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IDENTIFICATION AND CHARACTERIZATION OF HUMAN METABOLITES OF CAI [5-AMINO-1-1(4'-CHLOROBENZOYL-3,5-DICHLOROBENZYL)-1,2,3-TRIAZOLE-4-CARBOXAMIDE)

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ABSTRACT:

The calcium influx inhibitor and cytostatic agent, 5-amino-1-1(4'-chlorobenzoyl-3,5-dichlorobenzyl)-1,2,3-triazole-4-carboxamide (CAI), is in phase I clinical trial for patients with refractory cancer. Additional chromatography peaks were observed during HPLC analysis of patient samples. Identification and characterization of physiological metabolites were undertaken using HPLC techniques developed for their purification from blood, pleural fluid, and urine samples. A hydrophobic metabolite, M1, was purified and functionally characterized. Structural analysis of the purified compound indicated that it is a 3,5-dichloro-4(*p*-chlorobenzoyl)-benzoic acid. Quantitative analysis of M1 concentration during CAI administration indicated that the rise in M1 concentration lagged behind that of CAI and persisted after CAI was no longer detectable. No clear relationship between CAI or M1 and either toxicity or efficacy was observed. Chromatography of patient blood and urine samples under conditions favoring hydrophilic metabolite detection suggested the presence of a glucuronide compound; this was also indicated by sample treatment with β-glucuronidase. Attempts at purification did not yield a compound stable for structural analysis. The benzophenone metabolite, M1, was nonfunctional in assays of calcium influx inhibition or proliferation. No pharmacodynamic associations were observed for these metabolites, nor was there pharmacological activity of the M1 as an individual agent. These data suggest that CAI is processed into triazole and benzophenone moieties by phase I metabolism, and these metabolites or the parent compound may be conjugated for excretion by glucuronidation.

The novel anticancer agent, CAI[1], was originally identified for its antimetastatic, antiangiogenic, and antiproliferative effects *in vitro* and *in vivo* (1–4). CAI has been shown to inhibit stimulated calcium entry and downstream calcium-dependent signaling events (5–7). A structure–activity relationship analysis strongly indicated a link between inhibition of calcium influx by CAI and its anticancer activity (7). Specifically, it was demonstrated that both the benzophenone tail and the triazole head groups of CAI must be present for inhibition of the calcium-associated signaling and growth. Phase I clinical trials for patients with refractory cancer are nearing completion at the National Cancer Institute (8).

Oral formulations of CAI in PEG-400 liquid solution or gelatin capsules containing the PEG-400 solution have been well tolerated. Peripheral sensory neuropathy has been observed rarely (8%) and was reversible. Grade 1 and 2 nausea and vomiting occurred in over half of the patients, but was readily ameliorated by night time dosing or mild antiemetic agents (8). Orally administered CAI in PEG-400 liquid formulation yielded steady-state plasma concentrations in the range of 2.0–5.0 μg/ml, and was found to have a terminal half-life of 111 hr and a large volume of distribution ranging from 100 to >400 liters (Vd_{ss}/F) (9). CAI is a very hydrophobic compound and was assessed to be 99.4% protein bound (9). The CAI concentrations obtained in patient sera are in the concentration range shown to be effective in inhibiting calcium influx, calcium-regulated signaling, angiogenesis, and invasion and proliferation in laboratory and animal studies (3–5, 7).

Reversed phase HPLC has been used to quantitate CAI concentrations (10). Review of patient blood and urine chromatography profiles revealed development of peaks other than the known CAI peak, not present in pretreatment samples. The chromatographic separation was modified for analysis and purification of these putative metabolite peaks. Several possible peaks were identified in plasma, pleural fluid, ascites, and urine. We now report the identification, structure, and lack of function of a benzophenone metabolite (**M1**) and the presence of a probable glucuronide metabolite (**MG**) from patient samples.

Materials and Methods

Synthesized CAI and **M1** were obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). HPLC-grade ammonium acetate was purchased from Advanced Biotechnologies, Inc. (Columbia, MD). Maxiclean C_{18} cartridges were from Alltech (Deerfield, IL). XAD-2 Amberlite resin was obtained from Supelco (Bellefonte, PA). Gelman (0.2 μm) acrodisks and polytetrafluoroethylene filters were used for all sample preparation (Gelman Sciences, Ann Arbor, MI). Beckman Ultrasphere, spherical ODS-5, 10-μm C_{18} (10 × 250 mm) preparative column (San Romano, CA); a Chromantics irregular ODS-3 C_{18}, 10-μm (4.6 × 250 mm) analytical column (Thomson, Springfield, VA); or an Alltech ODS-3 C_8 (10 × 250 mm) preparative column was used on a Gilson HPLC system (Middleton, WI).

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[1] Abbreviations used are: CAI, 5-amino-1-1(4'-chlorobenzoyl-3,5-dichlorobenzyl)-1,2,3-triazole-4-carboxamide; **M1**, 3,5-dichloro-4(*p*-chlorobenzoyl)benzoic acid; CHOm5, Chinese hamster ovary cells stably transfected with the m5 muscarinic acetylcholine receptor; DMSO, dimethylsulfoxide; **MG**, glucuronidated metabolite.

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TABLE 1

Gradient chromatography methods used for metabolite separation

	C_{18} Analytical		C_{18} Preparatory		C_8 Preparatory	
Solvent A	95% 0.1 N NH_4OAc/5% acetonitrile		95% 0.1 N NH_4OAc/5% acetonitrile		90% 0.1 N NH_4OAc/10% acetonitrile	
Solvent B	100% methanol		100% methanol		90% methanol/10% acetonitrile	
Flow rate	1.0 ml/min		3.5 ml/min		4.0 ml/min	
Injection volume	50 ml		500 ml		750 ml	
Run time	30 min		75 min		60 min	
	Time	% B	Time	% B	Time	% B
	min		*min*		*min*	
	0	45	0	40	0	10
	3.3	45	5	40	4	10
	19	95	35	90	25	50
	24	95	45	90	44	50
	24.1	45	47	98	57	95
			52	98	62	95
			53	40	62.5	10

Fura-2AM was from Molecular Probes (Eugene, OR). Bovine liver, type B-10 β-glucuronidase was obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were analytical or HPLC grade.

