Effects of the calcium influx inhibitor carboxyamido-triazole on the proliferation and invasiveness of human prostate tumor cell lines

ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · OCTOBER 1996

Impact Factor: 5.09 · DOI: 10.1002/(SICI)1097-0215(19961009)68:2<259::AID-IJC20>3.0.CO;2-4

Int. J. Cancer: **68,** 259–264 (1996)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

EFFECTS OF THE CALCIUM INFLUX INHIBITOR CARBOXYAMIDO-TRIAZOLE ON THE PROLIFERATION AND INVASIVENESS OF HUMAN PROSTATE TUMOR CELL LINES

William J. WASILENKO[1,4,7], Ann J. PALAD[1,4], Kenneth D. SOMERS[1,4], Peter F. BLACKMORE[3,4], Elise C. KOHN[5], Johng S. RHIM[6], George L. WRIGHT, JR.[1,2,4] and Paul F. SCHELLHAMMER[2,4]

[1]*Departments of Microbiology and Immunology,* [2]*Urology and* [3]*Pharmacology, Eastern Virginia Medical School and* [4]*Virginia Prostate Center, Eastern Virginia Medical School and Sentara Cancer Institute, Norfolk, VA;* [5]*Laboratory of Pathology, National Cancer Institute, Bethesda, MD;* [6]*Laboratory of Molecular Oncology, National Cancer Institute, Frederick, MD, USA.*

Aberrant cellular signaling is a central feature of malignant cells and a potential target for anti-cancer therapy. Carboxyamido-triazole (CAI) is a calcium influx inhibitor that alters calcium-sensitive signal transduction pathways and suppresses the proliferative and metastatic potential of malignant cells. We have examined the effects of CAI on several tumor-associated parameters in human prostate cancer cell lines to evaluate the potential of CAI as a signal-transduction therapy agent for advanced-stage prostate cancer. Measuring anchorage-dependent cell growth, continuous application of CAI inhibited the growth of DU-145, PPC-1, PC3 and LNCaP tumor cells with 50% inhibitory concentrations ranging 10–30 μM. Direct cell enumeration assays revealed that the growth-suppressing activity of CAI toward DU-145 cells was reversible, indicating a cytostatic effect of the drug on tumor cells. The drug also inhibited the proliferation of several immortalized human prostatic epithelial cell lines. The proliferation of HaCaT- and RHEK-1-immortalized keratinocyte cell lines was relatively insensitive to CAI. Additionally, invasion by DU-145, PC3 and PPC-1 cells through Matrigel *in vitro* was reduced approximately 60–70% by 10 μM CAI. Other cellular effects of CAI included an attenuation of the elevation of intracellular free calcium in response to bombesin and carbachol in PC3 cells and a marked dose-dependent inhibition of prostate-specific antigen secretion in LNCaP cell cultures.

The high incidence of prostate cancer in North American males is growing with the extended life span of the male population. As in the normal prostate, many early-stage prostate tumors require androgens for survival, and therapies which interfere with steroid metabolism or availability are effective treatments for selected patients. Over time, however, most prostate cancer patients relapse and acquire an androgen-independent tumor that shows aggressiveness and poor response to conventional cytotoxic therapies (Issacs *et al.,* 1987). For patients with these androgen-insensitive tumors, alternative new therapies are urgently needed.

Considerable research has demonstrated that tumor cells exhibit altered signaling responses to factors that regulate cellular growth, differentiation, migration and apoptosis (Powis, 1991; Cole and Kohn, 1994). For this reason, drugs which manipulate or suppress signal transduction in malignant cells offer a potentially new approach to anti-cancer therapy (Levitzki, 1994; Kohn and Liotta, 1995). One prototype signal-transduction therapy agent is carboxyamido-triazole (CAI), which targets the homeostasis of cellular calcium, an important second messenger (Cole and Kohn, 1994; Kohn and Liotta, 1995). Originally developed as a coccidiostat, CAI is an inhibitor of non-voltage-gated calcium channels in electrically neutral cells like human carcinoma cells (Hupe *et al.,* 1991; Felder *et al.,* 1991). In pre-clinical studies with several types of rodent and human malignant cell, CAI has proven effective as an anti-proliferative, anti-migratory and anti-metastatic agent (Kohn *et al.,* 1992; Cole and Kohn, 1994). Additionally, CAI has shown anti-angiogenic properties in studies using human endothelial cells and in the chick chorio-allantoic membrane assay (Kohn *et al.,* 1995). Mechanistic studies in several cell types demonstrate that CAI interferes with selected calcium-sensitive signaling processes such as agonist-mediated tyrosine phosphorylation and arachidonic acid generation (Felder *et al.,* 1991; Kohn *et al.,* 1995), nucleotide biosynthesis (Hupe *et al.,* 1990) and MMP-2 matrix metalloproteinase (type IV collagenase) production (Kohn *et al.,* 1994*b,* 1995). In recently completed phase I clinical studies, CAI has shown promise at stabilizing disease progression in patients with several types of advanced solid tumor (Kohn *et al.,* 1996). Collectively, these findings underscore the importance of calcium in the biology of malignant cells and suggest a potentially valuable role for inhibitors of calcium homeostasis in the management of some advanced-stage cancers.

