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Centchroman inhibits proliferation of head and neck cancer cells through the modulation of PI3K/mTOR Pathway

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ABSTRACT

Centchroman (CC; 67/20; INN: Ormeloxifene) is a non-steroidal antiestrogen extensively used as a female contraceptive in India. In the present study, we report the anti-proliferative effect of CC in head and neck squamous cell carcinoma (HNSCC) cells. CC inhibited cell proliferation in a dose dependent manner at 24 h of treatment. Further studies showed that CC treatment induced apoptosis, inhibited Akt/mTOR and signal transducers and activators of transcription protein 3 (STAT3) signaling, altered proteins associated with cell cycle regulation and DNA damage and inhibited colony forming efficiency of HNSCC cells. In addition, CC displayed anti-proliferative activity against a variety of non-HNSCC cell lines of diverse origin. The ability of CC to serve as a dual-inhibitor of Akt/mTOR and STAT3 signaling warrants further studies into its role as a therapeutic strategy against HNSCC.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer accounting for over 500,000 new cases annually worldwide [1]. Despite the recent advances in diagnostic methods and improvement in treatment strategies, the prognosis of HNSCC patients in advanced stages remains largely unsatisfactory [2]. Simultaneous deregulation of multiple signaling pathways like PI3K/Akt/mTOR and STAT3 are often implicated in the pathogenesis of HNSCC [3–8]. Therefore the need for expedited development of effective HNSCC therapies is critical. At present major emphasis is being laid on designing new therapeutic strategies targeting multiple signaling pathways for more effective disease management in HNSCC [2].

At current rates, it takes a reasonably long period of time for a lead compound to be developed into a clinically approved drug. An arguably faster path to development is to reposition established drugs as anti-cancer agents. Successful examples of this approach include anti-inflammatory drugs (cyclooxygenase-2 inhibitors), drugs for morning sickness (lenalidomide, an analog of thalidomide) and other approved drugs that are currently under investigation as anti-cancer agents including the oral hypoglycemic rosiglitazone, the immunosuppressant rapamycin, the birth control hormone medroxyprogesterone acetate, HIV protease inhibitor nelfinavir and the antidiabetic drug metformin. Collectively, these examples illustrate how repositioning of existing drugs could complement *de novo* drug development.

Centchroman (CC) [$C_{30}H_{35}O_3N{\cdot}HCl$; trans-1-[2-{4-(7-methoxy-2,2-dimethyl-3-phenyl-3,4-dihydro-2H-1-benzopyran-4-yl)-phenoxy}-ethyl]-pyrrolidine hydrochloride, 67/20; INN: Ormeloxifene] is a non-steroidal antiestrogen [9,10] extensively used as a female contraceptive in India. CC has established anti-breast cancer activity [10,11] with excellent therapeutic index and is safe for chronic administration [12].

In the present study, we examined the effects of CC in HNSCC cells and investigated the molecular mechanisms involved in this process.

2. Materials and methods

2.1. Materials and reagents

Human head and neck squamous carcinoma cells (FaDu, CAL 27, SSC-27 and SSC-9) were obtained from the American Type Culture Collection (Manassas, VA, USA). The normal cell types were procured from commercial sources (Supplementary Table 1S). The test compound CC (Fig. 1A) was synthesised by Dr. D.P. Sahu at the medicinal process chemistry division of the Central Drug Research Institute (Lucknow, India). CC was dissolved in ethanol and stock solutions (10 mM) were further diluted in the culture media prior to the use in experiments. All antibodies and siRNAs for Akt, mTOR and STAT3 were from cell signaling technology (Danvers, MA,

