In Vivo Efficacy of a Novel Inhibitor of Selected Signal Transduction Pathways Including Calcium, Arachidonate, and Inositol Phosphates

Elise C. Kohn, Mary Ann Sandeen, and Lance A. Liotta

Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20892; Laboratory Animal Science Program, Program Resources, Inc./DynCorp, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

ABSTRACT

Aberrant signal transduction has been implicated in malignant transformation, growth, and progression. This has led to the proposal to use inhibitors of signal transduction pathways to treat cancer. One approach to circumventing potential toxicity and improving efficacy would be to target pathways upon which cancer cells selectively depend. Pathways associated with the malignant process involve calcium fluxes, the release of arachidonic acid, and the generation of phosphoinositides. In this report, CAI (L651582, NSC 609974), a substituted carboxyamido-imidazole and novel inhibitor of these selected signal transduction pathways, inhibits anchorage-dependent and -independent growth in a large series of human cancer cell lines. CAI pretreatment of HT-29 human colon cancer and 5R ras-transfected rat embryo fibroblast cells inhibits the formation and growth of experimental pulmonary metastases in nude mice. Oral administration of CAI in PEG-400 vehicle arrests growth and metastasis of transplanted human melanoma and ovarian cancer xenografts. No significant gross or histological toxicity was observed at CAI doses yielding blood levels in the concentration range demonstrated to inhibit select signal transduction pathways in vitro. These data indicate the feasibility and demonstrate a potential selectivity and sensitivity of using specific signal transduction inhibitors for the experimental treatment of cancer.

INTRODUCTION

Perpetual stimulation of GTP binding proteins coupled to second messenger pathways has been implicated in uncontrolled proliferation and oncogenesis (1, 2). Studies of cells transfected with members of the family of muscarinic receptors mAChR3 (3, 4), serotonin receptors (5), and the mas oncogene (6) have revealed candidate pathways selectively correlated with colony formation and tumorigenicity. mAChR m1, m3, and m5 receptor subtypes are coupled to phosphatidylinositol metabolism, arachidonic acid release, and the opening of calcium channels and have stimulatory effects upon adenylyl cyclase (7-9), whereas mAChR m2 and m4 subtypes are coupled to the inhibition of adenylyl cyclase. Only the former odd-numbered mAChR were capable of inducing NIH/3T3 cells to form foci of transformation in the presence of the muscarinic agonist carbachol. We have recently identified a synthetic compound which preferentially blocks second messenger pathways of calcium influx and arachidonic acid metabolism, which are selectively coupled to the odd-numbered mAChR (10). This agent, CAI (Fig. 1), constitutes a unique means of testing the general role of this group of second messenger pathways in tumor growth (11).

mAChR m5-transfected Chinese hamster ovary cells were used to identify signal transduction pathways inhibited by CAI treatment. In these studies, 10 μM CAI had an immediate effect on the release of arachidonic acid, generated by the muscarinic agonist carbachol, and receptor-mediated calcium influx. A minimal effect was seen on carbachol-stimulated inositol phosphate release; no effect was seen when the carbachol inhibition of adenyl cyclase function was tested in mAChR m2-transfected Chinese hamster ovary cells (10). These results were confirmed in neutrophils, in which CAI inhibited N-formylmethionyl-leucyl-phenylalanine-mediated arachidonic acid metabolism and calcium influx (12). In addition, we have demonstrated previously that preincubation of A2058 human melanoma cells with 4 μM CAI inhibited the generation of inositol phosphates after stimulation with autocrine motility factor (11). Together, these data suggest a select role for CAI in interrupting signals mediated through the specific receptor-linked pathways of phospholipases C and A2

MATERIALS AND METHODS

Growth Assays. For adherent monolayer growth assays, 5 x 10⁴ cells/well in 24-well plates were cultured in increasing concentrations of CAI or vehicle control. After 96 h of growth, cultures were washed and stained with crystal violet nuclear stain. Specifically bound stain was eluted, and the absorbance was determined. Results were determined as the percentage of vehicle control growth. Single cell suspensions of A2058 and OVCAR3 human tumor cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and either dimethyl sulfoxide vehicle or indicated concentrations of CAI in 0.3% agar plated over a basal layer of 0.5% agar in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Colony formation was quantitated at 10-12 days. Colony-forming efficiency was in the range of 1% for both cell lines.

