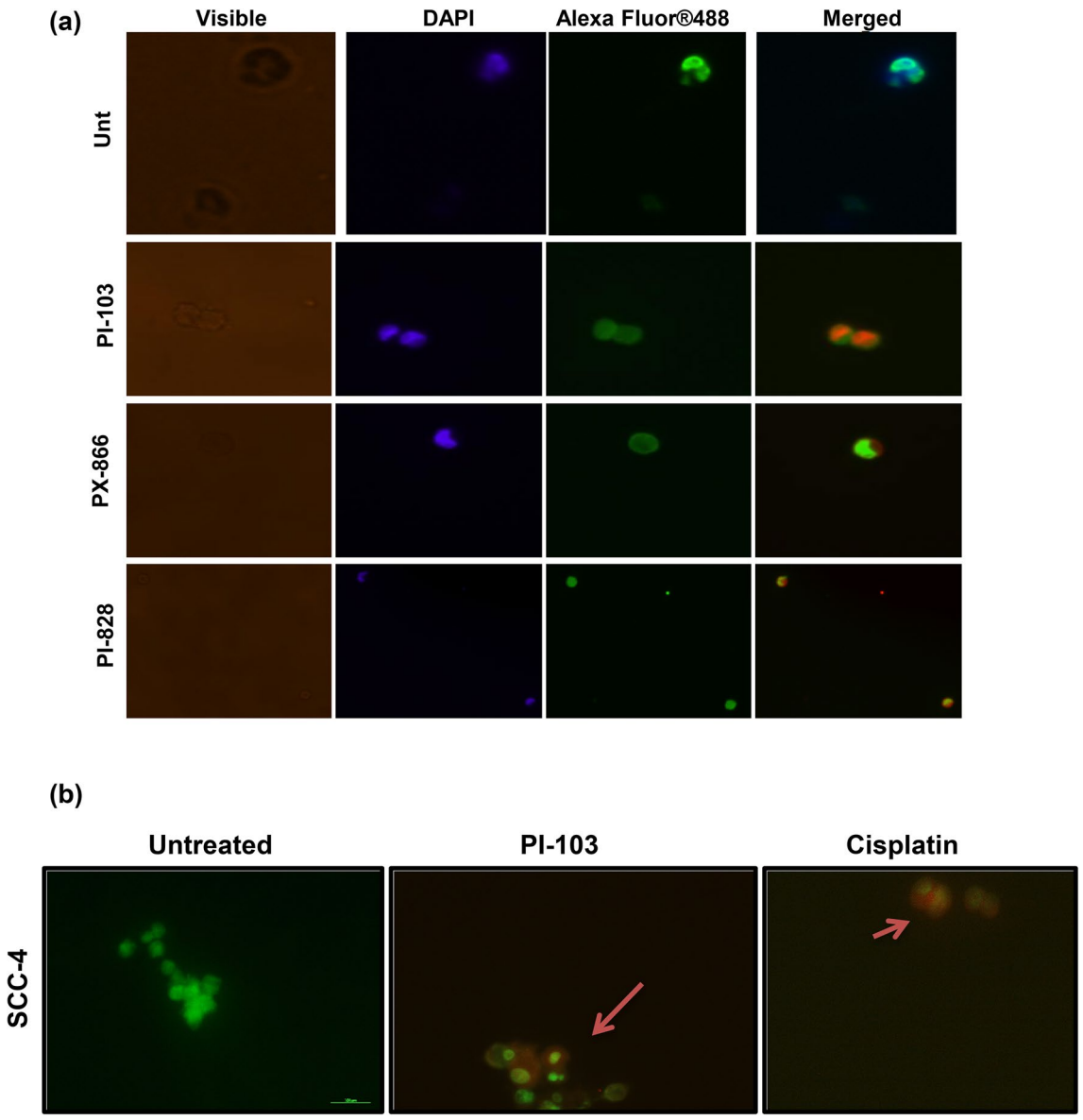

Fig. 5 Representative photomicrographs showing **a** localization of pNF-κB/p65 protein in SCC-4 cells, after PI-103, PI-828 and PX-866 treatment [blue: DAPI stain (nucleus), green: Alexa Fluor®488 (pNF-κB/p65 protein)]. **b** Induction of autophagy after PI-103, PI-828 and PX-866 treatment in acridine orange-stained SCC-4 cells. Arrows indicates the presence of bright red fluorescing autophagic vacuoles in the cell cytoplasm

PI3K inhibitor (PI-103) induced altered expression of various important proteins in oral cancer cells

Since the PI3K inhibitors showed the significant cytotoxicity in all OSCC cells, a representative experiment was performed to observe its effects on the expression of some regulatory proteins in SCC-4 cells after treatment with PI-103 using western blot assay. The results are shown in Fig. 6a, b. The expression levels of VEGF, Bcl-2, NF-κB and COX-2 proteins, but beclin-1, were observed to be downregulated in the oral cancer cells after PI-103 treatment as compared to the untreated cells. Blots also revealed the decreased expression of signaling proteins, i.e. P110α, Pan-Akt, total mTOR and p-mTOR proteins after PI-103 treatment in SCC-4 cells.