As prostate tumors develop androgen independence, a variety of stroma- and epithelium-derived autocrine and paracrine growth factors relay signals that influence the behavior of malignant prostate tissue (Chung, 1991; Ware, 1993). For this reason, inhibitors of signal transduction may be useful in the treatment of prostate cancer. We observed that calcium mobilization is a common signaling response in 3 advanced prostate tumor cell lines treated with a variety of neuropeptides, including bombesin and endothelin, which are associated with prostate tumor progression (Wasilenko *et al.,* 1996). This led us to hypothesize that calcium was an important component in signaling events underlying the development of prostate cancer. We therefore investigated the *in vitro* sensitivities of human prostate tumor cells to the anti-signaling drug CAI.

MATERIAL AND METHODS

Cell lines and cell culture

Human prostate tumor cells included the androgen-insensitive cell lines DU-145, PC-3 and PPC-1, as well as LNCaP cells, which are androgen-responsive. PPC-1 cells are a derived from PC-3 tumor cells (Chen, 1993). All prostate tumor cell lines were routinely cultured in RPMI 1640 medium supplemented with 5% FBS (GIBCO, Grand Island, NY). Non-tumorigenic human prostate epithelial cells were cultured in serum-free keratinocyte growth medium (GIBCO). PRNS-1-1 cells were derived from normal adult human prostatic epithelia immortalized with SV-40 virus (Lee *et al.,* 1994). FNC267B1 is a fetal human prostate epithelial cell line immortalized with SV-40 virus (Kaighn *et al.,* 1989). The RHEK-1 human epidermal keratinocyte cell line was immortal-

[7]To whom correspondence and reprint requests should be addressed, at Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, VA 23507, USA. Fax: (804) 624-2255.

Received: April 10, 1996 and in revised form July 8, 1996

ized with Ad12-SV40 hybrid virus (Rhim *et al.*, 1985) and grown in DMEM supplemented with 10% FBS. Spontaneously immortalized HaCaT epidermal keratinocytes were grown in DMEM with 10% FBS. MRC-5 human lung fibroblasts were grown in EMEM plus 10% FBS. NIH 3T3 cells were grown in serum-free DMEM plus glutamine, neomycin and 50 μg per ml of vitamin C for the collection of conditioned medium for use in the chemoinvasion assays.

Cell proliferation assays

CellTiter $96_{AQueous}$ (Promega, Madison, WI) colorimetric MTS assays for cell proliferation were performed with cells plated into 96-well plastic tissue culture dishes according to the manufacturer's recommendations. Cells were seeded into the appropriate medium as described above at a density of 5 to 7.5×10^3 cells. The number of viable cells was determined in quadruplicate generally after 5 days of drug treatment.

Direct cell enumerations were performed on the designated days after trypsinization of cells, followed by cell counting in a hemocytometer. Cells (5×10^4) were plated into 12-well dishes in growth medium to initiate the experiments.

For both of the above cell measurements, suspended cells were allowed to attach to the tissue culture plates overnight before drug treatment. CAI in the DMSO vehicle was then added, and media were changed thereafter every 2 days. The vehicle alone was found to have no effect on the growth of the cells under these assay conditions.

For anchorage-independent growth, 2.5×10^3 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 were counted in dishes after 14 days.

Measurement of intracellular free calcium

Nearly confluent cultures of cells were serum-starved for 4 hr and removed from flasks using a brief trypsinization. The removed cells were then washed and resuspended in phenol-red-free Hanks' balanced salt solution (HBSS) supplemented with 0.1% BSA, fraction V (Sigma, St. Louis, MO), pH 7.4. Cells were loaded with 4 μM, Fura2-AM (Molecular Probes, Eugene, OR) for 1 hr at 37°C, washed free of the dye and resuspended in the HBSS solution at approximately 10^7 cells/ml. Aliquots of the Fura2-AM-loaded cells were suspended in a small cuvette, gently stirred and then excited in a SPEX ARCM spectrofluorimeter at 330 and 380 nm. Emission was measured at 505 nm. All measurements were corrected for autofluorescence using an aliquot of cells which had been treated with 20 μM ionomycin plus 10 mM manganese chloride, which quenches the intracellular Fura 2 signal. Calibration was also performed in the presence of CAI to compensate for a small influence of CAI on the fluorescence values. Calcium levels in nanomolar values were then calculated according to Blackmore *et al.* (1992).

Chemoinvasion assays

Invasion of cells through Matrigel, a basement membrane matrix, was performed in a 48-transwell plate system using polycarbonate filters with 8 μm pore diameter (Nucleopore, Cabin John, MD). Filters were coated with type IV collagen (GIBCO), air dried, then over-layered with 25 μg/ml Matrigel. The Matrigel was obtained from a 500 μg/ml stock solution prepared in distilled water. Coated membranes were dried, then reconstituted in DMEM. Trypsinized tumor cells were suspended in DMEM plus 0.1% BSA before addition to the transwell apparatus. Approximately 2×10^6 cells per ml were added to the upper compartment; the NIH 3T3 cell conditioned medium, which served as a chemoattractant, was placed in the lower chamber. Tumor cell invasiveness was assessed following a 5-hr incubation at 37°C in a CO_2 incubator. CAI was present in both the upper and lower chambers. Non-invading cells were scraped from the upper surface of the filter, and cells that had invaded to the bottom surface of the membrane were stained with Diff-Quik (Baxter, McGaw Park, IL) and quantitated using an image analyzer.