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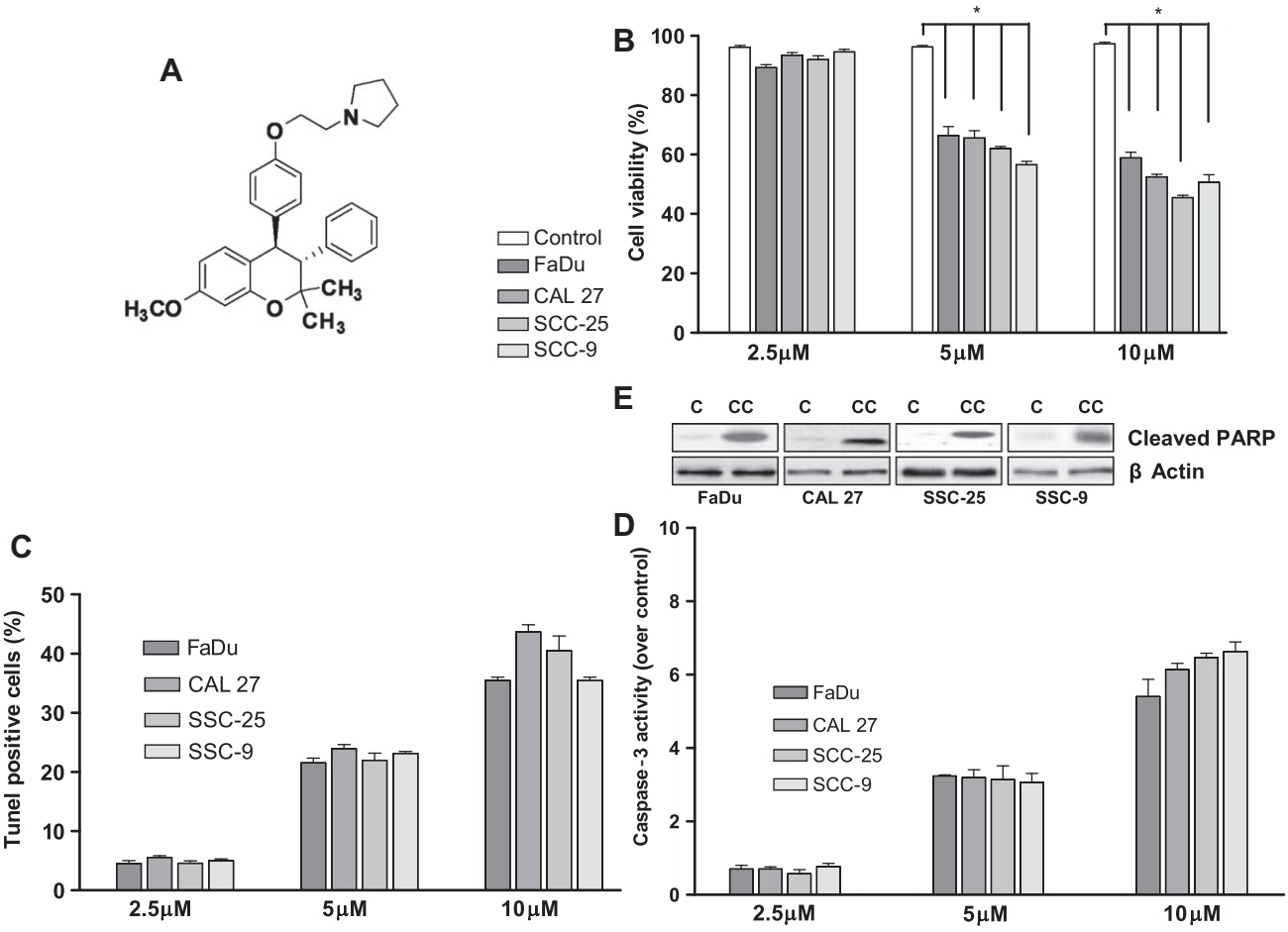