Animal Efficacy Studies. 5R (13), HT-29, A2058, and OVCAR3 (14) lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum until use. All animal data presented are indicative of three independent experiments; in general, 10 animals were used per treatment group. Experimental metastasis assays consisted of nude mouse tail vein inoculation of 5 x 10⁴ 5R or 10⁵ HT-29 cells which had been preincubated with vehicle or 20 μM CAI. Pulmonary metastases were counted at 14 and 21 days for 5R and HT-29 lines, respectively.

The antitumor effect of CAI was tested using groups of 10 nude mice/treatment condition, inoculated with either 10⁴ A2058 cells at two flank sites or i.p. with 30 million OVCAR3 cells. On day 3 after tumor introduction, CAI diluted in PEG-400 was administered by gavage at the indicated doses. Animals were monitored daily for toxicity, behavior, and extent of tumor. Weights, bidirectional measurements of A2058 tumor masses, and degree of ascites in OVCAR3-bearing mice were determined 3 times/week. At the indicated times, animals were sacrificed humanely, and specimens were taken for histological analysis.

The ability of CAI treatment to prevent initiation and growth of...
experimental metastases was determined by varying the time of initiation of CAI and vehicle treatment with respect to the time of inoculation with tumor cells as indicated. Orally administered CAI or PEG-400 vehicle was administered to nude mice (n = 20/group) for 5 days, and on day 6 \(10^4\) 5R cells were introduced via the tail vein. Following inoculation, animals were randomized into four groups (n = 10/group) to receive treatments as indicated in Fig. 3 for 10 days, after which the lungs were removed and pulmonary metastases were counted. Histological confirmation of the fibrosarcoma due to the 5R cells was obtained.

**Results**

**Inhibition of Tumor Cell Growth in Vitro.** The hypothesis that inhibition of selected important signal transduction pathways can impact on cancer proliferation in vitro was tested with adherent monolayer growth assays and soft agar colonization assays. The latter were used as a marker of transformation as well as for assessment of tumor proliferative capacity. Table 1 shows a profound effect of CAI (<20 µM) on the adherent proliferation of a wide spectrum of human tumor and rodent cell lines in culture, including lines carrying activating oncogenes such as the T24 human bladder cancer, which has activated H-ras. A range of sensitivity is evident. The 50% inhibitory concentrations for these cell lines varied between 2 and 10 µM. Studies are ongoing to address the impact of CAI on the growth of MCF 10A cells transfected with neu, transforming growth factor α, and ras. Fig. 2 presents the marked dose-dependent inhibitory effect of CAI on A2058 human melanoma and OVCAR3 human ovarian cancer cell colonization in soft agar; a representative photomicrograph demonstrates inhibition of colony size as well as colony number. The inhibitory concentrations for the soft agar colonization assays were 0.2–0.5 µM, at least one log more sensitive than suggested by the adherent monolayer growth assays. The soft agar growth inhibition results are within the range of physiologically attainable plasma concentrations in animal models (see below).
Inhibition of Primary Tumors and the Formation and Growth of Metastases in Nude Mice by p.o. Administration of CAI. The utility of inhibition of signal transduction as a new approach to cancer therapy requires that these in vitro effects translate to efficacy in vivo. The specificity of the effect of CAI on tumor cells was investigated by tumor cell preincubation with CAI and washing prior to introduction to nude mice via the tail vein. Only cell populations which excluded >95% trypan blue were used; no real effect on cell growth in culture was demonstrated with only a 24-h exposure to drug (data not shown). HT-29 human colon cancer cells or 5R ras-transfected rat embryo fibroblasts were cultured with 20 μM CAI prior to tail vein inoculation.