PSA measurements

LNCaP cells were cultured in T25 dishes at an initial seeding density of 1×10^5 cells using RPMI-1640 medium supplemented with 10% FBS. Cells were allowed to attach overnight, then treated the following day with or without the various doses of CAI. Supernatant fluid for each respective day of treatment was collected and frozen until prostate-specific antigen (PSA) quantitation. Cells remaining on the dishes were harvested and counted so that PSA levels could be expressed per cell number. The Imx immunoenzymatic assay (Abbott, Abbott Park, IL) was used to measure the concentration of PSA in the cell culture fluids.

Statistical analysis

For each cell line, Dunnett's test was used to compare each dose with control. Standard error (SE) and Student's *t* tests were determined using INSTAT software.

RESULTS

Anti-proliferative effects of CAI on various prostate tumor cell lines

To examine the sensitivity of prostate tumor cells to CAI, we measured the anchorage-dependent growth of 4 different human prostate tumor cell lines over a 5-day period of treatment with the drug. These analyses were performed using the MTS cell viability assay and included, as a negative control, treatments with a metabolite of CAI containing a substituted benzophenone tail group, which previously has been demonstrated to be ineffective at inhibiting the growth of other types of malignant cell (Kohn *et al.*, 1994*a*). Figure 1 demonstrates that CAI, in low micromolar concentrations, caused a dose-dependent inhibition of the growth of DU-145, PC3, LNCaP and PPC-1 tumor cells. The degree of inhibition varied between the cell lines, and the apparent IC_{50} values were generally between 10 and 30 μM. The inactive analog of CAI was found to have no effect on the growth of DU-145 and PC3 cells when assayed under these conditions.

The effects of CAI on prostate tumor cell growth were also evaluated by direct cell enumeration measurements over a 7-day period of treatment. A representative assay involving DU-145 tumor cells is shown in Figure 2*a*. In confirmation of the MTS growth data, CAI effectively inhibited the growth of this tumor cell line. The apparent IC_{50} value at day 7 by this assay was 0.7 μM.

The above data suggest that CAI causes cytostatic growth suppression. To examine this possibility, the ability of DU-145 cells to resume growth after the withdrawal of CAI from growth-inhibited cell cultures was evaluated (Fig. 2*b*). Proliferation of DU-145 cells began following the removal of CAI from the cell cultures after day 3. Similar results of reversible growth inhibition were obtained with PC3 cells (data not shown).

As another measure of tumor cell growth, we examined the effects of CAI on the capacity of prostate tumor cell lines to form colonies in soft agar suspension. A marked dose-inhibitory effect (IC_{50} values ranging 0.5–1.4 μM) was observed for the anchorage-independent growth of LNCaP, PC3 and DU-145 tumor cells (data not shown).

We also examined the effects of CAI on the growth of several immortalized but non-tumorigenic human cell lines to

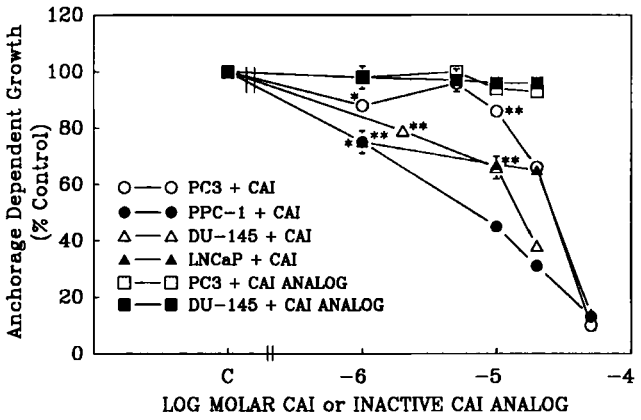

FIGURE 1 – Anti-proliferative effect of CAI on PC-3, DU-145, PPC-1 and LNCaP tumor cells measured by MTS viability assay; 5,000 cells were seeded in 96-well plates in medium containing 10% FBS without or with various concentrations of CAI and fed every 2 days with respective media. The number of viable cells was determined on day 5 by the MTS (Promega) cell proliferation assay. Points represent the mean ± SE of 2–3 experiments performed in quadruplicate. $^{*}p < 0.05$ from untreated control, $^{**}p < 0.01$ from untreated control (Dunnett's test). Higher doses were also significantly different from untreated control (asterisks not shown). For some points the standard error bar is not visible because SE is smaller than the symbol.

address the question of selectivity by CAI toward tumor cells. The immortalized cells included RHEK-1 and HaCaT squamous epithelial cells, an immortalized adult prostate epithelial cell line (pRNS-1-1), an immortalized fetal prostate epithelial cell line (FNC267B1) and MRC-5 lung fibroblasts. As shown in Figure 3, CAI inhibited the growth of these normal cells variably. Comparing Figure 3 with Figure 1, normal prostate epithelial cells (pRNS-1-1, FNC267B1) were less inhibited than PPC-1 or DU145 by 20 μM CAI and comparable in sensitivity to PC3 and LNCaP cells. Likewise, MRC-5 fibroblasts were less inhibited than PPC-1 or DU145 by 20 μM CAI but slightly more growth-inhibited than PC3 or LNCaP cells. Interestingly, compared with all of the other cell types, the 2 squamous epithelial cell populations, HaCaT and RHEK-1, were relatively resistant to the growth-inhibitory effects of low micromolar doses of CAI. These findings demonstrate the biological variability of cells to the anti-proliferative effects of CAI.

