Cancer Chemother Pharmacol (1994) 34: 515–521

Cancer Chemotherapy and Pharmacology



# CAI: effects on cytotoxic therapies in vitro and in vivo

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Received: 29 November 1993 / Accepted: 6 May 1994

**Abstract.** CAI (NSC 609974; L651582), a new agent that has demonstrated antimetastatic activity in vitro and in vivo, was not very cytotoxic toward EMT-6 mouse mammary carcinoma cells in culture or toward FSaIIC fibrosarcoma cells in vivo. Coexposure of EMT-6 cells to CAI and antitumor alkylating agents under various environmental conditions did not markedly increase the cytotoxicity of cisplatin (CDDP), melphalan, or carmustine (BCNU). However, the combination of CAI and 4-hydroperoxycyclophosphamide (4-HC) produced much greater than additive killing of EMT-6 cells. CAI also increased the sensitivity of hypoxic EMT-6 cells to X-rays. CAI increased the cytotoxicity of cyclophosphamide toward FSaIIC tumor cells when animals were treated with single doses of both drugs. The effect of CAI on tumor cell killing by cyclophosphamide was greatest at high doses of the antitumor alkylating agent. CAI administration appeared to result in increased serum levels of prostaglandin $E_2$ and leukotriene $B_4$ in animals bearing the Lewis lung tumor. Administration of CAI on days 4–18 did not alter the growth of the Lewis lung carcinoma but did result in an increase in the tumor-growth delay produced by treatment with CDDP, cyclophosphamide, melphalan, BCNU, and fractionated radiation. Although CAI did not reduce the number of lung metastases present in Lewis lung carcinoma-bearing mice on day 20, it did appear to reduce the number of large (vascularized) metastases present on that day.

**Key words:** L651582 – CAI – Combination therapy – Antitumor alkylating agents

## Introduction

CAI (NSC 609974; L651582), a carboxyamidotriazole originally prepared as a coccidiostat, has demonstrated antimetastatic activity in in vitro and in vivo model systems [2, 6, 12–17, 19, 45]. Although the mechanism of action of CAI remains to be fully elucidated, the effect of this molecule appears to occur at the level of the plasma membrane and to involve interference with signal-transduction pathways involving calcium influx and release of arachidonic acid and inositol phosphates [12, 14, 17].

Metastases and tumor growth are ongoing processes in malignant disease. It is therefore likely that agents such as CAI would be given along with cytotoxic therapies to eradicate the malignant cells. The current study was undertaken to examine combination of CAI with cytotoxic therapies, antitumor alkylating agents, and radiation in vitro and in vivo with the goal of determining whether additive or greater-than-additive tumor-cell cytotoxicity could be achieved.

## Materials and methods

*Drugs.* CAI (NSC 609974; L651582) was obtained as a gift from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, Md.). 4-Hydroperoxycyclophosphamide (4-HC) was obtained as a gift from Asta Medica Aktiengesellschaft (Frankfurt am Main, Germany). *cis*-Diamminedichloroplatinum(II) (CDDP), melphalan (L-phenylalanine mustard, L-PAM), *N,N*-bis-(2-chloroethyl)-*N*-nitrosourea (carmustine, BCNU), and cyclophosphamide were purchased from Sigma Chemical Co. (St. Louis, Mo.).

*Cell culture.* EMT-6 mouse mammary tumor cells have been widely used for the study of hypoxia [23–25]. Cultures were maintained in exponential growth in Waymouth's medium (I.S.I. Corp., Chicago, Ill.) supplemented with 15% newborn calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml; Grand Island Biological Co., Grand Island, N.Y.). The doubling time of these cultures, growing at 37° C in a humidified 5% $CO_2$/95% air atmosphere, was 16–19 h [26]. In vitro plating efficiencies of control cultures were 65%–80%.