Fig. 1. CC inhibits HNSCC cell proliferation through the induction of apoptotic cell death. (A) Structure of Centchroman (67/20; INN: Ormeloxifene). (B) CC treatment inhibits HNSCC cell proliferation. Cells were treated with various doses (2.5, 5 and 10 μM) of CC for 24 h and CCK-8 assay was used for the measurement of cell viabilities. (C) CC treatment induces DNA fragmentation. Cells were treated with various doses (2.5, 5 and 10 μM) of CC for 24 h and TUNEL assay was done using flow cytometry and the % TUNEL positive cells were measured. (D) CC treatment induces caspase-3 activation. HNSCC cells were treated with various doses of CC (2.5, 5 and 10 μM) for 24 h and measurement of caspase-3 activity was done using the CASPASE-3 Colorimetric Activity Assay kit. (E) CC treatment induces PARP activation. Cells were treated with 5 μM of CC for 24 h and the cell lysates were analyzed by western blotting for the detection of cleaved PARP, a marker for PARP activation. C: Control, CC: Centchroman. Data is expressed in means ± SEM and represent the results of three independent experiments (*$p < 0.05$).

USA). The Bradford assay kit was from Bio-Rad (RegentsPark, NSW, Australia). The Immobilion western chemiluminescence horseradish peroxidase kit and the caspase-3 Colorimetric Activity Assay kit was purchased from Milipore (Billerica, CA, USA). The CytoSelect™ 96-Well Cell Transformation Assay kit was procured from Cell Biolabs (San Diego, CA, USA). The TUNEL assay kit was from Roche (Mannheim, Germany). The Vybrant apoptosis assay kit was from Invitrogen (Eugene, OR, USA). The cell counting kit-8 (CCK-8), was from Dojinando Laboratory (Kumamoto, Japan). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.2. Cell culture and treatment

FaDu and CAL 27 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Sigma St. Louis, USA). SCC-25 and SCC-9 cells were maintained in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) with 10% FBS along with 1% antibiotic and antimycotic (Gibco, NY, USA). Cultures were maintained in 5% CO_2 and humidified in a incubator at 37 °C. Control cells (oral keratinocytes, astrocytes, and prostate epithelial cells) were cultured as per the suppliers instructions. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Histopaque density gradient centrifugation. Adherent monocytes were purified from PBMCs following adherence on glass petridish for 3 h after flushing the non-adherent cells by extensive washing with PBS. All experiments with human PBMCs were conducted under an approved institutional human ethics committee protocol. Cells were treated with increasing concentration of CC serial diluted in medium for 24 h for cell viability and apoptosis studies. For western blotting studies, cell lysates were prepared from cells treated with 5 μM of CC for 24 h.

2.3. Cell viability assay

Cell viability was assessed by the CCK-8 assay kit as per the manufacturer's instructions. The absorbance at 450 nm was read using a 96 well plate reader (Fluostar Omega Spectrofluorometer, BMG Technologies, Offenburg, Germany). All experiments were carried out in triplicate.

2.4. Flow cytometry

The TUNEL assay was done according to the manufacturer's instructions. Briefly, cells were harvested and fixed in freshly prepared 1% para-formaldehyde in PBS for 30 min at 4 °C and then in 70% ethanol for 1 h at 4 °C. Fixed cells were permeabilized using 0.2% Triton X-100 in 0.1% sodium citrate. The DNA labeling mixture

containing terminal deoxynucleotidyl transferase was then added. Cells were incubated overnight at room temperature and washed twice with PBS. Controls were resuspended in the TUNEL reaction mixture containing fluorescent dUTP without terminal deoxynucleotidyl transferase. Annexin-V labeling was performed as per the manufacturer's instructions using a Vybrant apoptosis assay kit. Briefly, cells after the respective treatments were washed with ice-cold phosphate buffer and suspended in annexin-V binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4) and incubated for 15 min with annexin-V conjugated to Alexa Fluor and 10 μg/ml propidium iodide (PI). Subsequently, cells were washed and resuspended in the same buffer. Both these analysis were carried out in a BD LSR flow cytometer (Becton–Dickinson, San José, CA).

2.5. Caspase activity assay

To measure caspase-3 activity, cell lysate were prepared from cells treated with CC for 24 h or with various treatments and analyzed using the caspase-3 Colorimetric Activity Assay kit as per the manufacturer's instructions.