Inhibition of invasive potential

To examine how other phenotypic properties of prostate tumor cells might be affected by CAI, we tested the effects of the drug on the invasion of tumor cells through Matrigel-coated membranes in modified Boyden chambers. The tumor cell lines PC3, PPC-1 and DU-145 readily invaded Matrigel-coated membrane filters, whereas LNCaP cells failed to invade (Fig. 4). Treatment of PC3, PPC-1 or DU-145 cells with CAI inhibited chemoinvasion in a dose-dependent manner (Fig. 4).

CAI inhibits agonist-induced calcium mobilization in prostate tumor cells

The anti-tumor effects of CAI have been correlated with alterations in cellular calcium homeostasis (Cole and Kohn, 1994). We therefore examined the influence of CAI on calcium mobilization in response to carbachol and bombesin, 2 agonists previously shown to rapidly elevate cellular calcium in prostate tumor cells (Wasilenko *et al.*, 1996). Using suspensions of PC3 cells loaded with the calcium fluoroprobe Fura-2, we observed that the ability of each of these agonists to elevate free intracellular Ca^{2+} was reduced approximately 30–50% by a pre-incubation of the cells with CAI (Fig. 5). The negative control metabolite (50 μM) had no effect on calcium elevation by bombesin or UTP, another calcium agonist (data not shown). Similar attenuations of calcium signaling were observed in CAI-treated DU-145 and PPC-1 cells (data not shown).

PSA production by LNCaP cells

Since LNCaP cells, but not PC3, PPC-1 and DU145, produce measurable PSA in cell culture, we assessed how the production of this important tumor biomarker might be influenced by CAI. For this experiment, LNCaP cells were continuously exposed to varying doses of CAI for over 4 days. The results shown in Figure 6 show that CAI causes a dose-dependent reduction in the level of PSA accumulating in the medium of the LNCaP cell cultures. This apparent PSA inhibition was observed after 1 day of treatment with the drug and continued for subsequent exposures to CAI.

DISCUSSION

Our investigation demonstrates a general sensitivity of proliferating prostate epithelial cells to CAI, a novel voltage-independent calcium influx antagonist that interferes with selected calcium-sensitive transmembrane signal-transduction events (Cole and Kohn, 1994). We found that CAI caused a dose-dependent inhibition of cellular proliferation and invasiveness in human prostate tumor cells *in vitro*. These findings not only couple the inhibition of prostate tumor cell growth and invasiveness to the actions of an inhibitor of cellular signal transduction but also suggest an important role for calcium in the biology of prostate tumor cells.

By uniquely comparing tumorigenic as well as non-tumorigenic cells from the same cell lineage, *i.e.*, prostatic epithelial cells, the results of this study extend earlier observations about the effect of CAI on growth inhibition. As reported for other types of tumorigenic cells (Kohn *et al.*, 1992), we observed that low micromolar concentrations of CAI were anti-proliferative. In addition, we found that growth inhibition by CAI was not necessarily selective toward tumor cells since the proliferation of non-tumorigenic prostate human cell lines was inhibited to varying degrees by continuous exposure to CAI. None of the tumorigenic cell lines was resistant to CAI. In contrast, proliferation of the 2 human keratinocyte cell populations was minimally affected by CAI, suggesting that cells from different lineages may vary in their sensitivity to this drug. These differences in CAI sensitivity could be due to variability in cell cycle kinetics, drug metabolism or a higher dependency on calcium-sensitive functions for cell proliferation in certain types of cell. Under *in vivo* conditions, these factors might contribute to the minimal toxicity reported for CAI in various pre-clinical tumor models and certain dosing regimens during phase I clinical trials (Kohn and Liotta, 1995; Kohn *et al.*, 1996).

The chemoinvasion assays allowed us to assess *in vitro* how CAI might affect the spread of prostate tumor cells through the basement membrane, an early and key step in the metastatic cascade (Kohn and Liotta, 1995). CAI has been shown to block the *in vitro* motility of a variety of malignant cells and human endothelial cells in culture (Cole and Kohn, 1994; Kohn *et al.*, 1995). The drug also decreases cell adhesiveness to matrix components (Kohn *et al.*, 1995) and reduces gelatinase (MMP-2) gene expression in malignant and endothelial cells (Kohn *et al.*, 1994*b*, 1995). Adhesion, motility and localized proteolysis are important factors in tumor cell invasion and metastasis (Kohn and Liotta, 1995). Although we did not directly evaluate the effect of CAI on these specific param-