Metabolite Isolation and Characterization. *Sample Preparation and HPLC Analysis.* Patient plasma, pleural fluid, or ascites samples from patients receiving daily CAI administration were prepared as described (10). Briefly, samples were extracted over C_{18} cartridges, washed with 0.1 M ammonium acetate (pH 6.5), then eluted with acetonitrile. Pleural fluid was used for bulk purification of hydrophobic metabolites with confirmation of peaks using plasma samples on analytical chromatography. For hydrophilic extraction, urine samples (400 ml) were loaded slowly over a prepared 3 × 18 cm bed volume XAD-2 column, washed with four bed volumes of water, and then eluted with one bed volume of acetonitrile. Small volume urines (25 ml) were also prepared and extracted in a analytical fashion using 1 × 5 cm bed volume XAD-2 columns. Eluates were then dried under nitrogen to near completion. An aliquot of the final product was resuspended in mobile phase.

Reversed-phase preparatory LC was performed using 1 of the 3 gradient separations described in table 1. The organic solvents were acetonitrile and/or methanol for chromatography on C_8 or C_{18} columns with 0.1 M ammonium acetate (pH 6.5) aqueous phase. The chromatographic methods were devised for detection and quantitation of patient samples (C_{18} analytical), fractionation and purification of M1 (C_{18} preparatory), and for assay and purification of the glucuronidated metabolite (C_8 preparatory). All chromatography was performed at ambient temperature (23°C) with detection at 263 nm. Selected peaks were collected and dried under nitrogen. The products were resuspended in water, desalted over XAD-2 columns, and eluted with acetonitrile. A sample of the final product was analyzed for purity and retention time using the analytical gradient HPLC.

β-Glucuronidase Hydrolysis In Vitro. To investigate the presence of glucuronidated compounds in patient urine samples, 25-ml urine aliquots were incubated with β-glucuronidase essentially as described (11). Control samples had either omission of enzyme or the concomitant addition of a specific β-glucuronidase inhibitor saccharo-1,4-lactone (1.0 mM) (12). The resultant samples were chromatographed and analyzed for change in peak profile indicating cleavage by the β-glucuronidase.

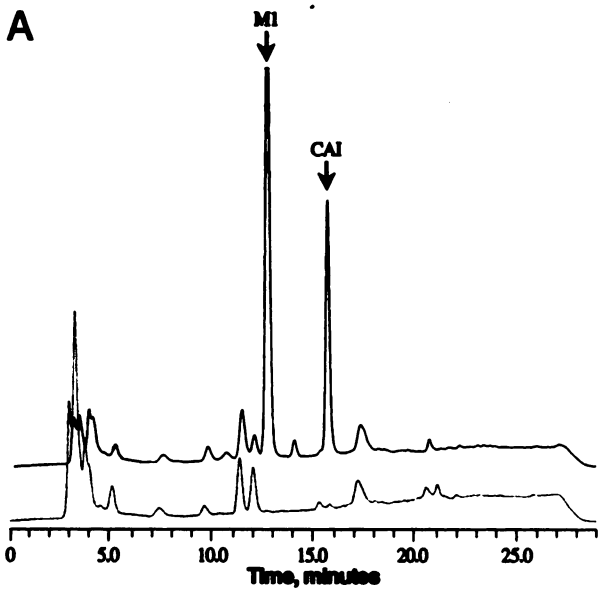
Functional Characterization. *Calcium Influx.* CHOm5 cells were used for the study of calcium influx, essentially as described (5, 6). CHOm5 cells were loaded with the acetoxy-ester of Fura-2 (2 μM), a fluorescent dye that is used to quantitate intracellular Ca^{2+} concentrations, after which cells were stimulated with the muscarinic agonist, carbachol (100 μM). The change in fluorescence was quantitated using the Image1/Fluor software package (Universal Imaging, Inc., West Chester PA). Data were obtained as the ratio of fluorescence emission at 510 nm after excitation at 340 and 380 nm, and calibrated as described (13). Cells were exposed to DMSO control (0.1%), CAI, or M1 (10 μM) for 10 min before and then during the carbachol stimulus. Data represent the mean of 30 imaged cells per experiment.

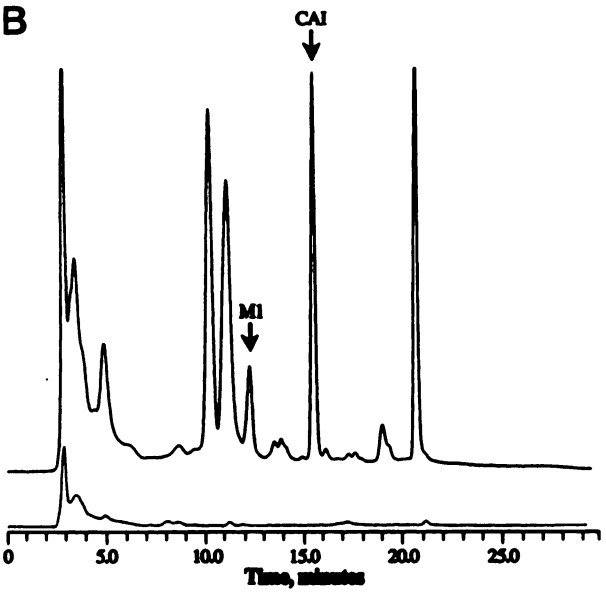
Arachidonic Acid Release. Subconfluent CHOm5 cells were loaded with [^{3}H]arachidonic acid and assayed as described (5, 7). Cells were stimulated with 100 μM carbachol in the presence of CAI (0–100 μM), M1 (0–100 μM), or DMSO control (0.1%) for 15 min. An aliquot of the supernate was removed, spun free of cells, and counted to measure carbachol-induced [^{3}H]arachidonic acid release. Data presented are the percentage of DMSO control-stimulated arachidonic acid release (mean ± SE, $N = 3$).

