Inhibition of Head and Neck Squamous Cell Carcinoma Growth and Invasion by the Calcium Influx Inhibitor Carboxyamido-triazole


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Abstract

Local invasion and lymph node metastasis are correlated with a decreased overall survival in head and neck cancer patients and warrant new strategies to intervene in the metastatic cascade. One approach is to focus on the intracellular signaling pathways underlying the metastatic process. A common regulatory point in several signal transduction pathways is intracellular calcium homeostasis. We assessed the effect of a novel calcium influx inhibitor, carboxyamido-triazole (CAI), on the growth and invasive phenotype of cell lines derived from head and neck squamous cell carcinoma (HNSCC). CAI inhibited the growth of FaDu and EVSCC17M cells in a dose-dependent (IC_{50} 13–15 μM) and reversible manner. CAI also caused a generalized attenuation of receptor-mediated calcium elevation to several calcium mobilization agonists, including epidermal growth factor and bradykinin. The effects of CAI on the invasive phenotype of HNSCC cell lines were assessed by a chemoinvasion assay. HNSCC cell lines exhibited a range of invasive potential as measured by the capacity of tumor cells to penetrate a reconstituted basement membrane of Matrigel. HNSCCs were classified as highly invasive (EVSCC14M and EVSCC17M) or weakly invasive (EVSCC18, EVSCC19M, UMSCC10A, and FaDu). Treatment of HNSCC cell lines with 10 μM CAI for 24 h reduced invasion 2–14-fold in a dose-dependent manner. HNSCCs also exhibited different motilities as measured by a chemotaxis assay. EVSCC14M and EVSCC17M were highly motile, whereas EVSCC18, EVSCC19M, UMSCC10A, and FaDu were less motile. CAI reduced the migration of all cell lines. Conditioned medium from HNSCC cell lines was analyzed by zymography for production of M_{r} 72,000 type IV collagenase [matrix metalloproteinase (MMP)-2] and M_{r} 92,000 type IV collagenase (MMP-9). All HNSCC cell lines secreted MMP-2 and/or MMP-9 into conditioned medium. Treatment of cells with 10 μM CAI for 24 h resulted in a reduction of both MMP-2 and MMP-9 production. The results demonstrate that CAI blocks cellular proliferation, migration, chemoinvasion, and MMP production by HNSCC in vitro and identify calcium-dependent signaling as a new target for inhibition of the malignant phenotype of HNSCC.

Introduction

Nearly 42,000 new cases of HNSCC\(^3\) and approximately 13,000 HNSCC deaths are estimated in the United States in 1997 (1). Globally, HNSCC accounts for 500,000 new cases annually and is the fifth most frequent cancer overall, comprising 8% of the total malignancies. Despite improvements in current therapies, including surgery, radiation, and conventional chemotherapy, 5-year survival rates have remained unchanged over the past 20 years in whites and have decreased in African Americans (1–3). Moreover, increased rates of oral cancers were reported in males ages 15–34 in the United States (4) and in young males and females in Scotland (5). Taken together, these statistics emphasize the need to identify new targets and to develop innovative therapeutic approaches for HNSCC.

Accumulating evidence demonstrates that calcium homeostasis and calcium-regulated cellular events are important in the generation and maintenance of the malignant phenotype (6). Thus, interruption of calcium-dependent signaling pathways represents a novel target for treatment and prevention of cancer development and dissemination (6). CAI is a novel inhibitor of non-voltage-gated calcium influx and has shown antiproliferative, antimigratory, and antimetastatic properties in a range of human cancer cell lines (7–11). The actions of CAI are related to a disruption of calcium homeostasis, which inhibits downstream calcium-sensitive signaling pathways, such as agonist-mediated tyrosine phosphorylation of phospholipase C-γ, inositol phosphate, and arachidonic acid generation (12–14); nucleotide biosynthesis (7); and MMP-2 production (15). In a recently completed Phase I clinical trial, CAI administered p.o.

\(^{3}\) The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; CAI, carboxyamido-triazole; MMP, matrix metalloproteinase; FBS, fetal bovine serum; CM, conditioned medium; EGF, epidermal growth factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.
stabilized tumor progression in approximately 50% of patients with advanced disease with low toxicity (16), indicating a potential new approach in the management of advanced-stage cancers.