This work was supported by NIH grants PO1-19589 and RO1-50174

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The pH of the medium was adjusted using a sodium bicarbonate ($NaHCO_2$)/5% $CO_2$ buffer system [10]. To produce hypoxia, the plastic flasks, containing exponentially growing monolayers in complete medium plus serum, were fitted with sterile rubber septa and exposed to a continuously flowing 95% $N_2$/5% $CO_2$ humidified atmosphere for 4 h at 37° C as previously reported [35, 36]. Parallel flasks were maintained in 95% air/5% $CO_2$. At the end of 4 h, the drug or vehicle was added to the flasks by injection through the rubber septum without disturbing the hypoxia. EMT-6 cells were exposed to various concentrations (5, 10, 50, 100, 250, or 500 μ*M*) of CAI under normally oxygenated conditions for 19 h. In combination-treatment experiments, EMT-6 cells were exposed to 50 μ*M* CAI for 4 h prior to and during 1-h exposure to melphalan, 4-HC, CDDP, and BCNU or during radiation delivery and for an additional 19 h. In the combination studies, hypoxia was maintained for the first 5 h of drug exposure.

Cell viability was measured by the ability of single cells to form colonies as described previously [35, 36]. Each experiment was repeated three times, and each data point per experiment represents the results obtained for three different dilutions of cells plated in duplicate.

*Tumor excision assay.* The FSaII fibrosarcoma [22] adapted for growth in culture (FSaIIC) [37, 38] was carried in C3H/He male mice (Jackson Laboratories, Bar Harbor, Me.). For the experiments, $2\times10^6$ tumor cells prepared from a brei of several stock tumors were implanted s.c. into the legs of 8 to 10 week-old C3H/He male mice. On day 7 after tumor implantation when the tumors measured about 100 $mm^3$ in volume, the mice were given CAI (100, 300, 400, or 500 mg/kg) by i.p. injection or CAI (100 mg/kg) followed by cyclophosphamide (100, 300, or 500 mg/kg, i.p.) On day 8 the mice were euthanized.

The tumors were excised under sterile conditions in a laminar flow hood and minced to a fine brei using two scalpels. Four tumors were pooled for each treatment group. Approximately 500 mg tumor brei was used to make each single-cell suspension. All reagents were sterilized with 0.22-μm Millipore filters and were added aseptically to the tumor cells. Each sample was washed in 20 ml α-MEM, after which the liquid was gently decanted and discarded. The samples were resuspended in 450 units collagenase/ml (Sigma, St. Louis, Mo.) and 0.1 mg DNase/ml (Sigma) and were incubated for 10 min at 37° C in a shaking water bath. The samples were resuspended as described above and incubated for another 15 min at 37° C, following which the samples were filtered through two layers of sterile gauze. The samples were washed twice, then resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS).

These single-cell suspensions were counted and plated in duplicate at three different cell concentrations for the colony-forming assay. No significant difference was observed in total cell yield from the pooled tumors in any treatment group. After 1 week, the plates were stained with crystal violet and colonies of >50 cells were counted. The untreated tumor-cell suspensions had a plating efficiency of 10%-16%. The results are expressed as the surviving fraction (±SE) of cells from treated groups as compared with untreated controls [37, 38].

*Bone marrow toxicity.* Bone marrow was taken from the same animals used for the tumor-excision assay. A pool of marrow from the femurs of two animals was obtained by gently flushing the marrow through a 23-gauge needle, and a granulocyte-macrophage colony-forming units (CFU-GM) assay was carried out [37, 38]. Bone marrow cells were suspended in supplemented McCoy's 5A medium containing 15% FBS, 0.3% agar (Difco, Detroit, Mich.), and 10% conditioned media as a source of colony-stimulating activity. The colony-stimulating activity supplement was prepared by incubating L-929 mouse fibroblasts (2,500 cells/ml; Microbiological Associates, Bethesda, Md.) with 30% FBS in McCoy's 5A medium for 7 days at 37° C in a humidified atmosphere the containing 5% $CO_2$. The supernatant containing the colony-stimulating activity was obtained by centrifugation of the medium at 10,000 *g* for 10 min at 4° C followed by filtration under sterile conditions. The bone-marrow cell cultures were incubated for 7 days at 37° C and were fixed with 10% glutaraldehyde. Colonies of at least 50 cells were scored on an Acculite colony counter (Fisher Scientific, Springfield, N.J.). The results of three experiments, in which determinations for each group were made in duplicate at three cell concentrations, were averaged. The results are expressed as the surviving fraction from treated groups as compared with untreated controls.