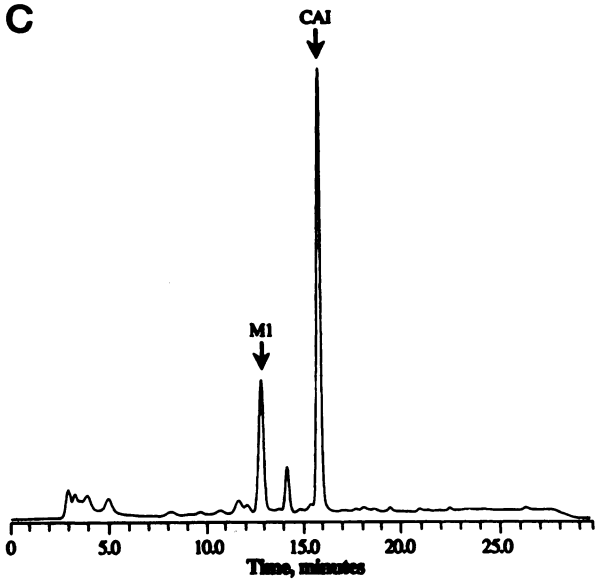
Cell Proliferation Assays. The growth inhibitory effects of CAI and M1 were tested in A2058 human melanoma cells as described (7). Cells were cultured in replicates with CAI or M1 (0–30 μM) or DMSO (0.1%) for 96 hr. Monolayers were stained with crystal violet, and optical density of the elutes was measured at 540 nm. Results are presented as percentage of control (mean ± SE, $N = 3$).

Results

Identification and Chemical Characterization. Patient CAI concentration and peak profiles were determined using a gradient reversed-phase chromatography designed to optimize resolution of peaks near the void volume. Figure 1 shows a representative peak profile of patient CAI plasma samples before CAI administration (fig. 1*A*, *bottom trace*) and after 28 days of daily administration (fig. 1*A* *upper trace*), and urine sample pre-CAI and after 28 days of dosing (fig. 1*B*, *bottom* and *top traces*, respectively). The predominant peak observed at 12.5 min (M1) was not detected in predrug plasma or urine samples, and increased in magnitude over the time of daily CAI administration. This peak was present after CAI administration in all patient fluids analyzed; an example pleural fluid is shown in fig. 1*C*. The UV absorbance spectrum of M1 observed with a diode array UV detector was similar to that obtained for the CAI parent compound (data not shown). A C_{18} column preparative chromatography method was developed to collect and further purify this peak for structural analysis after initial extraction over a C_{18} column. Pleural fluid samples were subjected to hydrophobic extraction and preparative chromatography for purification of putative metabolites. Observed peaks were confirmed in extracted plasma samples using analytical chromatography. Hydrophilic metabolites were studied by extraction of urine samples over XAD resin, followed by a different gradient







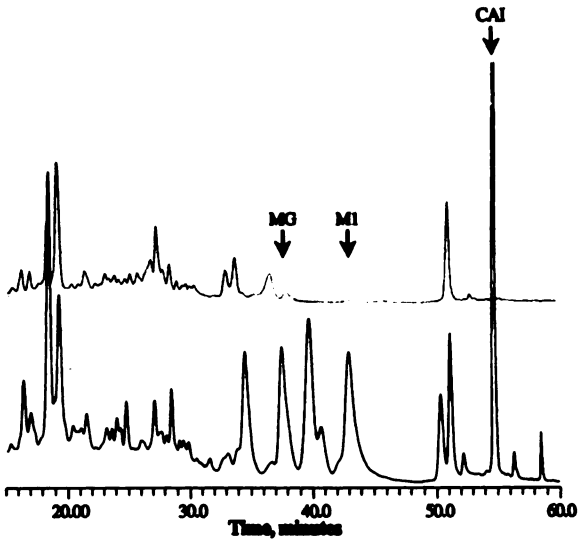


FIG. 2. *Peak profile of samples prepared with XAD extraction and C_8 chromatography.*

This approach was taken to enrich for more soluble metabolites expected in urine, such as glucuronides. Pre-CAI, *top trace*; post-28 days of treatment, *bottom trace*; MG, 38 min; M1, 43 min; CAI, 55 min.

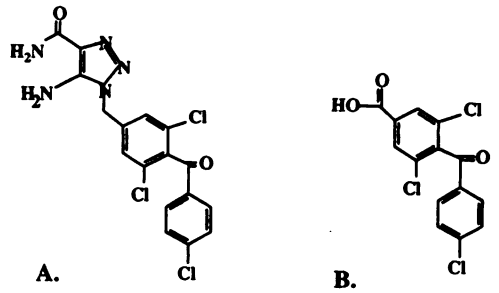


FIG. 3. *Structure of CAI and M1.*

(*A*) CAI; (*B*) M1.

chromatography using C_8 columns. Chromatographs indicated the presence of peak(s) not present in predrug urine or plasma samples (fig. 2).