Because CAI selectively inhibits calcium-dependent signaling pathways (6), we hypothesized that CAI might block aberrant signaling in HNSCCs and be useful for the management of advanced-stage HNSCCs. The present study was conducted to test the effects by CAI on the growth and invasive phenotype of human head and neck cancer cell lines. Because proteolytic degradation of extracellular matrix and cell migration are two important steps in tumor invasion (17), we also assessed the effect of CAI on MMP production and cell migration. The results demonstrate that CAI inhibited the proliferation, migration, and invasive potential of HNSCC cells in vitro and blocked production of MMPs. Collectively, the data extend the signal transduction inhibition effects of CAI to HNSCC and identify Ca2+-dependent signaling pathways as a new therapeutic target in the treatment of HNSCC.

Materials and Methods

Cell Lines and Cell Culture. HNSCC cell lines derived from carcinoma of the tongue (EVSCC14M, EVSCC17M, and EVSCC19M) and larynx (EVSCC18) were established at Eastern Virginia Medical School (18, 19). Additional HNSCC cell lines, UMSCC10A and FaDu, were derived from SCC of the larynx and hypopharynx, respectively (18). All HNSCC cell lines were cultured in EMEM supplemented with 10% FBS and nonessential amino acids. HaCaT is a spontaneously immortalized keratinocyte cell line (20), and Beas-2B is a SV40 T antigen-immortalized bronchial epithelial cell line (21). HT1080 human fibrosarcoma cells were used as a positive control for invasion and MMP production. NIH3T3 cells were used as a source of CM for migration and chemoinvasion assays. HaCaT, HT1080, and NIH3T3 cells were grown in DMEM supplemented with 10% FBS; Beas-2B cells were grown in 2% FBS supplemented with LHC-8 (Biofluids Inc., Rockville, MD). All cells were cultured at 37°C in 5% CO2/95% air.

Cell Proliferation Assays. Celltiter 96 Aqueous (Promega, Madison, WI) colorimetric MTS assays for cell proliferation were performed in 96-well tissue culture plates as recommended by the manufacturer. Cells were plated at 5 x 10^3 cells/well in growth medium, and the number of viable cells was determined in quadruplicate after 5 days of drug treatment.

Cell proliferation was also assessed by direct cell enumeration. Cells (5 x 10^6) were plated into 12-well dishes in growth medium and allowed to attach overnight before initiation of drug treatment. Growth media containing CAI or DMSO vehicle control were added to duplicate wells, and media were changed thereafter every 2 days. Cells were counted in a hemocytometer on designated days after trypsinization.

For anchorage-independent growth, 2.5 x 10^3 cells were seeded into 24-well cluster dishes in 0.35% agar-containing growth medium supplemented with the designated amount of CAI. Colonies >0.1 mm in diameter in duplicate wells were counted after 14 days incubation at 37°C.

Measurement of Intracellular Free Calcium. Nearly confluent cultures of cells grown in T75 flasks were serum starved for 4 h and removed by brief trypsinization. Cells were washed and resuspended in phenol red-free HBSS supplemented with 0.1% BSA, fraction V (Sigma Chemical Co., St. Louis, MO). Cells were loaded with 4 μmol of the calcium indicator dye Fura-AM (Molecular Probes, Eugene, OR) for 1 h at 37°C as described previously (10, 22). Calcium mobilization was assessed in Fura-2AM-loaded cells using a SPEX ARCM spectrofluorimeter at 330 and 380 nm. Calcium levels in nanomolar values were calculated as described previously (22), using calibration in the presence of CAI to compensate for any influence of CAI on fluorescence values.

Chemoinvasion and Migration Assays. Chemoinvasion and cell migration assays were performed in a 48-well modified Boyden chamber. Polycarbonate filters (pore size, 8 μm; Nucleopore, Cabin John, MD) were first coated with type IV collagen (5 μg/filter; Life Technologies, Inc., Grand Island, NY) and air dried. Matrigel (1 mg/ml) in cold distilled water was layered onto each filter (50 μg/filter). Filters were dried with an equivalent amount of DMSO vehicle. Untreated cells were incubated with an equivalent amount of DMSO vehicle.

Fig. 1 Effect of CAI on agonist-induced elevation of [Ca2+]i in EVSCC17M (A) and FaDu (B) cells. EVSCC17M and FaDu cells were loaded with Fura-2 and preincubated with 20 μM CAI for 30 min before agonist (EGF 200 ng/ml and bradykinin 1 μM addition (arrows). Untreated cells were incubated with an equivalent amount of DMSO vehicle.
Fig. 2  Antiproliferative effect of CAI and its reversibility on EVSCC17M tumor cells measured by cell counting. A. cells (5 × 10^5) were plated in 12-well dishes in growth medium containing 5% FBS without (Control) or with the designated CAI concentration. Cell cultures were then fed every 2 days with the respective media. On the designated days, cells were harvested with trypsin and counted. B. cells were grown in medium without (Control) or with 10 μM CAI. After 3 days of treatment, the CAI medium in one set of cell cultures was removed and replaced with regular growth medium (REV Day3). On the designated days, cells were counted as in A. Data are from one representative experiment of two.