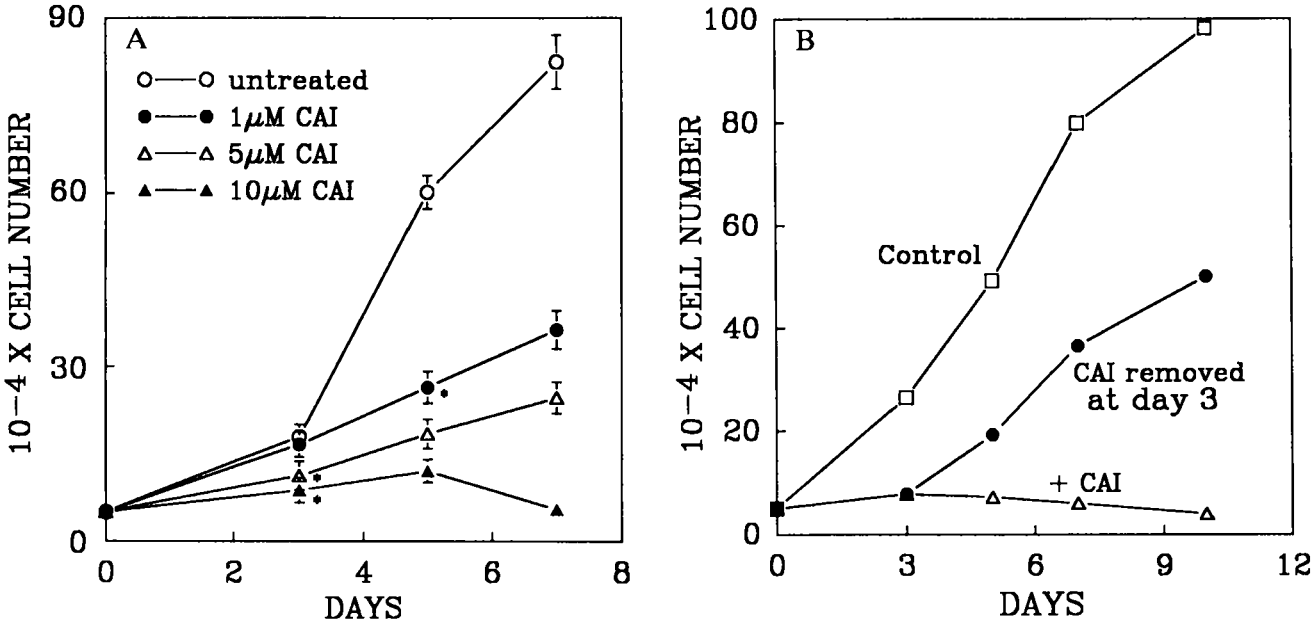

FIGURE 2 – Anti-proliferative effect of CAI and its reversibility on DU-145 tumor cells measured by cell counting. *(a)* Cells (50,000) were plated in 12-well dishes in growth medium containing 5% FBS without or with the designated CAI concentration. Cell cultures were fed every 2 days with respective media. On the designated days, cells from replicate wells were harvested with trypsin and counted. Values are the mean ± SE of duplicate experiments performed with 2 or 3 samples per point. $*p < 0.01$ from non-treated control at representative time point (Student's *t* test). Latter time points were also significantly different from untreated control (asterisks not shown). For some points the standard error bar is not visible because SE is smaller than the symbol. *(b)* Cells were grown in medium without or with 10 μM CAI. After 3 days of treatment, the CAI medium in one set of cells was removed and replaced with regular growth medium. On the designated days, cells were counted as in *(a)*. Points are the mean of duplicate samples from a typical experiment performed 3 times. Mean variance < 10%.

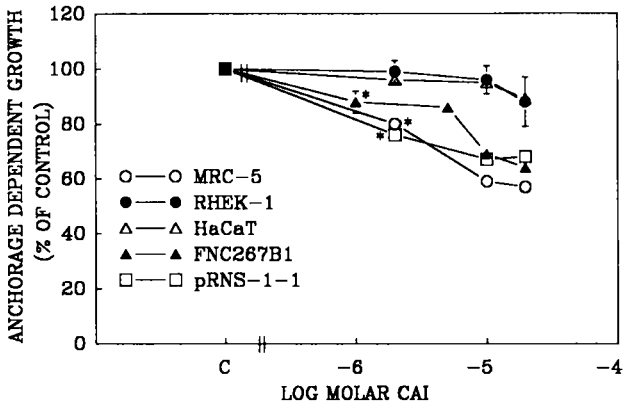

FIGURE 3 – Effect of CAI on the proliferation of immortalized human cell lines measured by anchorage-dependent growth. Cells (7,500) were seeded in 96-well plates in medium containing 10% FBS without or with 0.2, 2.0 or 20 μM CAI and fed every 2 days with respective media. The number of viable cells was determined on day 5 by the MTS cell proliferation assay. Points are the mean ± SE of 2–3 independent experiments performed in quadruplicate. $*p < 0.05$ from untreated control (Dunnett's test). Higher doses were also significantly different from untreated control (asterisks not shown). For some points the standard error bar is not visible because SE is smaller than the symbol.