The M1 hydrophobic fraction isolated from plasma of CAI patients by HPLC was evaporated to dryness with nitrogen gas and then subjected to structural characterization by MS and NMR spectroscopy. Solid-probe electron impact mass spectral analysis (VG model 70E) of the M1 fraction displayed a molecular ion peak of *m/z* 328 ($C_{14}H_7Cl_3O_3$+) with a characteristic cluster for three chlorines. The spectrum also displayed mass spectral fragment ion peaks of *m/z* 217 ($C_8H_3Cl_2O_3$+) and *m/z* 139 (C_7H_4ClO+), with characteristics of two and one chlorine atom(s), respectively. Additional peaks were present at *m/z* 287, 234, and 86 caused by impurities. The impurity peaks were

Fig. 1. *Chromatographic profiles on C_{18} column.*

Samples were prepared and chromatographed as discussed in *Materials and Methods*. A representative peak profile of patient plasma samples (*A*) and urine samples (*B*) are shown for samples obtained before CAI administration (*bottom trace*) and after 28 days of daily CAI administration at 125 mg/m^2 in PEG-400 liquid formulation (*top trace*). A representative pleural fluid sample chromatogram is shown in (*C*). Sample was obtained after 28 days of daily CAI administration at 150 mg/m^2 in PEG-400 liquid formulation. Retention times: CAI, 15.5 min; M1, 12.5 min.

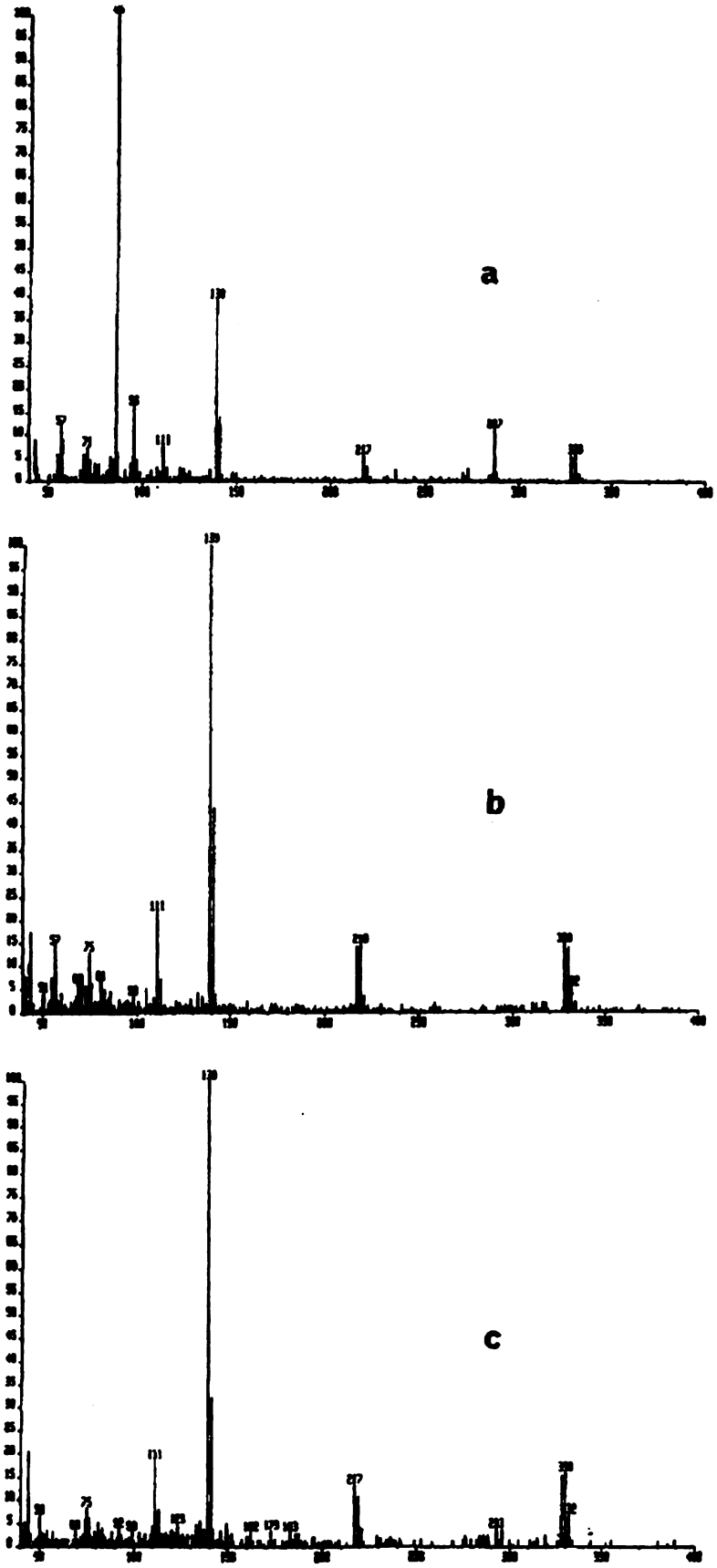


FIG. 4. *Mass spectra of the* M1 hydrophobic fraction by (a) electron-impact solid probe and (b) GC/MS. (*c*) Mass spectrum of the synthetic standard, M1, by GC/MS.