*Tumor-growth delay experiments.* The Lewis lung tumor [28, 31, 33] was carried in male C57BL mice (Taconic Laboratories, Germantown, N.Y.). For the experiments, $2\times10^6$ tumor cells prepared from a brei of several stock tumors were implanted s.c. into the legs of male mice aged 8–10 weeks.

By day 4 after tumor-cell implantation, Lewis lung tumors have begun neovascularization [8, 9]. Animals bearing Lewis lung tumors were treated daily on days 4–18 following tumor implantation with i.p. CAI (50 mg/kg) alone or in combination. When the tumors had reached a volume of approximately 100 $mm^3$ (day 7 after tumor-cell implantation), cytotoxic therapy was initiated. CDDP (10 mg/kg) or melphalan (10 mg/kg) was injected i.p. on day 7 after tumor implantation. Cyclophosphamide (150 mg/kg) or BCNU (15 mg/kg) was given i.p. on days 7, 9, and 11 following tumor implantation. Radiation was delivered locally to the tumor-bearing limb as 2, 3, or 4 Gy given daily on days 7–11.

The progress of each tumor was measured thrice weekly until the lesion had reached a volume of 500 $mm^3$. The tumor-growth delay was calculated as the number of days required for each treated tumor to reach a volume of 500 $mm^3$ as compared with untreated control tumors. Each treatment group comprised six animals and each experiment was repeated three times. Tumor-growth delay data are presented as the mean values ±SE calculated for the treatment group as compared with the control group [40].

*Lung metastases.* The number of external lung metastases detected on day 20 in animals treated as described above after tumor implantation were counted manually and scored as measuring ≥ 3 mm in diameter. The data shown are the mean values for 6–12 pairs of lungs. Parentheses indicate the percentage of large (vascularized) metastases [40].

*Radioimmunoassay.* Serum levels of prostaglandin $E_2$ and leukotriene $B_4$ were measured in normal and Lewis lung tumor-bearing mice using radioimmunoassay kits (NEK-020 and NEK-037, Dupont NEN Research Products, Boston, Mass.). Blood from five animals was pooled for each assay. Animals received no treatment or received daily i.p. injections of CAI (50 mg/kg) beginning on day 4 following tumor-cell implantation. Each assay was performed four independent times.

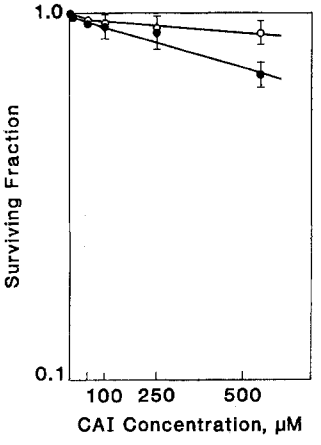


**Fig. 1.** Survival of exponentially growing normally oxygenated (●) and hypoxic (O) EMT-6 cells exposed to various concentrations of CAI for 24 h. Hypoxia was maintained for the first 5 h of drug exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

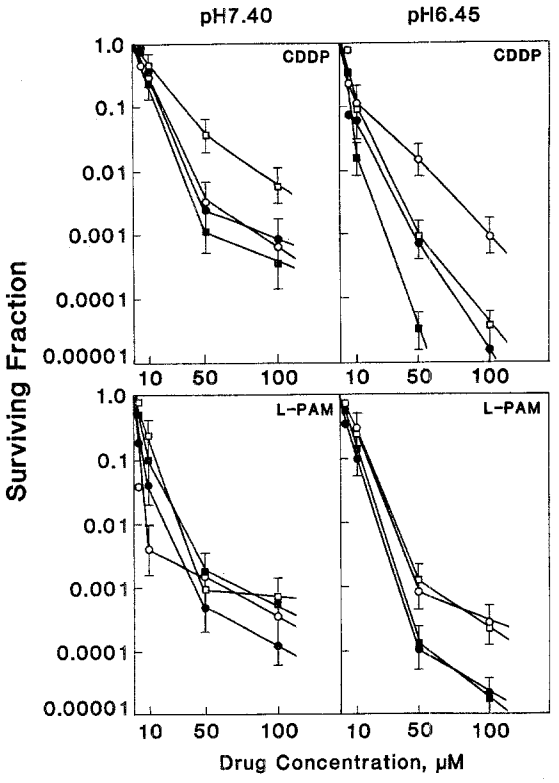


**Fig. 2.** Survival of exponentially growing normally oxygenated and hypoxic EMT-6 cells exposed to various concentrations of CDDP or L-PAM alone (●, O) or in combination with CAI (50 μ*M*, 24 h; ■, ❑) at pH 7.40 or pH 6.45 under normally oxygenated (●, ■) or hypoxic (O, ❑) conditions during the 5th h of modulator exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

*Data analysis.* Additivity/synergy was determined by isobologram methodology [11, 39]. Statistical significance was determined by the Dunn multiple-comparisons test.