Discussion

Increasing incidence of oral cancer still remains a matter of serious concern around the world, especially in Asian countries. The mutations in the class IA PI3K catalytic subunit, homozygous deletion PTEN and '*Akt*' overexpression,

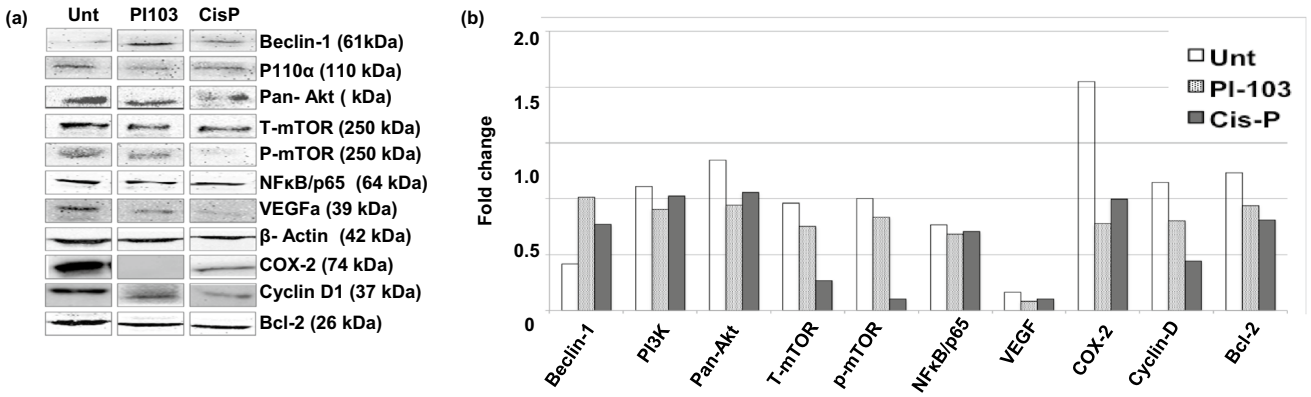

Fig. 6 Altered expressions of various important proteins SCC-4 cells after PI-103, PI-828 and PX-866 treatment. **a** Western blots showing alteration in expression of various important proteins and β-actin in SCC-4 cells lines after PI-103 (IC50) treatment. **b** Densitometric analysis of the blots showing fold change expression of test proteins to β-actin. (*p value < 0.05)

etc. correspond to carcinogenesis [25–30]. Therefore, the increasing evidence of the deregulation of PI3K signaling pathway in cancers suggests it to be a lucrative target for cancer drug discovery and also to overcome chemo and radiotherapy resistance. In breast cancers with *PIK3CA* mutations, PI3K pathway inhibitors seem to have single-agent activity in breast cancers with *ERBB2* amplifications [31]. This suggests that these cancers rely on the PI3K signaling pathway for their establishment and spread. In addition, when breast cancers with *ERBB2* amplifications become resistant to anti-ERBB2 therapies, they still seem to require PI3K signaling for growth and survival [32]. Therefore, there is enthusiasm for the development of PI3K/Akt/mTOR inhibitors for the treatment of cancers. Some of the earliest PI3K inhibitors include wortmannin and LY294002 [33, 34]. The PI3K inhibitors used in the present study include PI-103, PX-866 and PI-828.

We found that PI-103 and PI-828 exhibited dose-dependent cytotoxic effect on SCC-4, SCC-9 and SCC-25. Similarly, many studies showed anti-proliferative effects of PI3K inhibitors in several cancers [35, 36]. We also observed that PX-866 treatment did not induce any significant cytotoxicity in the oral cancer cell lines. However, Ihle et al. 2005 reported concentration of PX-866 cytotoxicity (IC_{50} = 0.1–3 nm) in non-small cell lung cancer xenografts [19]. Another study showed cytotoxic effects of PX-866 treatment (0.4 µM and 0.8 µM) with respect to inhibition of invasion, induction of apoptosis and mediation of cell cycle arrest in glioblastoma cells [24]. We observed that PI-103 exhibits highest cytotoxicity on the cell lines when compared to PI-828 and PX-866. In concordance with MTT assay, PI-103, PI-828 and PX-866 significantly reduced colony growth formation in oral cancer cells. PI-103 and PX-866 have been reported to arrest the cell cycle in G0/G1 phase [37] and G1 phase [24] in glioma cells, respectively. We observed that the PI-103 induced S-phase and PX-866 induced S- and G0/G1 phase arrest in cell cycle of oral cancer cells leading to delayed mitotic cycle. Analyzing the alteration in expression of cyclin-D1 protein in drug-treated lysates of OSCC cells further substantiated the results obtained from cell cycle analysis. PI-103, PI-828 and PX-866 treatment induced downregulation of cyclin-D1 expression in oral cancer cells.