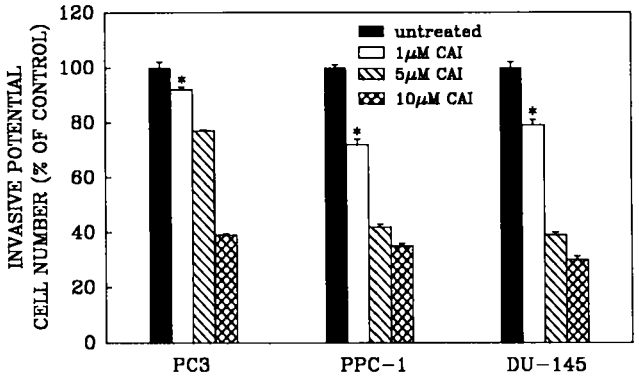

FIGURE 4 – Effect of CAI on invasive potential of prostate tumor cell lines PC-3, PPC-1 and DU-145. Invasion assays were conducted using Matrigel-coated PVP membranes in modified Boyden chambers. Cells were exposed to CAI for approximately 18 hr prior to and during the assays. Invasion was measured after 5 hr of exposure to 3T3 cell conditioned medium. Columns are the mean ± SE of 3 independent experiments performed with 6 samples per treatment. $*p < 0.01$ from untreated control (Student's *t* test). Higher doses were also significantly different from untreated control (asterisks not shown).

eters, an inhibition in any one of these events could have led to the markedly decreased chemoinvasion observed in this study.

Since calcium is a pleiotropic second messenger in cell regulation and function, it is likely that CAI will affect several cellular events simultaneously. Hupe *et al.* (1990) reported that phosphoribosyl pyrophosphate synthetase, an enzyme indirectly regulated by calcium and involved with the first step in nucleotide biosynthesis, was inhibited in MDBK bovine kidney cells treated with CAI. Inhibition of this enzyme resulted in a decrease in nucleotide biosynthesis that correlated with the inhibition of calcium influx and proliferation by these cells. Subsequent work has demonstrated that CAI attenuates ago-

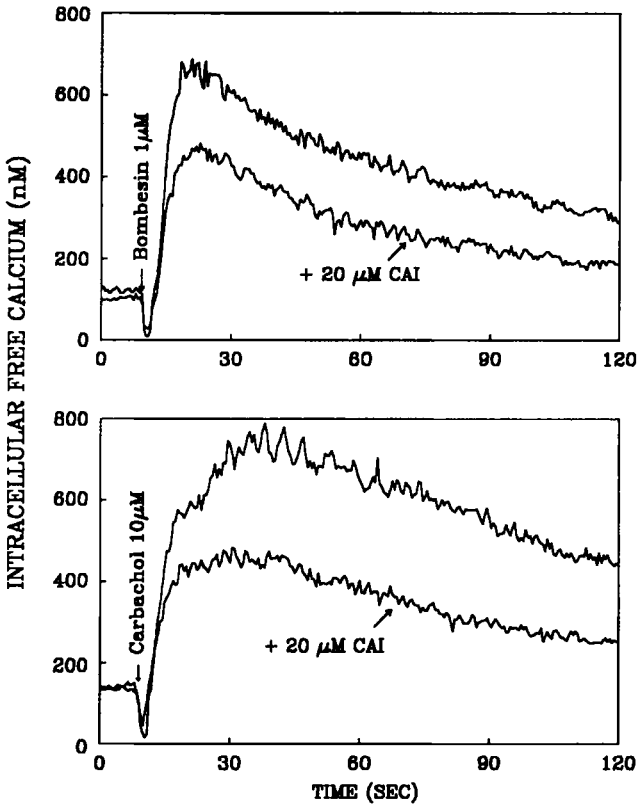

FIGURE 5 – Effect of CAI on agonist-induced elevation of $[Ca^{2+}]$ in PC-3 cells. PC-3 cells were loaded with Fura-2 and pre-incubated with 20 μM CAI for 30 min before agonist (bombesin 1 μM, carbachol 10 μM) addition, indicated by the arrow. Untreated cells were incubated with an equivalent amount of vehicle (DMSO). The results shown are representative of 5–7 experiments. Variability between replicates generally was less than 15% in a given experiment.

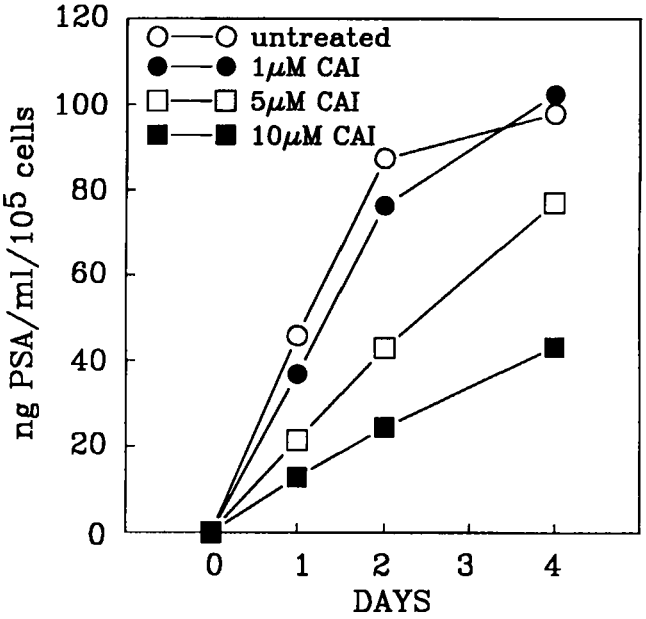

FIGURE 6 – Effect of CAI on cumulative PSA production by LNCaP tumor cells. Cells (10^5) were seeded in T25 flasks in growth medium without or with the designated CAI concentration. At indicated time intervals, medium from duplicate wells was assayed for PSA levels by the IMX immunoassay (Abbott) and the cell number determined by counting cells in a hemocytometer. Points are means from a representative experiment performed 3 times in duplicate, with variance generally < 10% of mean values. On day 4, the mean number of cells in the various samples was control (2.4×10^5), 1 μM CAI (1.7×10^4), 5 μM CAI (1.3×10^4) and 10 μM CAI (1.2×10^4). A 48-hr treatment with the non-active CAI analog (20 μM) had no effect on PSA production (data not shown).