## Results

CAI was not very cytotoxic toward normally oxygenated or hypoxic exponentially growing EMT-6 cells upon 24 h exposure to drug concentrations of up to 500 μ*M* (Fig. 1). CDDP was equally cytotoxic toward normally oxygenated and hypoxic EMT-6 cells at pH 7.40 but was less cytotoxic toward hypoxic EMT-6 cells when the pH of the medium was 6.45 (Fig. 2). The addition of CAI (50 μ*M*, 24 h) to treatment with CDDP decreased the cytotoxicity of the drug toward hypoxic cells at pH 7.40 but increased its cytotoxicity toward cells maintained under both oxygenation conditions at pH 6.45. Melphalan showed no cytotoxic selectivity in terms of cellular oxygenation at pH 7.40 but was more cytotoxic toward normally oxygenated cells at pH 6.45. The addition of CAI to treatment with melphalan did not alter the cytotoxicity pattern of that drug. BCNU was equally cytotoxic toward normally oxygenated and hypoxic EMT-6 cells at pH 7.40 but at pH 6.45 was less cytotoxic toward hypoxic EMT-6 cells (Fig. 3). CAI protected hypoxic EMT-6 cells at both pH values from the cytotoxicity of the drug toward normally oxygenated cells.

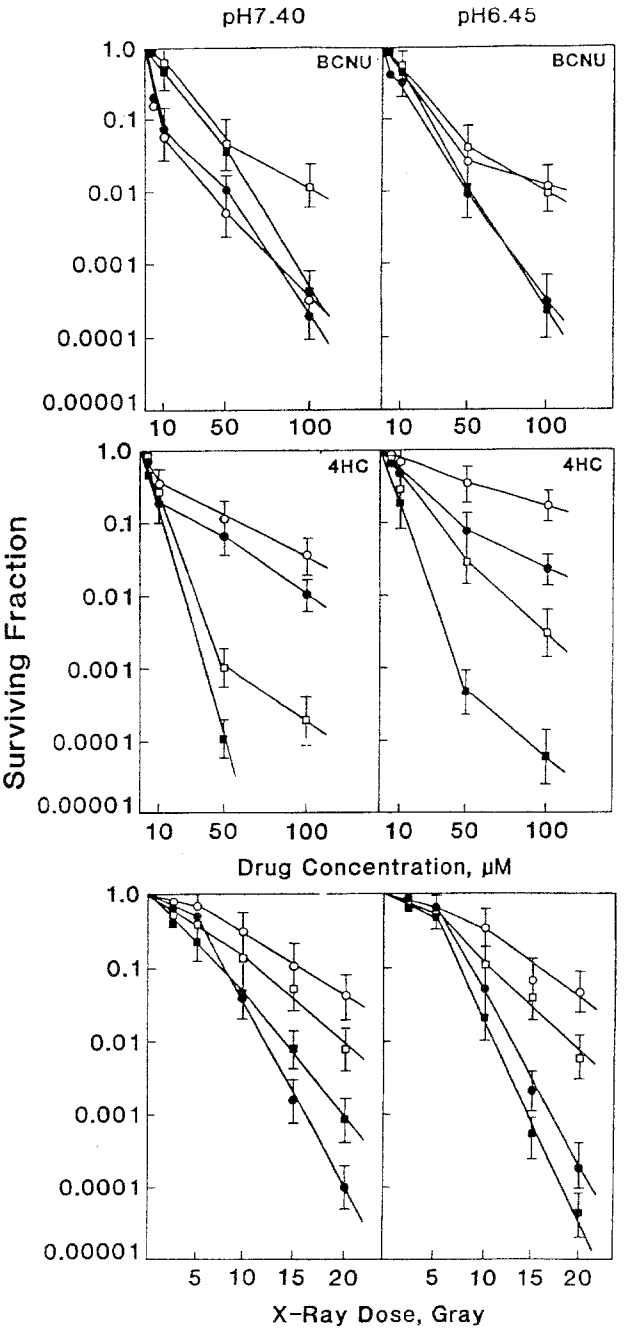


**Fig. 3.** Survival of exponentially growing normally oxygenated and hypoxic EMT-6 cells exposed to various concentrations of BCNU or 4-HC or various doses of radiation alone (●, O) or in combination with CAI (50 μ*M*, 24 h; ■, ❑) at pH 7.40 or pH 6.45 under normally oxygenated (●, ■) or hypoxic (O, ❑) conditions during the 5th h of modulator exposure. *Points*, Mean values for 3 independent determinations; *bars*, SEM

4-Hydroperoxycyclophosphamide (4-HC) was equally cytotoxic toward normally oxygenated and hypoxic EMT-6 