CO₂, 95% air for 5 h. At the end of the incubation, the cells on the upper surface of the filters were removed mechanically. Filters were fixed and stained with Diff-Quik (Baxter, McGaw Park, IL), and the number of invasive cells was counted using a microscope with an image analyzer. Cell migration assays in response to NIH3T3 CM were performed similarly in modified Boyden chambers using polycarbonate filters coated with 0.01 mg/ml gelatin, which is permissive for cell attachment and migration.

Zymography. Gelatin zymography was performed as described previously (23). Briefly, 2 × 10^6 cells in T25 flasks were incubated at 37°C for 24 h; washed with insulin, transferrin, and selenium medium (Sigma); and incubated in 3 ml of insulin, transferrin, and selenium medium at 37°C for 24 h. The CM was collected, centrifuged at 400 × g for 10 min at 4°C to remove cells and cell debris, and stored at -70°C. Aliquots of CM (40 μl) were loaded onto 10% acrylamide gels containing 1 mg/ml gelatin. After electrophoresis, gels were washed twice for 30 min with 2.5% Triton X-100 and incubated in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, 0.02% Brij-35, and 0.1% Triton X-100 overnight at 37°C. The gels were stained with 0.5% Coomassie Blue, and gelatinolytic activity, visualized as cleared regions of the gel, was quantitated by scanning densitometry of individual lanes.

Results

Inhibition of Agonist-induced Calcium Mobilization by CAI. We showed previously that CAI attenuated agonist-induced elevation of cellular calcium in prostate tumor cell lines (10). To confirm that CAI acted to alter calcium homeostasis in HNSCC, we evaluated the calcium signaling properties of non-treated versus CAI-treated EVSCC17M and FaDu cells. Using cells loaded with the calcium fluoroprobe Fura-2, we observed a rapid elevation of intracellular calcium levels in EVSCC17M and FaDu cells treated with two general calcium agonists, EGF and bradykinin, respectively. The EGF effect on calcium in EVSCC17M cells was dose dependent and was attenuated >70% by EGTA in the assay buffer, indicating a significant calcium influx component involved with calcium mobilization by EGF in HNSCC cells (data not shown). Preincubation with 20 μM CAI markedly attenuated calcium elevation in both cell lines (Fig. 1, A and B). Similar results were obtained with other agonists (data not shown), confirming that CAI blocks agonist-induced calcium influx in HNSCC cells. We also examined the dose dependency of CAI to attenuate bradykinin-induced elevations of intracellular free calcium. CAI at a concentration of 1 μM produced a 10% inhibition, whereas 10 μM CAI produced a maximum 50% inhibition (data not shown). Increasing the concentration of CAI to 20 μM and 50 μM did not produce any additional inhibition.
Effects on Head and Neck Tumor Cells

The effect of CAI on HNSCC cell proliferation was assessed by seeding 0.1 mm in diameter in duplicate wells were counted after 14 days. Data results demonstrate that CAI inhibits growth of EVSCC17M in a representative assay using EVSCC17M is shown in Fig. 2A. The direct cell enumeration over a 7-day period of treatment. A two. are mean colony counts (bars, SE) of a representative experiment of two.

**Antiproliferative Effects of CAI on HNSCC Cell Lines.**

The effect of CAI on HNSCC cell proliferation was assessed by direct cell enumeration over a 7-day period of treatment. A representative assay using EVSCC17M is shown in Fig. 2A. The results demonstrate that CAI inhibits growth of EVSCC17M in a dose-dependent manner. Cell growth arrest at 5 μM or 10 μM CAI over a 7-day period (Fig. 2A) suggested cytostatic rather than cytotoxic growth inhibition by CAI. To distinguish between these possibilities, we tested the ability of EVSCC17M cells to resume growth after withdrawal of CAI from growth-inhibited cells (Fig. 2B). Growth inhibition of EVSCC17M cells by 10 μM CAI was reversible following removal of CAI from the culture medium on day 3 (Fig. 2B). Resumption of cell proliferation was delayed 2 days following drug reversal but then proceeded at a rate similar to that of untreated cells. The data indicate cytostatic, rather than cytotoxic, growth inhibition by CAI.