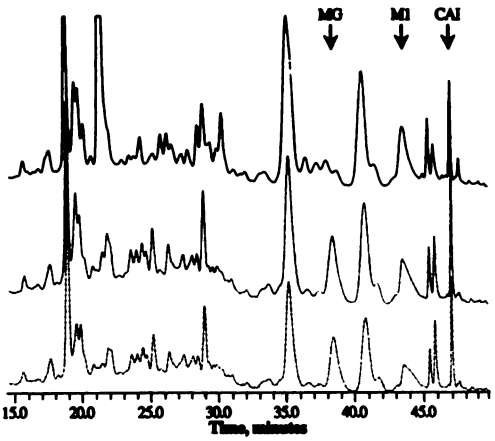


FIG. 5. *Demonstration of a glucuronide metabolite.*

Urine samples were treated with β-glucuronidase as described (*top trace*) (11). Control samples had either omission of enzyme (*middle trace*) or the concomitant addition of a specific β-glucuronidase inhibitor (*bottom trace*). Peak profile was shifted in the presence of β-glucuronidase with the loss of peak MG (38 min) and gain of peaks at 22 and 31 min. This shift was not observed in the absence of enzyme or in the presence of inhibitor.

not present when the M1 fraction was analyzed by GC/MS (Supelco 3 meter, He 100°C to 280°C at 20°C/min, sp 2330, 0.25 mm i.d.). Solid-probe chemical ionization/MS with ammonia and deuteroammonia (Finnigan model 4600) indicated that the molecule contains one exchangeable hydrogen. These spectral data are consistent with a molecular structure as shown in fig. 3*B*. The ^{1}H-NMR spectrum (Varian VXR-500S, in $CDCl_3$) of the M1 fraction displayed an aromatic signal at 8.09 ppm (s, 2H) and a pair of coupled signals (J 8.5 Hz) at 7.75 (d, 2H) and 7.50 (d, 2H) ppm, which are also consistent with the structure shown in fig. 3*B*. The spectrum also displayed impurity signals dispersing over the region between 1–5 ppm. The mass and NMR data obtained on the CAI metabolite are in good agreement with that obtained from the synthetic compound, 3,5-dichloro-4-(*p*-chlorobenzoyl)-benzoic acid (fig. 4).

Glucuronide Metabolite. The probability of a glucuronide metabolite was investigated using enzymatic methods. Urine samples were prepared and then incubated with β-glucuronidase as described in *Materials and Methods*. Chromatographic analysis of these experimental samples revealed loss of a peak that could be recovered by inclusion of a specific inhibitor of β-glucuronidase, D-saccharic acid 1,4-lactone (fig. 5). The peak profile of the urines incubated with both enzyme and inhibitor was identical to that of the control patient urine specimens. MG disappeared when β-glucuronidase activity was uncontested, and new peaks were observed at 21 and 28 min. The probable glucuronide metabolite(s) were not detected in plasma, pleural fluid, or predrug urine.

Analysis of CAI and M1 in Patient Samples. Standard curves were generated for both CAI and synthetic M1 using analytical gradient chromatography on the C_{18} column (range: 0.06–10.0 μg/ml; $r^2 = 0.998$). Samples from randomly selected patients were analyzed to investigate the relationship between plasma concentrations of CAI and M1 over the period of daily CAI administration. Figure 6*A* shows the progressive increase in concentration of M1 in urine after a single oral dose of CAI (test dose). The concentration *vs.* time curve for CAI and M1 over the test dose sampling period is shown in fig. 6*B*. CAI is absorbed and is detectable in plasma within 1–3 hr postdose and falls to undetectable concentrations within 48–120 hr. The plasma concentration *vs.* time curves of CAI and M1 were analyzed for several patients over a period of at least 28 days of daily CAI

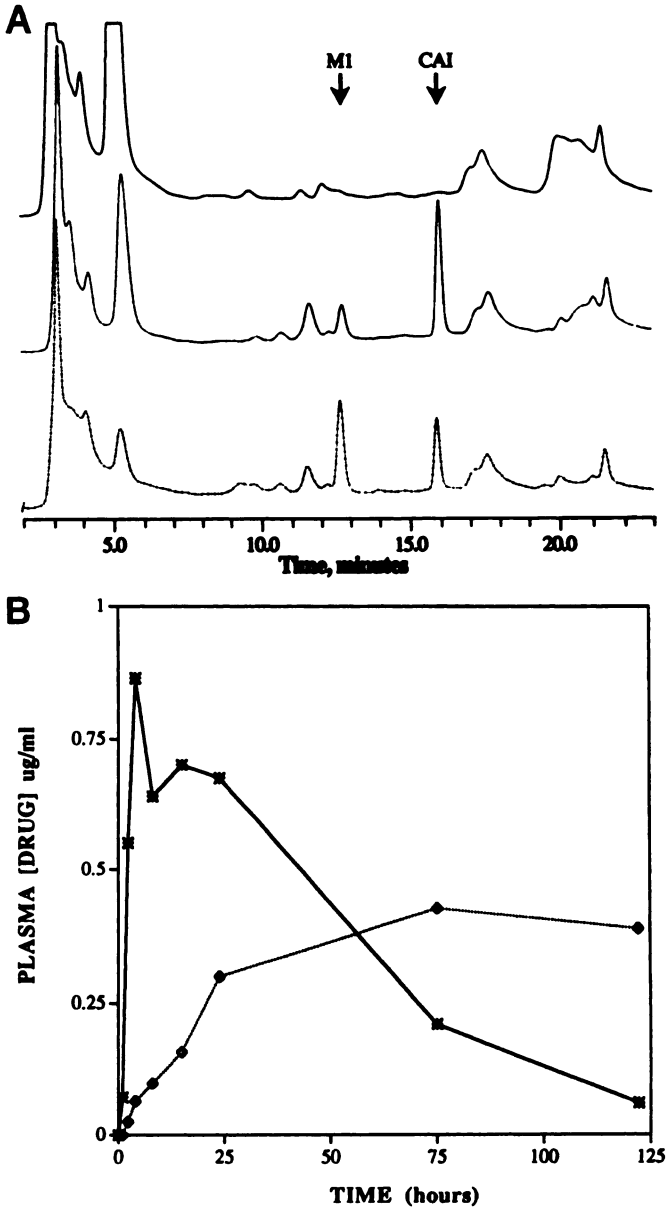


FIG. 6. *Progressive increase in M1 concentration after a single dose of CAI.*

(*A*) Plasma samples taken before CAI administration (*top trace*), 12 hr (*middle trace*), and 24 hr (*bottom trace*) after a single test dose of CAI (330 mg/m^2 in PEG-400 liquid formulation) and prepared according to *Materials and Methods*. (*B*) Concentration *vs.* time curve for CAI (*) and M1 (◆) after a single administered dose of CAI (125 mg/m^2 in PEG-400 liquid formulation).