nist-induced elevations of cellular calcium through a mechanism involving inhibition of non-voltage-gated calcium channels (Hupe *et al.*, 1991; Felder *et al.*, 1991). This property of CAI has been linked to the inhibition of calcium-dependent signal-transduction steps required for arachidonic acid release and selected tyrosine kinase phosphorylations (Felder *et al.*, 1991; Kohn *et al.*, 1994*a*, 1995). In agreement with these earlier studies, we observed an attenuation of agonist-induced calcium elevation in several prostate tumor cell lines treated with CAI. These data, considered along with the observation that an analog of CAI unable to inhibit calcium signaling also failed to inhibit prostate tumor cell proliferation, strongly suggest that perturbations in calcium homeostasis contribute to the modulation of prostate tumor cell phenotype by CAI. As with most drugs, however, CAI may affect other cellular processes in addition to calcium homeostasis.

Another key finding was that CAI markedly reduced the measurable level of PSA recovered from the culture fluids of LNCaP cells. PSA is a serine protease and glycoprotein with similarities to glandular kallikrein. Production of this protease, which is elevated in prostate adenocarcinomas, is often used as an indicator of disease progression (Stamey and Kabalin, 1989). Because CAI inhibited both cell proliferation and the expression of PSA in LNCaP cells, it may be possible to utilize PSA production for monitoring the response to therapy by CAI in pre-clinical or clinical studies on prostate cancer. Additionally, the mechanism by which CAI modulates PSA production in prostate tumor cells deserves further study. Since the PSA levels were normalized to the number of viable cells on each sampling day, it is possible that an inhibition in either the synthesis and/or secretion of PSA may underlie the reduced PSA production in CAI-treated LNCaP cell cultures. The latter may be due to some general but poorly understood effect of CAI on cellular protein expression or modulations of signal-transduction pathways required for PSA expression.

In contrast to conventional chemotherapeutic agents, many inhibitors of cellular signal transduction often cause cytostatic rather than cytotoxic responses in malignant cells (Powis, 1991; Kohn and Liotta, 1995; Levitzki, 1994). We observed that CAI was cytostatic against the prostate tumor cell line DU145 *in vitro*. This finding is in agreement with previous results showing that CAI has cytostatic effects against some tumor cells (Cole and Kohn, 1994; Kohn and Liotta, 1995). Current mainstays for the treatment of localized prostate cancer include radical prostatectomy and X-irradiation. Androgen deprivation has usually been reserved for metastatic disease, and cytotoxic drugs have been employed to treat hormone-refractory disease. Treatments with cytostatic agents like CAI may be beneficial as adjuvant therapies with androgen deprivation to delay the appearance of androgen-independent metastatic disease and/or combined with cytotoxic agents to improve efficacy in hormone-refractory disease. In support of these possibilities, we have observed in preliminary studies that CAI synergizes with doxorubicin to inhibit the growth of PC3 cells *in vitro*. Synergistic interactions between CAI and quercetin

have also been observed in human breast carcinoma cells (Yeh *et al.*, 1995).

In summary, our findings indicate that an inhibitor of cellular signaling can have multiple effects on the malignant properties of human prostate tumor cells. CAI and other signal-transduction therapies may be useful in the design of new strategies for the treatment of advanced-stage prostate cancer as well as for cancers at earlier stages. Further studies with CAI alone or in combination with other therapies appear warranted in appropriate pre-clinical animal tumor models of prostate cancer.

ACKNOWLEDGEMENTS

We thank Dr. P. Kolm for assistance with statistical analysis. We thank Drs. H. Kleinman and M. Kibbey, NIDR, Bethesda, MD, for Matrigel and Dr. Y. Wu for help with invasion assays. This study was supported by a grant from CaP CURE.

REFERENCES

BLACKMORE, P.F., OAKES, S.G. and SOMERS, K.D., Altered H1 histamine receptor signaling in BALB/3T3 cells transformed by v-K-*ras* and v-H-*ras* oncogenes. *Oncogene,* **7,** 2053–2057 (1992).

CHEN, T.R., Chromosome identity of human prostate cancer cell lines PC-3 and PPC-1. *Cytogenet. Cell Genet.,* **62,** 183–184 (1993).

CHUNG, L.W.K., Fibroblasts are critical determinants in prostatic cancer growth and dissemination. *Cancer Metastasis Rev.,* **10,** 263–274 (1991).

COLE, K.A. and KOHN, E.C., Calcium-mediated signal transduction: biology, biochemistry, and therapy. *Cancer Metastasis Rev.,* **13,** 33–41 (1994).

FELDER, C.C., MA, A.L., LIOTTA, L.A. and KOHN, E.C., The antiproliferative and antimetastatic compound L651582 inhibits muscarinic acetycholine receptor-stimulated calcium influx and arachidonic acid release. *J. Pharmacol. Exp. Ther.,* **257,** 967–971 (1991).