2.6. Western blotting experiments

The protein content of the control and CC treated cell extracts was determined by the Bradford assay. For each of the samples 40 μg of protein were electrophoresed on 10–15% SDS–PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary antibodies at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:5000 dilution). Labeled bands were detected by Immobilion western chemiluminescence horseradish peroxidase kit and images were captured. Densitometric analysis for determination of relative protein expression was done using a Bio-Rad VersaDoc image system (Bio-Rad, Laboratory, UK) with β-actin as loading control.

2.7. Colony formation assay

The soft agar colony formation assay was performed using CytoSelect™ 96-Well Cell Transformation Assay kit as per the manufacturer's instructions. Values are expressed as a percentage of control over treated. Data of three independent experiments was presented as mean ± SEM.

2.8. Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis of the results was done using the statistical functions of the Graphpad Prism 4.0 software (GraphPad Prism Software Inc., San Diego, California, USA). All comparisons between groups were performed using two-tailed paired student's *t*-test. Differences were considered significant at a 0.05 level of confidence.

3. Results

3.1. Centchroman inhibits proliferation of HNSCC Cells

HNSCC cells (FaDu, CAL 27, SCC-9 and SCC-25) were treated with various doses of CC for 24 h. The results of the CCK-8 cell viability assays conducted at the end of the treatment period, showed that the IC_{50} dose of CC was ~7.5 μM in FaDu and CAL 27 cells while it was ~10 μM in SSC-9 and SSC-25 cells. While no significant ($p > 0.05$) effect on cell viability was observed in cells treated with 2.5 μM of CC, a significant (~35%, $p < 0.05$) decrease in the cell viability was observed in all the HNSCC cell lines treated with 5 μM of CC (Fig. 1B). Soft agar colony formation assays, further validated these results as a ~25% colony inhibition was observed (Supplementary Fig. 1S) post CC treatment. Since TUNEL positive cells are characteristic of DNA fragmentation apoptotic signaling, we next assayed CC treated HNSCC cells by flowcytometry based TUNEL assay. The results depicted a significant ($p < 0.05$) increase in TUNEL positive cells (Fig. 1C) post CC treatment. Since, the activation of caspase-3 like proteases play a crucial role in apoptotic cell death [13], we next determined the caspase-3 activities in CC treated HNSCC cells. A ~3.5-fold increase in the caspase-3 activities over control cells was observed across HNSCC cell lines (Fig. 1D; $p < 0.05$). As caspase-3 activity was elevated in CC treated cells, we next determined the PARP activation in these cells treated with a selected dose of 5 μM of CC. Immunoblotting experiments clearly showed the PARP activation characterized by the cleaved PARP fragment in HNSCC cells (Fig. 1E). These results collectively suggested that CC induced inhibition of HNSCC cell proliferation was through apoptotic cell death.

3.2. Centchroman inhibits Akt/mTOR signaling in HNSCC cells

Activation of PI3K/Akt plays an important role in carcinogenesis by maintaining cancer cell proliferation, preventing apoptosis, and supporting the process of metastasis. As aberrant activation of the PI3K/Akt occurs frequently in HNSCC [6,7] recent efforts have focused on developing novel antitumor agents targeting this pathway [8]. Therefore, we next studied the effects of CC treatment on the PI3K/Akt/mTOR signaling pathway. CC (5 μM) treatment decreased Akt phosphorylation in HNSCC cells (Fig. 2). As inhibition of PI3 kinase p110α blocks Akt phosphorylation [14] we further investigated whether this decrease in pAkt was the consequence of reduced p110α levels. However, results showed that CC had no effect on p110α levels (Fig. 2). As CC inhibited pAkt, we investigated its effect on Akt downstream target mTOR. CC treatment downregulated phospho-mTOR in HNSCC cells (Fig. 2). mTOR activation results in phosphorylation of effector molecule p70S6K and S6 ribosomal protein, which subsequently leads to mTOR-dependent gene transcription that regulates cell proliferation, protein synthesis, and metabolism [15]. We therefore, determined the effect of CC on the status of p70S6K and pS6 kinase. CC inhibited phosphorylation of mTOR targets 70S6K and ribosomal protein S6 (Fig. 2). siRNA mediated silencing of Akt or mTOR (Supplementary Table 2S) or pretreatment of HNSCC cells with specific inhibitors of PI3/AKT (LY294002 and wortmannin) or mTOR (rapamycin) further increased the potency of CC treatment (Supplementary Table 3S). These results indicated that CC acts as a dual inhibitor of Akt/mTOR pathway.