administration (fig. 7). The concentration of M1 rose more slowly than that of CAI. M1 concentrations remained elevated for several days after CAI was no longer detectable both in test doses and in patients wherein samples were obtained after the discontinuation of CAI administration (figs. 6*B* and 7*D*; data not shown) and ranged from 1 to 5 μg/ml at the time when patients had attained steady-state CAI concentrations. CAI concentrations measured in pleural fluid and ascites were similar to those measured in concomitant plasma samples (ref. 8 and unpublished data).

M1 Does Not Inhibit Calcium Influx or Calcium-Mediated Arachidonic Acid Release. CAI has been shown to alter both basal cellular calcium concentrations and ligand-induced calcium influx (refs. 5–7 and unpublished observations). CHOm5 cells stably ex-

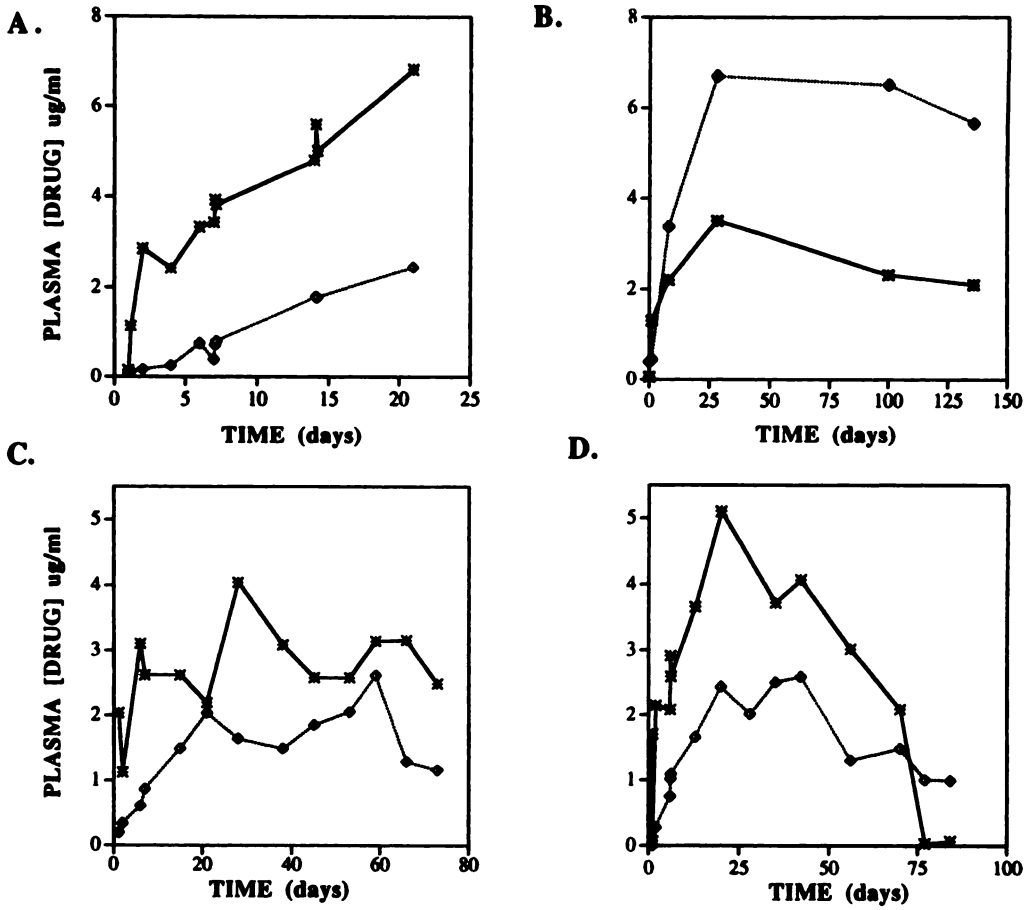


FIG. 7. *Concentration vs. time curves for CAI and M1 over daily CAI administration.*

Samples from four randomly selected patients (*A–D*) were prepared and analyzed according to *Materials and Methods*. CAI doses for patients represented in (*A–D*) were 150 mg/m^2/day, 100 mg/m^2/day, 330 mg/m^2/every other day, and 150 mg/m^2/day in liquid formulation, respectively. CAI, *; M1, ◇.

pressing the muscarinic acetylcholine receptor were used to assay for the ability of the major metabolite, **M1**, to inhibit calcium influx and calcium-mediated signal transduction. When CHOm5 cells were exposed to CAI immediately before stimulation with carbachol, a stable acetylcholine analog, receptor-mediated calcium entry was abrogated (fig. 8*A*). In contrast, pretreatment of CHOm5 cells with **M1** had no effect on stimulated calcium entry. No inhibition of calcium influx by partially purified **MG** was observed (data not shown). Release of arachidonic acid by phospholipase A2 in this system has been shown to require calcium influx (14). We have shown previously that CAI exposure markedly reduces arachidonic acid release in CHOm5 cells (5, 7). Figure 8*B* confirms the inhibitory effects of CAI on arachidonic acid release and shows the lack of effect of **M1** over a similar concentration range.