cells at pH 7.40 but was less cytotoxic toward hypoxic EMT-6 cells than toward normally oxygenated EMT-6 cells at pH 6.45. The addition of CAI to treatment with 4-HC resulted in increased cytotoxicity toward both normally oxygenated and hypoxic cells at both normal and acidic pH. The cytotoxicity observed with the combination of CAI and

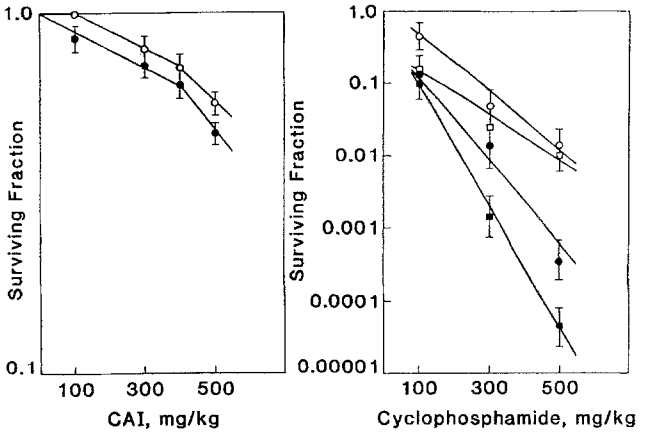


**Fig. 4.** Survival of FSaIIC tumor-cells from tumors (●, ■) and bone-marrow CFU-GM (O, ❑) from the same animals after treatment with various doses of CAI, cyclophosphamide (●, O) or CAI (100 mg/kg) along with cyclophosphamide (■, ❑). Data represent mean values for 3 independent experiments ±SEM

4-HC was much greater than additive for the two agents. At a concentration of 50 μ*M* 4-HC at normal pH a 2-log increase in cytotoxicity was obtained with the combination as compared with 4-HC alone for both normally oxygenated and hypoxic cells. Under acidic pH conditions at a concentration of 50 μ*M* 4-HC, the 2-log increase in cytotoxicity seen at pH 7.40 was maintained in the normally oxygenated cells but under hypoxic conditions only a 1-log increase in cytotoxicity was obtained with the combination as compared with 4-HC alone.

The radiation-survival curves generated for the EMT-6 cells were similar at normal and acidic pH and, as expected, radiation was selectively cytotoxic toward the normally oxygenated cells. Although the addition of CAI to treatment with radiation did not markedly alter the response of these cells to treatment, there was a trend (2.5- to 3-fold increase in cytotoxicity) toward increased cytotoxicity of radiation in the hypoxic cells at both pH values in the presence of CAI.