3.3. Centchroman downregulates STAT3 phosphorylation in HNSCC cells

mTOR inhibitors block STAT activation [16] and STAT3 inhibition is known to induce apoptosis in HNSCC cells [17]. We therefore determined the status of STAT3 activation in CC treated cells. A decrease in pSTAT3 Tyr705 was observed upon CC (5 μM) treatment (Fig. 3A). Further siRNA mediated silencing of STAT3 expression (Supplementary Table 2S) or pretreatment of HNSCC cells with specific STAT3 inhibitor (stattic) increased the potency of CC treatment (Supplementary Table 3S). These results indicated that CC inhibits STAT3 activation in HNSCC cells.

3.4. Centchroman alters expression of proteins involved in cell cycle regulation and DNA damage response

Inhibition of PI3K/Akt/mTOR signaling affects cell cycle progression [18,19]. mTOR inhibitors induce cell cycle arrest through the

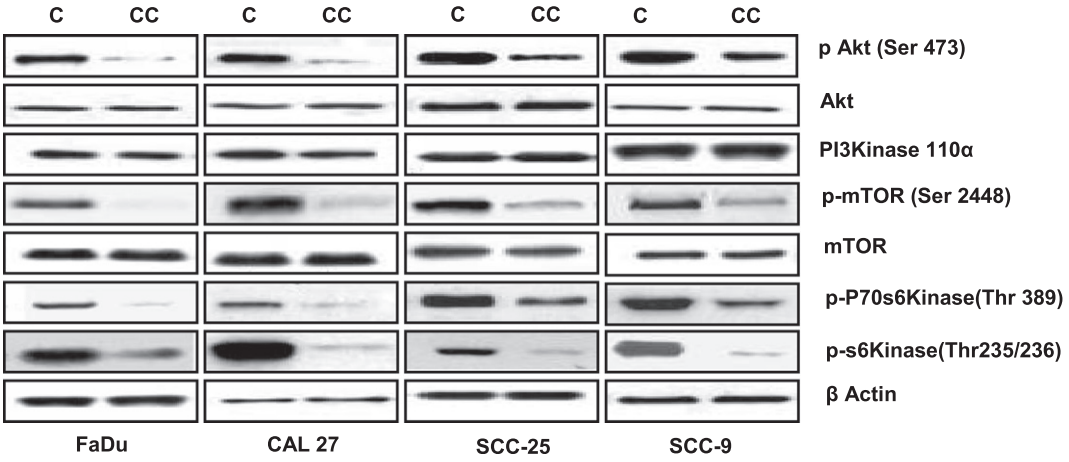

Fig. 2. CC treatment inhibits PI3K/mTOR pathway. HNSCC cells were treated with 5 μM of CC for 24 h. Total cell extracts were prepared and expression of p-Akt, Akt, PI3 kinase 110α, p-mTOR, mTOR, p-70s6Kinase and p-s6Kinase was assayed by western immunoblotting. β-Actin was employed as a loading control. C: Control, CC: Centchroman. The results are representative of three independent experiments.