We assessed the generality of CAI growth inhibition on HNSCC cell lines compared to immortalized, nontumorigenic squamous epithelial cell lines. A representative experiment is shown in Fig. 3. CAI inhibited FaDu and EVSCC17M growth in a dose-dependent fashion but was without affect on human bronchial epithelial (Beas-2B) or epidermal keratinocyte (HaCaT) cell lines at comparable concentrations. The half-maximal inhibitory concentrations (IC50) for FaDu and EVSCC17M were 13 μM and 15 μM CAI, respectively. In contrast, Beas-2B and HaCaT were relatively resistant to CAI, in confirmation of earlier work (10), thus demonstrating CAI selectivity for HNSCC cell growth inhibition.

To determine the effect of CAI on anchorage-independent growth, FaDu cells were plated in soft agar, and colony formation was assessed with or without CAI treatment (Fig. 4). FaDu cells formed colonies in soft agar with plating efficiency of ~10% (240 colonies/2500 cells plated). CAI treatment resulted in a concentration-dependent inhibition in the number and size of colonies in soft agar with an apparent IC50 of 1.5 μM. Interestingly, the IC50 for anchorage-independent growth inhibition of FaDu by CAI was about 10-fold less than inhibition of adherent cell proliferation by the MTS assay, a reflection of the increased sensitivity of the clonogenic assay.

**Effect of CAI on Chemoinvasion and Cell Migration by HNSCC Cell Lines.**

We used the Matrigel chemoinvasion assay to characterize the invasive potential of HNSCC cell lines and to examine the effects of CAI on invasion. HNSCC cell lines exhibited a range of invasive potential and were classified as highly invasive (EVSCC14M and EVSCC17M) or weakly invasive (EVSCC18, EVSCC19M, UMSCC10A, and FaDu; Fig. 5). Treatment of HNSCC cell lines with 10 μM CAI for 24 h completely blocked invasion by EVSCC17M cells and reduced invasion of other HNSCC cells 2–14-fold (Fig. 5). Inhibition of chemoinvasion by CAI was concentration dependent, with an IC50 of 5.8 μM and 3.4 μM for EVSCC14M and EVSCC17M, respectively (data not shown). As with invasion, HNSCC cell lines displayed different motilities as measured by the chemotaxis assay (Fig. 6). EVSCC14M and EVSCC17M were highly motile, whereas FaDu, UMSCC10A, EVSCC18, and EVSCC19M were decreasingly less motile in response to a chemotactic stimulus. Cell migration by HNSCC cells correlated closely with chemoinvasion. CAI (10 μM) inhibited the migration of all cell lines, with the most potent inhibition observed with EVSCC17M cells.

**Effect of CAI on MMP Production by HNSCC Cell Lines.**

To better define the inhibitory effect of CAI on the invasive phenotype of head and neck tumor cells, we assessed the effects of CAI on MMP production and activity by zymog-
Fig. 6 Effect of CAI on migration of HNSCC cell lines. Migration assays were performed as described for the chemoinvasion assay, except that polycarbonate membranes were coated with gelatin. The results are the means (bars, SE) of two independent experiments. Inset, expanded scale of HNSCC cells with reduced motility. Statistical analysis demonstrated that CAI significantly inhibited cell migration of cell lines (P < 0.05).

Fig. 7 Effect of CAI on MMP production by HNSCC cells. Cells were treated without (−) or with (+) 10 μM CAI for 24 h, and MMP activity in CM was assessed by gelatin zymography as described in “Materials and Methods.” Shown is a representative zymogram of CM from EVSCC19M, EVSCC14M, and EVSCC18. CM from HT1080 was used as a positive control for MMP-9 and MMP-2 activity. The Mr 92,000 and 72,000 MMP activities are indicated (kD).

Fig. 8 Quantitative densitometry of MMP production by HNSCC cell lines. Cells were treated without (Control) or with 10 μM CAI for 24 h, and MMP activity of CM was assessed by gelatin zymography as described in “Materials and Methods.” MMP activity was quantitated by densometric scanning of individual lanes and expressed in arbitrary units.