Pretreatment of HT-29 cells inhibited pulmonary colonization by 96%, from 40 ± 32 (SD) (0–89) to 1.5 ± 2 (0–6) (n = 9/group; P < 0.003). Experiments with 5R demonstrated a similar profound effect with >250 pulmonary metastases/lung from vehicle-treated cells compared with <10 colonies/lung from CAI-treated cells. This direct effect of CAI on tumor cells led into experiments of p.o. administration of CAI to test its effects on host and tumor.

CAI is a lipophilic compound for which solubility and good oral bioavailability was achieved without vehicle-related toxicity using PEG-400 vehicle. A2058 cells were inoculated s.c. into the flanks of nude mice, after which measurable tumors were observed within 14 days. CAI in PEG-400 was given by gavage beginning on day 3 after tumor introduction. There was significant growth arrest (Fig. 3A) as well as a marked delay in tumor incidence in CAI-treated animals. Marked necrosis in tumors from the CAI-treated animals was seen at histological evaluation of necropsy specimens (Fig. 3C). No gross toxicity was observed during the 24 days of CAI administration (food and water intake, weight loss, inactivity) or at necropsy and histological review of major organs (lungs, liver, spleen, kidney, gastrointestinal tract, bone marrow, skin, diaphragm).

OVCAR3, a human ovarian cancer line which produces extensive intraperitoneal disease with diaphragmatic and serosal implants, liver metastases, and malignant ascites (14), was introduced i.p., followed 3 days later by daily CAI, 100 mg/kg p.o., or PEG-400. Statistically significant differences were seen in time to development of ascites (P < 0.005; not shown), body weight as a marker of total body tumor burden (Fig. 3B; P <
0.001), and volume of ascites at necropsy (PEG-400, 0.7 + 0.6 ml, versus CAI, 0.3 + 0.2 ml; P< 0.005). Histology of necropsy specimens (Fig. 3C) showed CAI-induced tumor necrosis and inhibition of pulmonary micrometastases.

CAI prevention of the formation and growth of experimental metastasis was tested by oral pretreatment of mice for 5 days, inoculation with untreated 5R cells, and then continuing treatment as indicated (Fig. 4). CAI prior to tumor cell injection prevented metastatic colonization as effectively as CAI treatment given only after tumor cell inoculation or when given continuously (Fig. 4). This is in keeping with the known sustained circulating plasma levels of CAI (Table 2). No toxicity was noted during treatment or at necropsy.

Determination of Circulating Plasma Concentrations of CAI. A cohort of nude mice was inoculated with A2058 cells and treated with 100 or 250 mg/kg/day as described for the experiment in Fig. 4A. These animals were sacrificed humanely, at 4-day intervals 18 h after p.o. administration of CAI, and plasma was frozen until evaluation by HPLC (15). Table 2 shows that the CAI plasma levels ranged from 1 to 5 µg/ml in the 100 mg/kg/day cohort and from 5 to 10 µg/ml in the 250 mg/kg/day group. These plasma levels are in the range determined to be biologically significant in the inhibition of signal transduction and proliferation in in vitro studies.

DISCUSSION

In this report, we have demonstrated the in vivo anticancer efficacy of a novel compound whose mechanism of action is to inhibit selected signal transduction pathways. This drug, CAI (L651582, NSC609974), is effective in inhibiting growth and colony formation in vitro as well as in experimental and spontaneous metastasis systems and in animal tumor xenograft models. These in vitro and in vivo results were obtained with similar concentrations of CAI either in the culture media or demonstrated in the plasma after simple p.o. drug administration.