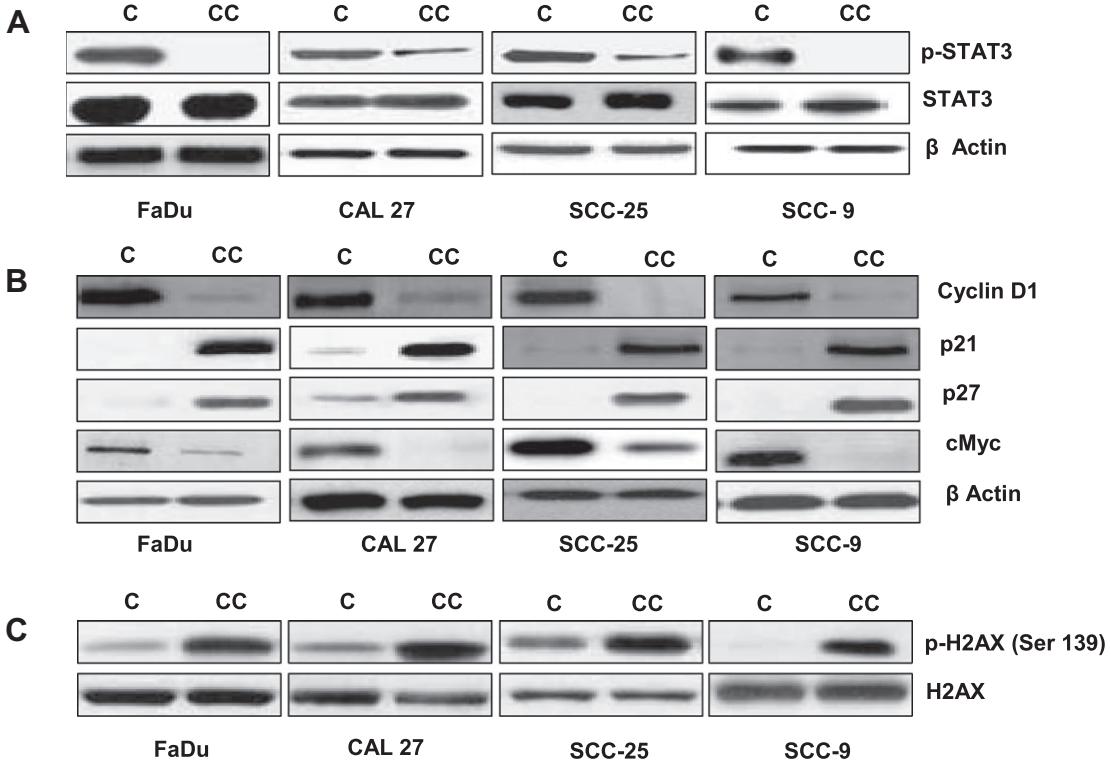

Fig. 3. CC treatment inhibits STAT3 activation and alters expression of proteins associated with cell cycle regulation and DNA damage response. (A) HNSCC cells were treated with 5 μM of CC for 24 h and the expression of p-STAT3 and STAT3 was assayed by western immunoblotting. β-Actin was employed as a loading control. (B) HNSCC cells were treated with 5 μM of CC for 24 h and the expression of cell cycle regulatory proteins Cyclin D1, p21, p27 and cMyc was assayed using western immunoblotting. β-Actin was employed as a loading control. (C) HNSCC cells were treated with 5 μM of CC for 24 h and the expression of proteins associated with the DNA damage response (p-H2AX and H2AX) were assayed using western immunoblotting. C: Control, CC: Centchroman. The results are representative of three independent experiments.

down regulation of Cyclin D1 and upregulation of p27 [18]. Since CC inhibited HNSCC cell proliferation, we determined the expression of molecules associated with cell cycle progression. An increase in p21 and p27, and decrease in Cyclin D1 and c-Myc levels was observed in HNSCC cells upon CC treatment (Fig. 3B). As maintained DNA breaks induce apoptosis [19] and since H2AX is phosphorylated at sites of DNA double-strand breaks [20], we determined the expression of γ-H2AX in CC treated cells. While an increased γ-H2AX expression was observed in CC treated cells (Fig. 3C), the levels of total H2AX was unaffected (Fig. 3C). These results indicate that CC altered expression of cell cycle regulatory and DNA damage response proteins in HNSCC cells.