M1 Does Not Inhibit Proliferation. CAI inhibits the proliferation of multiple cancer cell lines with IC_{50} values of 1–10 μM (2, 7). Growth assays with the A2058 human melanoma cell line (fig. 9) and CHOm5 cells (data not shown) were performed using CAI or **M1** over the concentration range of 0–30 μM. No inhibitory effect of **M1** on the proliferation of either cell line was observed. Higher concentrations, up to 100 μM, also did not effect growth potential (data not shown).

Discussion

We have purified and identified a prominent plasma and urinary metabolite of the calcium influx inhibitor, CAI. The observation of newly developing HPLC peaks after administration of CAI led to the isolation and chemical characterization of the metabolite. Previous *in vitro* studies using human liver microsomes and human liver slices indicated the probability of a glucuronide metabolite (11), necessitating development of different chromatographic separation to favor isolation and purification of this more hydrophilic compound. The hydrophobic metabolite, **M1**, was readily purified and shown to be a 3,5-dichloro-4(*p*-chlorobenzoyl)-benzoic acid coming from cleavage of the halogenated benzophenone tail of CAI from its substituted triazole head. **M1** was detectable in patient plasma, pleural fluid, ascitic fluid, and urine. The appearance of **M1** in plasma was delayed compared to that of CAI, and the concentration of **M1** remained elevated after CAI could no longer be detected chromatographically. No relationship between the concentration of **M1** and patient CAI side effects was found. Synthetic **M1** was used to study function. As suggested on the basis of previous structure function studies (7), **M1** did not inhibit calcium influx, calcium influx-dependent release of arachidonic acid, or proliferation *in vitro*. Whereas the glucuronide compound could be separated and partially purified, it seemed to be unstable and could not be collected intact in adequate amounts to verify structure and assay for biological activity. β-Glucuronidase assays confirmed the presence of glucuronide metabolites in the urine of CAI patients. These results suggest that there are several CAI metabolites produced by different metabolic pathways. There is no evidence to suggest that these metabolites may have independent activity or toxicity.

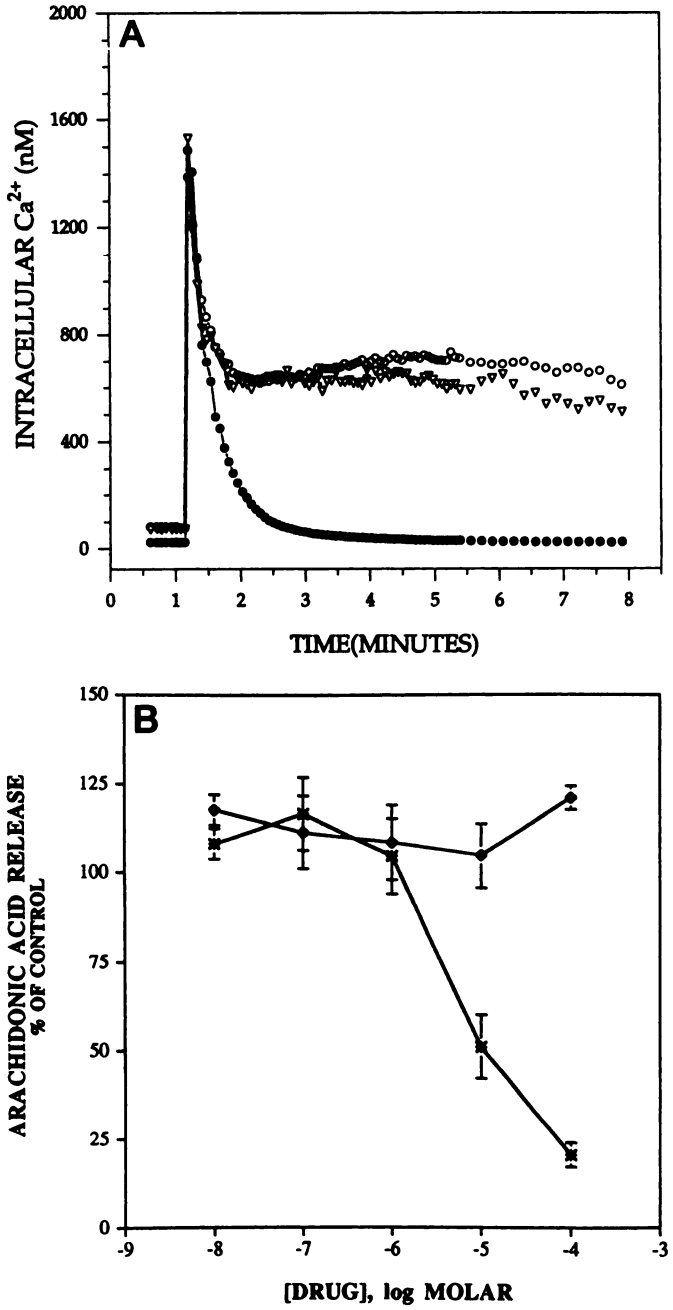


FIG. 8. *M1 does not inhibit calcium influx or arachidonic release in stimulated CHOm5 cells.*

(*A*) Calcium influx. CHOm5 cells were stimulated with carbachol (100 μM) in the presence of DMSO control (0.1%), CAI (10 μM), or M1 (10 μM) and intracellular calcium concentrations measured as described. Results presented are the mean of at least 30 cells, representative of two separate experiments. CAI, *; M1, ◇; DMSO, ●. (*B*) Arachidonic acid release. Carbachol-stimulated arachidonic acid release was quantitated in the presence of DMSO control (0.1%), CAI (0–100 μM), or M1 (0–100 μM). Data are presented as percentage of DMSO control release (mean ± SE, $N = 3$). CAI, *; M1, ◇.