The effect of CAI on tumor-cells and bone-marrow CFU-GM was assessed in animals bearing the FSaII fibrosarcoma (Fig. 4). CAI was injected i.p. as a single dose and its cytotoxicity to FSaII tumor-cells and bone-marrow CFU-GM was assessed 24 h after drug administration. CAI was not very cytotoxic to either FSaII tumor-cells or bone-marrow CFU-GM; 50% of the tumor cells were killed by a dose of 475 mg/kg CAI. The effect of adding CAI to treatment with cyclophosphamide was also assessed in animals bearing FSaII fibrosarcoma. Cyclophosphamide killed increasing numbers of both tumor-cells and bone-marrow CFU-GM with increasing doses of the drug. The selectivity for tumor-cell killing as compared with killing of bone-marrow CFU-GM ranged from 3 to 40 orders of magnitude over the range of cyclophosphamide doses tested. Administration of CAI (100 mg/kg, i.p.) immediately prior to cyclophosphamide did not alter the tumor-cell killing by cyclophosphamide in the standard-dose area (100 mg/kg) but increased tumor-cell killing by the drug at higher cyclophosphamide doses, reaching an 8-fold increase in cytotoxicity at 500 mg/kg cyclophosphamide. Overall, no significant increase in the toxicity of cyclophosphamide to bone-marrow CFU-GM was achieved by the addition of CAI to treatment with the drug.

For tumor-growth delay studies, CAI (50 mg/kg) was given daily by i.p. injection for 2 weeks beginning on day 4 after s.c. implantation of the Lewis lung carcinoma in the hind legs of C57BL male mice. Untreated control animals bearing this tumor will die from lung metastases within 22–25 days following s.c. tumor implantation. On this 2-week treatment schedule, CAI did not alter the tumor growth or the numbers of lung metastases detected, although the percentage of large metastases decreased (Table 1). Daily administration of CAI increased the tumor-growth delay produced by a single dose of CDDP (10 mg/kg) by a factor of 1.2 and increased the tumor-growth delay produced by a single dose of melphalan by a factor of 1.8. CAI administration increased the tumor-growth delay produced by cyclophosphamide by a factor of 1.3, which corresponded to a 7.2-day increase. Similarly, the combination of CAI and

**Table 1.** Growth delay of the Lewis lung tumor and number of lung metastases on day 20 after treatment with various anticancer therapies alone or in combination with CAI

| Treatment group | Tumor-growth delay, days[a] | | Mean number of lung metastases (% large) | |
|---|---|---|---|---|
| | Alone | +CAI[b] | Alone | +CAI |
| – | – | 0.4±0.3 | 21 (62) | 21 (43) |
| CDDP (10 mg/kg) | 4.5±0.3 | 5.4±0.5 | 13 (58) | 13 (38) |
| Cyclophosphamide (3×150 mg/kg) | 21.5±1.7 | 28.7±2.4* | 12 (40) | 12 (33) |
| Melphalan (10 mg/kg) | 2.7±0.3 | 4.9±0.4** | 15 (48) | 16 (38) |
| BCNU (3×15 mg/kg) | 3.6±0.4 | 4.8±0.4 | 16 (53) | 17 (35) |
| X-rays (5×2 Gy) | 3.1±0.4 | 4.8±0.3* | – | 17 (41) |
| (5×3 Gy) | 4.4±0.4 | 5.7±0.4* | 17 (40) | 17 (35) |
| (5×4 Gy) | 6.3±0.6 | 6.8±0.5 | – | 17 (35) |

[a] Tumor-growth delay is the difference in the number of days required for treated tumors to reach a volume of 500 $mm^3$ as compared with untreated control tumors. Untreated control tumors reach a 500-$mm^3$ volume in about 14 days. Data represent mean values ± SE for 18 animals

[b] CAI (50 mg/kg) was injected i. p. daily on days 4–18. CDDP and melphalan were given i. p. on day 7. Cyclophosphamide and BCNU were injected i. p. on days 7, 9, and 11. X-rays were delivered daily on days 7–11 locally to the tumor-bearing limb