There are three different pathways by which cells can undergo death: apoptosis, autophagy and necrosis. Here, we observed that the PI3K inhibitor treatment induced apoptosis in oral cancer cells as evaluated microscopically, by AnnexinV/PI and DAPI staining. PI-103 and PI-828 were found to be more potent in inducing apoptosis than PX-866. Earlier studies report the PI3K inhibitor treatment induced apoptosis in tumour cells, e.g. PI-103 in leukemia [38] and PX-866 in glioblastoma cells [39]. Apoptotic effects of PI3K inhibitors on oral cancer cells were further confirmed by observing the downregulation of an anti-apoptotic protein, Bcl-2. Bcl-2 serves as a guardian of outer mitochondrial membrane, in preserving its integrity by opposing Bax and Bak [40].

Tumour cells with constitutively activated PI3K/AKT/mTOR pathway have been shown to inhibit autophagy and promoting their growth and survival [41]. In addition to apoptosis, PI-103 also induced autophagy in SCC-4 cells as observed by acridine orange staining and increased the expression of beclin-1 protein that promotes autophagy. Another study has shown induction of autophagy after PI-103 treatment in SF268 glioblastoma cells [42].

Inhibition of NF-kB/p65 has been found to be associated with the characteristics of an apoptotic cell death. Introduction of NFκB (Bay11-7085) and PI3K (LY294002) survival pathway inhibitors have been reported to decrease expression of p65, induce dephosphorylation of AKT and synergistic apoptotic response in primary effusion lymphoma

(PEL) cells [43]. This concurrent activation or inhibition of PI3K and NF-κB components or activity strongly suggests the crosstalk between these two survival pathways [43, 44]. Here, we observed that the exposure of oral cancer cells to PI3K inhibitors did not only down regulated the expression of phosphorylated-NF-κB/p65 protein, but also inhibited its nuclear translocation. Syed's group in 2006 also showed that cigarette smoke results in increased expression of pNF-κB/p65, which prevents apoptosis by mediating cell survival signal [45].

PI-103, PI-828 and PX-866 exhibited significant decrease in the invasive ability of the oral cancer cell lines. Inhibition of invasion by PI3K inhibitors has been reported previously in glioblastoma cell [24, 39]. Again, PI-103 was found to be the most effective inhibitor of invasion and metastatic ability of the oral cancer cells.

Vascular endothelial growth factor (VEGF) in cancer induces angiogenesis, increases vascular permeability and it also contributes to the various other key aspects of tumourigenesis [46–48]. We have previously reported the elevated levels of VEGFa in peripheral circulation and at the tumour site of OSCC patients [49]. Investigating the altered VEGFa protein expression in SCC-4, SCC-9 and SCC-25 cells further substantiated the results obtained from the matrigel assay. VEGFa expression was down regulated in all the three cell lines after treatment with PI-103, PI-828 and PX-866.

Cyclooxygenases (COX-1 and COX-2) are the enzymes involved in the synthesis of prostaglandins, which are mediators of inflammation and have been implicated in carcinogenesis [50]. Previous study from our lab has reported over expression of COX-2 in oral cancer patients and that the peptide-mediated COX-2 inhibition resulted in growth inhibition in oral cancer cell lines [51]. The present study affirms the previous results as the COX-2 expression was down regulated in all the oral cancer cell lines after PI-103, PI-828 and PX-866 treatment.

Somatic mutations in PI3KCA have been identified in a variety of human tumours, including breast, colon, and endometrial cancers and glioblastomas [25]. We have previously shown threefold increase in circulating PI3K110α in OSCC patients as compared to the healthy subjects that also positively correlated with the disease progression [52]. Activated Akt further phosphorylates mTOR at serine 2448. This critical phosphorylation reportedly causes activation of mTORC1, which further signals for growth and protein synthesis by activating proteins like PRAS40, 4EBP1, etc. [53]. In the current study, PI3K inhibitor resulted in the reduced expression of Pan-Akt (Akt1, Akt2 and Akt3) in SCC-4 cells. PI-103 also reduced the expression of Total-mTOR (mTORC1 and mTORC2) and phosphorylated-mTOR in the oral cancer cells. It indicates that PI3Ka inhibitor also binds to and disrupts the various other downstream components of the pathway to achieve tumour growth inhibition.

Our study depicts that targeting PI3K/Akt signalling by specific PI3Kinase inhibitor, i.e. PI-103, PI-828 or PX-866 may lead to the discovery of an effective targeted therapy for treatment of oral cancer patients in combination with other available chemotherapies. It appears that the PI3K inhibitors show anti-tumour activity by inhibition of inflammation, cell cycle arrest, angiogenesis and enhancement of apoptosis in oral squamous cell carcinoma. Hence, PI-103, PI-828 and PX-866 may be developed as potential therapeutic agent for treatment of OSCC patients associated with hyper activation of the PI3K/Akt pathway.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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