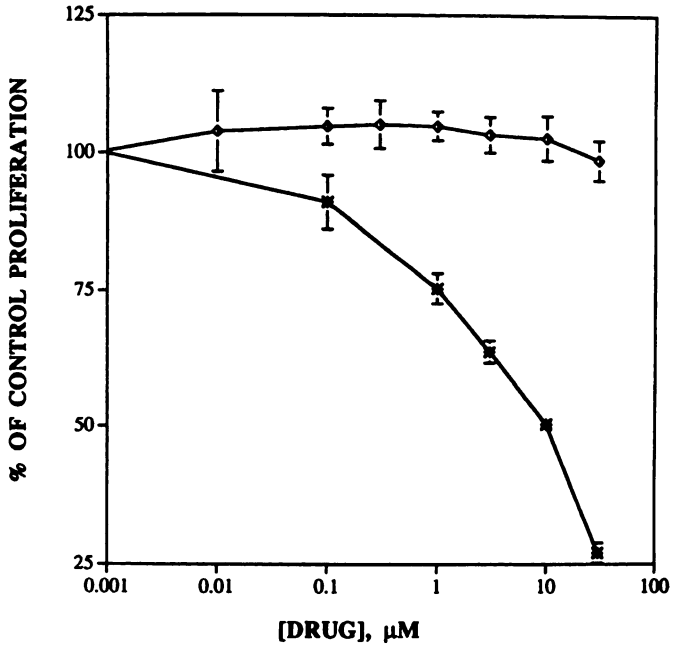


FIG. 9. *M1 does not inhibit proliferation.*

Monolayer growth assays with the A2058 human melanoma cell line were performed with DMSO control (0.1%) and CAI, or M1 over the concentration range of 0–30 μM. Adherent cells were stained with crystal violet and eluted as described. Results presented are the mean percentage of control ± SE of three individual experiments. CAI, *; M1, ◇.

Drug metabolism and resultant detoxification have classically been divided into two phases. Phase I metabolism (such as reduction, oxidation, and hydrolysis) often results in cleavage of the parent compound, exposing reactive groups for phase II reactions and facilitating elimination (15). Phase II reactions proceed by conjugation of highly polar molecules (such as compound sugars) onto exposed reactive groups of the parent drug or phase I metabolite. The conjugation of hydrophilic moieties greatly increases the solubility of many otherwise insoluble compounds, such as CAI, enabling rapid excretion in the urine and bile (12, 16). Hepatic glucuronidation has been demonstrated to be the principal conjugation pathway in vertebrates. Our previous studies suggested that CAI could be metabolized in the liver through hepatic cytochrome P450 pathways (11). Inclusion of ketoconazole in liver microsome assays prevented production of CAI metabolites in those studies. Those *in vitro* studies also suggested phase I metabolism of CAI is needed to produce a compound with the structure of M1. In the present study, use of β-glucuronidase helped to confirm the presence of CAI glucuronide(s) in patient samples, although instability prevented isolation and purification for structural analysis.

Halogenated benzophenone compounds and substituted triazole and imidazole moieties were included in our previous structure–activity studies in anticipation that these structures might be produced by phase I metabolic pathways in the patients (7). The calcium influx inhibitory and antiproliferative activity of CAI was lost when the compound was cleaved between the triazole and the benzophenone, between the chlorobenzoyl groups, or upon dehalogenation. No inhibitory effects were seen in cells incubated with a combination of the substituted triazole and the chlorobenzoyl benzoic acid, suggesting that the cancer cells could not produce a functional molecule from the sum of parts. Based on those studies, it was hypothesized that a structure such as M1 would have no biological or biochemical activity. As shown, no inhibition of calcium influx or calcium-dependent arachidonic acid release was observed with concentrations of M1 as high as 20 μM. No inhibition of proliferation was seen in the same concentration range. Furthermore, the ability of CAI to reduce gelatinase A activity was not observed when A2058 or CHOm5 cells were

incubated with **M1** (data not shown). These studies do not rule out alternative mechanisms of action for either CAI or **M1**. Alternative biochemical actions may result in as yet undetected efficacy or toxicity.

Quantitative analysis of **M1** in plasma and urine did not demonstrate a stoichiometric relationship that was consistent between patients, suggesting that **M1** might be a substrate for further metabolism. Most patients developed steady-state, plateau plasma concentrations of **M1** that were lower than their plasma CAI concentrations. The half-life of **M1** was longer than that of CAI, with **M1** remaining elevated after CAI was no longer detectable both after a single CAI test dose and in samples obtained after discontinuation of CAI. Plasma and urinary concentrations of **M1** did not seem to correlate directly with observed patient toxicity. Most of the toxicity in the >60 patients treated to date has been minimal to mild gastrointestinal intolerance (Clinical Therapy Evaluation Program grades 1 and 2), frequently immediately postdose (8). This would not be expected to be caused by hepatic metabolism of CAI. Only six episodes of nongastrointestinal toxicity of grade 2 or higher have been observed (sensory neuropathy, 4; myelosuppression, 1; myalgia and fatigue, 1). Where samples were available, no correlation between symptoms and signs with **M1** concentration or CAI concentration was observed.

These results characterize physiological metabolism of CAI and demonstrate that a prominent phase I metabolite, **M1**, is neither biologically active nor related to toxicity observed *in vitro*. Although the presence of CAI metabolites had been demonstrated *in vitro* and *in vivo*, no characterization of the chemical structure or functional activity had been shown (11). This demonstration of phase I metabolism *in vivo* is consistent with the *in vitro* findings from the previous hepatic microsome and liver slice experiments. Identification of an inactive benzophenone cleavage product is consistent with the expected metabolic pathways of CAI and its previously characterized structure–activity profile.

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This work is dedicated to the memory of Edward Robert Soltis.

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