3.5. Centchroman inhibits proliferation of cancer cells of diverse origin in vitro

We next evaluated whether CC exhibits anti-proliferative property against other cancer cells, by testing its effects against a panel of human cancer cell lines in vitro. Treatment with varying concentrations of (1–10 μM) CC reduced viability of A172 (brain

glioblastoma cells), PC-3 (prostate cancer cell), T47D (breast cancer cells) and THP-1 (acute monocytic leukemia cells) by ~10–60%, as compared to their respective controls (Fig. 4A). These findings indicate that CC not only inhibits proliferation of HNSCC, but also exhibits anti-proliferative activity against a wide variety of human cancer cell lines. To show the selectivity of CC for tumor cells, the effects of CC was also investigated in normal human cell types (oral keratinocytes, human astrocytes, PrEC, HMEC and PBMC cells). Treatment with the maximum dose of CC (10 μM) decreased viability in these nontransformed cells by ~15% (Fig. 4B), suggesting that the anti-proliferative ability of CC is selective for transformed cells.

4. Discussion

Studies in the clinical setting [10] and in vitro screening [11] have identified CC for its anti-tumorigenic activity in breast cancer cells. CC is a non-steroidal antiestrogen with favorable pharmacokinetic, pharmacodynamic and cytotoxic profiles with an excellent therapeutic index [12]. Apart from breast cancer cells nothing was known regarding the effects of CC in other cancer cells. Earlier studies have established that CC induces caspase dependent apoptosis in both estrogen receptor positive (ER positive) and estrogen receptor negative (ER negative) breast cancer cells through the mitochondrial pathway [11]. However not much is known regarding the ER independent effects of CC. Recent studies have revealed that chemotherapeutic drugs with antiestrogenic properties like Tamoxifen exert ER independent anti-cancer activities by targeting the protein kinase C, calmodulin-dependent cAMP phosphodiesterase and telomerase activities [21]. In this context the results of the present study suggest that CC inhibits HNSCC cell proliferation through the inhibition of the Akt/mTOR pathway (Fig. 2) and induction of apoptosis (Fig. 1). Moreover, the dual inhibition of Akt and mTOR by CC could be an effective therapeutic strategy as both these signaling pathways are aberrantly activated in HNSCC [4–8], suggesting that dual Akt/mTOR inhibitor can effectively overcome the effects of feedback loop efficiently than a single inhibitor selectively targeting mTOR. The unique ability of CC to inhibit both Akt and mTOR could possibly be exploited as novel anti-cancer therapy in HNSCC. We found that in addition to inhibiting the Akt/mTOR axis, CC also inhibited STAT3 signaling (Fig. 3A). PKC inhibitors attenuate Ras activation and this attenuation correlates with an inhibition of RasGRP3 phosphorylation [22]. Interestingly, PKCα regulates mTOR as well as STAT3 activation [23]. It is possible that CC effects Akt/mTOR and STAT3 signaling pathways through its ability to bind PKC (Figs. 2 and 3A).

CC mediated decrease in STAT3 activation was concurrent with decreased Cyclin D1 and increased p21 expression (Fig. 3B), while Cyclin D1 overexpression and STAT3 activation are mutually exclusive events [24], p21 is known to inhibit STAT3 signaling [25]. Inhibition of mTOR signaling is known to induce cell cycle arrest through the regulation of Cyclin D1 and p27 [18]. Besides HNSCC, CC also decreased the viability of several other cancer cell types (Fig. 4A) possibly through different mechanisms, currently under