* $P < 0.01$, ** $P < 0.001$ vs anticancer therapy alone

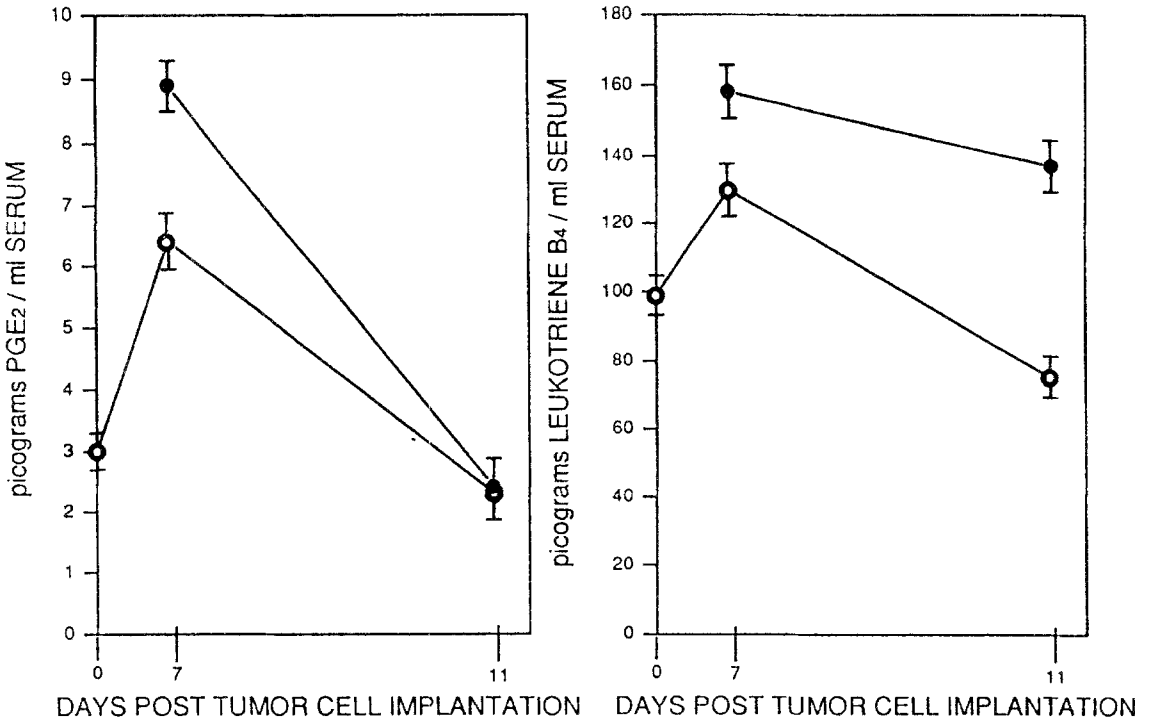


**Fig. 5.** Serum levels of prostaglandin $E_2$ (*$PGE_2$*) and leukotriene $B_4$ in animals implanted s.c. with Lewis lung carcinoma in the hind leg on day 0. Animals received no treatment (O) or were injected daily beginning on day 4 with CAI (50 mg/kg, i.p.; ●. Prostaglandin $E_2$ and leukotriene $B_4$ levels were determined by radioimmunoassay. Each point represents the mean value for 4 independent determinations

BCNU resulted in a tumor-growth delay that was 1.3-fold that of BCNU alone.

The effect of the administration of CAI on the tumor-growth delay produced by fractionated radiation was greater with low-dose-per-fraction radiation than with high-dose-per-fraction radiation. The radiation dose-modifying factor calculated from the radiation doses needed to produce 5–6 days of tumor-growth delay in the presence of CAI was 1.3–1.5 as compared with radiation alone.

The numbers of lung metastases detected on day 20 were decreased by each of the cytotoxic therapies. The daily administration of CAI along with the cytotoxic therapies did not affect the numbers of lung metastases observed on day 20; however, the percentages of lung metastases that were large (vascularized) on day 20 were decreased by the combination treatment.

It has been recognized for some time that the Lewis lung carcinoma secretes prostaglandin $E_2$ [46–48], and alteration in arachidonic acid metabolism has been implicated in the mechanism of action of CAI [6, 13, 14, 17]. Radioimmunoassay was used to determine the serum levels of prostaglandin $E_2$ and leukotriene $B_4$ in mice bearing the Lewis lung tumor with and without treatment with CAI (Fig. 5). The presence of the Lewis lung tumor in the animals resulted in an increase in serum levels of both prostaglandin $E_2$ and leukotriene $B_4$ as compared with non-tumor-bearing animals. As the tumor became large, levels of both prostaglandin $E_2$ and leukotriene $B_4$ decreased. Treatment with CAI (50 mg/kg) beginning on day 4 resulted on day 7 in serum levels of prostaglandin $E_2$ and leukotriene $B_4$ that were higher than those seen in tumor-bearing controls. On day 11, the prostaglandin $E_2$ levels detected in CAI-treated animals were equal to those observed in the control animals but leukotriene $B_4$ levels remained elevated in treated mice as compared with controls.