HUPE, D.J., BEHRENS, N.D. and BOLTZ, R., Anti-proliferative activity of L-651,582 correlates with calcium-mediated regulation of nucleotide metabolism at phosphoribosyl pyrophosphate synthetase. *J. cell. Physiol.,* **144,** 457–466 (1990).

HUPE, D.J., BOLTZ, R., COHEN, C.J., FELIX, J., HAM, F., MILLER, D., SODERMAN, D. and VAN SKIVER, D., The inhibition of receptor-mediated and voltage dependent calcium entry by the antiproliferative L-651,582. *J. biol. Chem.,* **266,** 10136–10142 (1991).

ISAACS, J.T., SCHULZE, H. and COFFEY, D.S., Development of androgen resistance in prostatic cancer. *In:* G.P. Murphy, S. Khoury, I.R. Kuss, C. Chatelain and L. DENIS (EDS.), *Prostate cancer, part A: research, endocrine treatment, and histopathology,* pp. 21–31, A.R. Liss, New York (1987).

KAIGHN, M.E., REDDEL, R.R., LECHNER, T.J., PEEHL, D.M., CAMALIER, R.F., BRAH, D.E., SAFFIOTTI, U. and HARRIS, C.C., Transformation of human neonatal prostate epithelial cells by strontium phosphate transfection with a plasmid containing SV40 early region genes. *Cancer Res.,* **49,** 3050–3056 (1989).

KOHN, E.C., ALESSANDRO, R., SPOONSTER, J., WERSTO, R.W. and LIOTTA, L.A., Angiogenesis: role of calcium-mediated signal transduction. *Proc. nat. Acad. Sci. (Wash.),* **92,** 1307–1311 (1995).

KOHN, E.C., FELDER, C.C., JACOBS, W., HOLMES, K.A., DAY, A., FREER, R. and LIOTTA, L.A., Structure–function analysis of signal and growth inhibition by carboxyamidotriazole, CAI. *Cancer Res.,* **54,** 935–942 (1994*a*).

KOHN, E.C., JACOBS, W., KIM, Y.S., ALESSANDRO, R., STETLER-STEVENSON, W.G. and LIOTTA, L.A., Calcium influx regulates matrix metalloproteinase-2 (72 kDa type IV collagenase, gelatinase A) expression. *J. biol. Chem.,* **269,** 21505–21511 (1994*b*).

KOHN, E.C. and LIOTTA, L.A., Molecular insights into cancer invasion: strategies for prevention and intervention. *Cancer Res.,* **55,** 1856–1862 (1995).

KOHN, E.C., REED, E., SAROSY, E., CHRISTIAN, M., LINK, C.J., COLE, K.A., FIGG, W.D., DAVIS, P.A., JACOB, J., GOLDSPIEL, B. and LIOTTA, L.A., Clinical investigation of a cytostatic calcium influx inhibitor in patients with refractory cancers. *Cancer Res.,* **56,** 569–573 (1996).

KOHN, E.C., SANDEEN, M.A. and LIOTTA, L.A., *In vivo* efficacy of a novel inhibitor of selected signal transduction pathways including calcium, arachidonate, and inositol phosphates. *Cancer Res.,* **52,** 3208–3212 (1992).

LEE, M.S., GARKOVENKO, E., YUN, J.S., WEIJERMAN, P.C., PEEHL, D.M., CHEN, L.S. and RHIM, J.S., Characterization of adult human prostatic epithelial cells immortalized by polybrene-induced DNA transfection with a plasmid containing an origin-defective SV 40 genome. *Int. J. Oncol.,* **4,** 821–830 (1994).

LEVITZKI, A., Signal-transduction therapy: a novel approach to disease management. *Europ. J. Biochem.,* **226,** 1–13 (1994).

POWIS, G., Signaling targets for anticancer drug development. *Trends Pharmacol. Sci.,* **12,** 188–194 (1991).

RHIM, J.S., JAY, G., ARNSTEIN, P., PRICE, F.M., SANFORD, K.K. and AARONSON, S., Neoplastic transformation of human epidermal keratinocytes by Ad12-SV40 and Kirsten sarcoma viruses. *Science,* **227,** 1250–1252 (1985).

STAMEY, T.A. and KABALIN, J.N., Prostate specific antigen in the diagnosis and treatment of adenocarcinoma of the prostate. I. Untreated patients. *J. Urol.,* **141,** 1070–1075 (1989).

WARE, J.L., Growth factors and their receptors as determinants in the proliferation and metastasis of human prostate cancer. *Cancer Metastasis Rev.,* **12,** 287–301 (1993).

WASILENKO, W.J., COOPER, J., PALAD, A.J., SOMERS, K.D., BLACKMORE, P.F., RHIM, J.S., WRIGHT, G.L. and SCHELLHAMMER, P.F., Calcium signaling in prostate cancer cells: evidence for multiple receptors and enhanced sensitivity to bombesin/GRP. *Prostate* (In press) (1996).

YEH, Y.A., HERENYIOVA, M. and WEBER, G., Quercetin: synergistic action with carboxyamidotriazole in human breast carcinoma cells. *Life Sci.,* **57,** 1285–1292 (1995).