Discussion
CAI is a novel inhibitor of calcium influx that selectively blocks calcium-mediated signal transduction events (24). Previous reports have shown that CAI inhibits the proliferation and invasive behavior of malignant cells in vitro and in animal
tumor models (9–11). The effect of CAI on the proliferation and invasiveness of head and neck tumor cells, however, has not been examined previously. In the present study, we demonstrate that CAI inhibited the growth of cell lines derived from HNSCC in a dose-dependent and reversible manner, suggesting an important role for calcium in the biology of HNSCC. These findings raise the interesting possibility that CAI, acting as a cytostatic agent, may be useful for targeting calcium-mediated signal transduction pathways as a treatment for HNSCCs.

The limiting factor for conventional treatments of cancer is the toxicity to normal tissues. The goal of successful cancer therapy is to selectively kill tumor cells or arrest metastatic spread while sparing normal tissue. One therapeutic approach capitalizes on the accumulating evidence that the malignant tumor cell phenotype is the result of aberrant signal transduction. Drugs that intercept cell signaling pathways are predicted to function as cytostatic agents by inhibiting cell proliferation and reversing the malignant cell phenotype. Calcium represents a signaling molecule essential in many signal transduction pathways and is known to regulate a number of biochemical processes, including cell proliferation, invasion, angiogenesis, and secretion (25). In the present study, we found that CAI at clinically achievable levels blocked the growth of several head and neck tumor cell lines but was without effect on the proliferation of nontumorigenic, immortalized HaCaT and Beas-2B cells. This finding suggests that the antiproliferative activity of CAI is selective against squamous tumor cell lines as compared with immortalized squamous epithelial cell lines of the same lineage. The results are consistent with in vivo preclinical studies and Phase I clinical trials (9, 16, 26, 27), showing that CAI exhibits minimal toxicity to skin, bone marrow, and the digestive system. The mechanism(s) for the selective effect of CAI on HNSCC is unclear. Variability in cell cycle kinetics, drug metabolism, or a higher dependency on calcium-sensitive functions for cell proliferation in HNSCC cells as compared to nontumorigenic epithelial cells may account for the differences observed. Regardless of the mechanism(s) of CAI action, the dependency of the malignant tumor cell on calcium signaling has exposed an Achilles’ heel and renders the malignant cell more vulnerable to agents that inhibit or modulate calcium influx.

Tumor invasion of the basement membrane is one early and key step in the metastatic cascade (6). We demonstrated that CAI inhibited the invasiveness of HNSCC cell lines in vitro by the chemoinvasion assay, a method that assesses the ability of tumor cells to penetrate a reconstituted basement membrane of Matrigel. Comitantly, CAI was found to block the in vitro motility of HNSCC cell lines. These results are consistent with previous reports showing CAI is an antimitotic agent (8, 24). Because migration was correlated with invasive potential of HNSCC in the present study, our results suggest that inhibition of the invasiveness of HNSCC cell lines was mediated partially by an inhibition of cell motility. Localized proteolysis is another important factor in tumor cell invasion and metastasis (6). We demonstrated that CAI inhibited both MMP-2 and MMP-9 production by HNSCCs, indicating that calcium homeostasis is important in regulation of MMP secretion. This supports and extends previous studies showing that CAI inhibits MMP-2 production by fibrosarcoma and endothelial cell lines (15, 28). Whether CAI inhibits the production of MMPs at the level of transcription or translation/secretion in HNSCC is not clear. In a recent study, CAI was found to reduce MMP-2 mRNA transcription (15). Additional work is necessary to determine whether CAI also inhibits transcription of MMPs in HNSCCs. The reduction of MMP activity by CAI suggests that inhibition of the invasive potential of HNSCCs also involves an inhibition of MMP production.

In contrast to conventional chemotherapeutic agents, CAI has been found to cause cytostatic rather than cytotoxic responses in malignant cells and disease stabilization in patients with advanced malignancies (6, 16, 27, 29). In agreement with these studies, we observed that CAI was cytostatic against HNSCC cell lines in vitro, suggesting that CAI may be beneficial for the management of HNSCCs. Inasmuch as CAI reversibly inhibited growth and invasiveness of HNSCCs, it may prove useful as an adjunctive treatment with surgery and radiation to prevent the recurrence and establishment of metastasis and/or in combination with effective chemotherapeutic agents to reduce toxicity and improve treatment efficacy in metastatic disease. Observation of synergistic interactions between CAI and quercetin in growth inhibition of human breast carcinoma cells supports this possibility (30).

In summary, the novel signal transduction drug, CAI, displayed multiple effects on malignant properties of HNSCC cells, including inhibition of proliferation, invasion, migration, and MMP production. The results indicate that CAI may be useful in the design of new strategies for the treatment of HNSCCs as well as chemoprevention.

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References


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