## Discussion

In the search for new antitumor agents with unique mechanisms of action, agents that block or alter inter- and intracellular signal transduction may be envisioned to inhibit the proliferation of tumor-cells or of normal cells critical to tumor growth and may even lead to cell death. CAI is one of the family of 1,2,3-triazoles developed against the coccidiosis-inducing intracellular parasitic protozoan *Eimeria tenella*. CAI appears to inhibit calcium uptake by infected cells, thereby inhibiting proliferation of the cells and of the parasite [2, 6, 12, 17]. This effect on calcium uptake has been demonstrated in tumor-cells, Madin-Darby bovine kidney cells, and rat polymorphonuclear leukocytes [6, 12, 17]. CAI did not appear to inhibit cyclooxygenase or 5-lipoxygenase, but the release of arachidonic acid was partially or fully inhibited [6, 14, 17]. Several lines of evidence indicate that CAI may function as an inhibitor of signal transduction involving a G-protein intermediate [13, 14].

In cell culture, CAI was relatively noncytotoxic toward EMT-6 cells and did not markedly alter the cytotoxic activity of any of the antitumor alkylating agents or radiation except 4-HC. CAI in combination with 4-HC produced synergistic cytotoxicity under each of the four environ-

mental conditions tested. In vivo single doses of CAI were not very toxic toward FSaII tumor-cells or toward bone-marrow CFU-GM. A single dose of 100 mg/kg CAI given prior to cyclophosphamide resulted in a dose-dependent increase in tumor-cell killing by the drug.

For tumor-growth delay studies, CAI was given daily by i.p. injection at 50 mg/kg. At peak levels this dose should reach about 100 $\mu M$ in the plasma, which is 10-fold the 10-$\mu M$ concentrations needed to produce activity in several in vitro assays [6, 13–17]. A dose of 100 mg/kg CAI given daily by gavage inhibited the growth of the A2058 human melanoma implanted s.c. in nude mice [17].

It has been recognized for some time that the murine Lewis lung carcinoma is an overproducer of prostaglandin and that inhibitors of prostaglandin synthesis can influence the growth of that tumor [46–48]. Arachidonic acid metabolites are involved in inflammatory processes [20, 27, 29]. In cancer, tumor-cell production of prostaglandins has been associated with cell growth, mutagenesis and promotion, immune suppression and metastasis [4, 5, 21, 46–48]. The effect of arachidonic metabolites on these processes may be indirect [1, 3, 7, 18]. For example, collagenase, an enzyme that degrades collagen IV, is critical to tumor invasion in that it allows degradation of the basement membrane [30, 32, 34, 42–44]. Recently it has been reported that metabolites of arachidonic acid formed via both the cyclooxygenase and lipoxygenase pathways are required for the production of collagenase IV and that inhibition of these pathways renders human HT-1080 tumor-cells noninvasive and nonmetastatic [21]. These studies indicate an important role for the metabolites of arachidonic acid in the production of collagenase IV. Administration of several nonsteroidal anti-inflammatory drugs (NSAIDS), including indomethacin, sulindac, diflunisal, and mefenamic acid as well as the pyrazoline lipoxygenase inhibitor phenidone, both alone and in combination reduces serum levels of prostaglandin $E_2$ and leukotriene $B_4$ in animals bearing the Lewis lung carcinoma [41] and, in combination with cytotoxic therapies, lead to increases in tumor-growth delay. CAI given as described above resulted in increased serum levels of prostaglandin $E_2$ on day 7 and in increased serum levels of leukotriene $B_4$ on days 7 and 11 in tumor-bearing mice as compared with untreated tumor-bearing animals. These levels reflect the effects of CAI on both tumorous and normal tissues.

CAI administration did not alter the growth of the Lewis lung tumor when treatment was begun on day 4 following tumor-cell implantation. However, CAI in combination with cytotoxic therapies increased the tumor-growth delay produced by all of the treatments and was most effective in combination with melphalan, an amino acid analog known to enter cells through an active transport mechanism, and low-dose radiation therapy. The present results indicate that further study of CAI as a modulator of cytotoxic anticancer therapies is warranted.

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