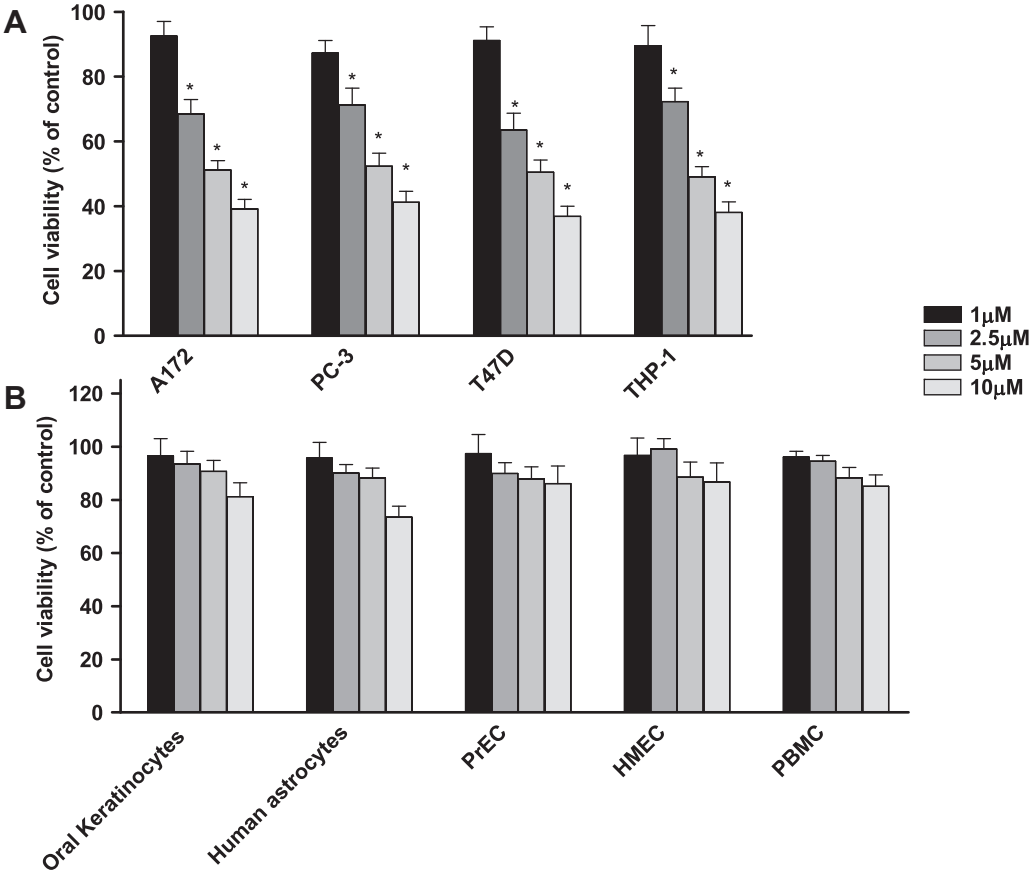

Fig. 4. Effect of CC treatment on cell viabilities of non-HNSCC cancer cells and normal cell types. (A) Transformed cells of diverse origin (A172: brain glioblastoma cells, PC-3: human prostate cancer cells, T47D: human breast cancer cells and THP-1: acute monocytic leukemic cells) were treated with various doses of CC (1, 2.5, 5 and 10 μM) for 24 h and the cell viabilities were assayed using CCK-8 assay. (B) Nontransformed cells of diverse origin, i.e. oral keratinocytes; human astrocytes; human prostate epithelia cells (PrEC); human mammary epithelial cells (HMEC); peripheral blood mononuclear cells (PBMCs) were treated with various doses of CC (1, 2.5, 5 and 10 μM) for 24 h and the cell viabilities were assayed using CCK-8 assay. Data is expressed in means ± SEM and represent the results of three independent experiments (*$p < 0.05$).

investigation in our laboratory. It is established that cytotoxic responses is a reflection of an integrated readout of all targets and/or biochemical pathways affected upon drug treatment [26]. As strong a correlation exists between chemo-sensitivity and gene expression [26], it is likely that differential expression of cellular pathways in cancer cell types of diverse origin could have resulted in differences in sensitivity to CC.

5. Conclusion

Taken together our studies suggest that (i) CC induces HNSCC apoptosis and (ii) inhibits Akt/mTOR pathway. This ability of CC to act as a multi-inhibitor that blocks Akt/mTOR and STAT3 pathways suggest that its potential as a chemotherapeutic agent against HNSCC should be further evaluated. Importantly, CC could be a promising candidate for the treatment of a wide variety of malignancies, since it elicits cell death in many tumor cell types.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.049.

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