Table 2 CAI plasma levels (µg/ml) from nude mice bearing A2058 human xenografts

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>100 mg/kg/day</th>
<th>250 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.33 ± 1.45</td>
<td>5.51 ± 2.07</td>
</tr>
<tr>
<td>5</td>
<td>1.12 ± 0.48</td>
<td>6.17 ± 1.23</td>
</tr>
<tr>
<td>9</td>
<td>1.55 ± 0.33</td>
<td>4.96 ± 0.34</td>
</tr>
<tr>
<td>13</td>
<td>2.16 ± 0.88</td>
<td>7.70 ± 2.30</td>
</tr>
<tr>
<td>17</td>
<td>5.34 ± 1.50</td>
<td>10.0 ± 0.82</td>
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</tbody>
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Aberrant signal transduction has been linked to tumorigenesis and metastatic spread (1–5, 13, 16). These abnormalities in signal transduction may be due to inappropriate expression and activation of receptors (3, 5, 6), point-mutated transducers such as G proteins (2), or oncogene activation (1, 2, 17, 18). Inositol phosphate and arachidonic acid production and calcium influx appear to be closely associated with many receptor-driven events, including mitogenesis (4, 19), vision (20), olfaction (21), and both normal and malignant cellular locomotion (16, 22, 23). Ligand-driven transforming events have been demonstrated for the mACHR and serotonin receptor subtypes specifically linked to these pathways (3, 5). These activities are important subsets of the common signal transduction pathways which have been linked to cancer biology. The common theme is the coupling of the abnormal signal with the transforming events(s).

We have identified this linkage as a target for anticancer drug development. This specificity identifies a subset of signal transduction pathways important in cancer development and dissemination and underscores both the importance of understanding these pathways and the need to target them for investigation. These ligand-dependent events offer models from which to demonstrate the biological importance of these specific biochemical pathways by using the selective inhibitor CAI. We have demonstrated that CAI can inhibit receptor-mediated calcium influx, arachidonic acid release, and the generation of inositol phosphates in vitro in concentrations which are physiologically attainable in vivo (10, 11, 15).

One other agent with selectivity for RCME has been studied. SKF 96365 is a substituted imidazole which inhibits RCME in platelets, endothelial cells, and neutrophils and blocks voltage-gated L-type calcium channels in vascular smooth muscle cells under patch clamp (24); based upon the anticancer properties of CAI, SKF96365 becomes a candidate anticancer drug. Hupe et al. (12) demonstrated that CAI (L651582) also inhibited L- and T-type voltage-gated channels in guinea pig atrial cells and RCME due to the formyl peptide, N-formyl-methionyl-leucyl-phenylalanine, in neutrophils. Neither study evaluated the effect of its respective agents on malignant cells. We demonstrated that CAI inhibited RCME due to muscarinic agonist stimulation of Chinese hamster ovary m5 mAChR-bearing cells. Preliminary results in our laboratory have shown that CAI inhibits RCME in A2058 melanoma cells. Another question for study is the specificity of CAI for different effector enzyme subtypes, notably phospholipase C-γ, which is regulated by tyrosine phosphorylation (25, 26). The role of ligand-generated calcium as a potential regulator of phospholipase C-γ has not been defined; CAI may be useful as a tool in those experiments.

As with any agent, other mechanisms of action may account for the therapeutic effects which are observed. Hupe and colleagues (27) showed that CAI inhibited precursor incorporation into nucleotide pools in MDBK cells; in comparable CAI concentrations, inhibition of MDBK cells, HeLa cells, and fibroblast proliferation in monolayer culture was seen. Our cell line
screen found that CAI inhibited in vitro proliferation of the normal human skin fibroblast line CCD27.sk (50% inhibitory concentration \( \sim 1.0 \mu g/ml \)). The growth-inhibitory effects seen against normal and malignant cell lines in culture were not borne out by studies of CAI's effects in vivo. No toxicity to normal tissues including connective tissue, mucosal surfaces, and bone marrow was demonstrated when necropsy sections from nude mice receiving oral CAI were reviewed. These observations underscore the selectivity demonstrated by the in vivo effects of CAI.

Signaling events are so primary in cellular function that any agent which interferes with signal-effector coupling should be uniformly toxic to normal cells and tissues as well as to malignant and metastatic cells and tissues. Yet, CAI is not toxic to the animal grossly or at more detailed histological evaluation. This seeming dichotomy defends the hypothesis that malignant cells have a higher state of dependence upon certain second messenger pathways, rendering them selectively sensitive to CAI. Thus, we have targeted a pathway which is important in malignant growth and metastatic dissemination and have demonstrated that interruption of the coupling of this pathway to the end effect may be accomplished by an orally active, nontoxic novel compound, CAI. A Phase I clinical study of CAI for refractory solid tumor patients has opened for accrual.

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REFERENCES

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