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ORIGINAL ARTICLE

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Biliary excretion of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) and metabolites by Fischer 344 rats

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Abstract *Purpose*: 17-(Allylamino)-17-demethoxygeldanamycin (17AAG), an analogue of the benzoquinone ansamycin geldanamycin, has been extensively studied preclinically and is being evaluated clinically. Studies were performed to define the biliary excretion of 17AAG after i.v. delivery to rats, and to characterize the metabolites of 17AAG observed in rat bile. *Materials and methods*: In vivo studies were performed in bile-duct-cannulated Fischer 344 rats given a 10 mg/kg i.v. bolus dose of 17AAG. In vitro studies were performed with cloned human CYPs and microsomal epoxide hydrolase. Biliary excretion of 17AAG and metabolites was quantified by HPLC and followed for 4 h after drug delivery. 17AAG metabolites in bile and in in vitro reaction mixtures were identified with LC/MS/MS. *Results*: By 15 min after i.v. delivery of 17AAG, bile contained at least 15 biotransformation products with absorbance spectra similar to that of 17AAG. Of these, metabolites eluting at 2.7, 2.9, and 8.6 min were present in sufficient concentrations to be quantified, although the lack of authentic standards resulted in their being expressed as 17AAG equivalents. Within the first 4 h after drug delivery, biliary excretion accounted for $28.9 \pm 6.1\%$ of the 10-mg/kg 17AAG dose. 17AAG and 17-(amino)-17-demethoxygeldanamycin (17AG) accounted for $4.1 \pm 1.0\%$ of the delivered dose, with 17AAG accounting for $2.0 \pm 0.5\%$ and 17AG accounting for $2.1 \pm 0.5\%$. The metabolites eluting at 2.7, 2.9, and 8.6 min accounted for $10.6 \pm 2.0\%$, $9.8 \pm 1.2\%$, and $1.0 \pm 0.2\%$, respectively, of the administered dose. LC/MS/MS analysis of bile demonstrated major metabolites with molecular weights of 545 and 619, corresponding to 17AG and the diol previously described as resulting from metabolism of 17AAG by CYP3A and microsomal epoxide hydrolase. Of the remaining proposed metabolites, ten had a mass and MS/MS spectrum consistent with mono-oxygenated 17AAG metabolites. One of these metabolites has been identified as the epoxide previously described as resulting from CYP3A oxidation of the allyl double bond. Two other proposed metabolites had a mass and MS/MS spectrum consistent with demethylated 17AAG metabolites, and one had a mass and MS/MS spectrum consistent with a di-demethylated 17AAG metabolite. An analogous series of demethylated and oxidized metabolites was also observed for the 17AG metabolite. *Conclusions*: Biliary excretion of 17AAG represents a

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major route of elimination, although most of the material excreted is in the form of metabolites. Bile of rats dosed with 17AAG contained a number of metabolites not previously identified in the plasma or urine of mice treated with 17AAG, but analogous to metabolites described in bile of rats treated with 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17DMAG, NSC 707545), another geldanamycin analogue undergoing preclinical evaluation in preparation for subsequent clinical trials.

Keywords Geldanamycin · Ansamycin · HSP90 · Biliary excretion

Introduction

The benzoquinone ansamycin antibiotic geldanamycin [11] (Fig. 1) has potent antiproliferative activity [2, 22, 27, 28, 30, 31, 32, 40]. Although the exact mechanism by which geldanamycin inhibits cell growth continues to be characterized, it is thought to be related to the ability of geldanamycin to bind specifically to heat-shock protein 90 and its homologue, GRP94, and thereby interfere with the heteroprotein complexes they form with oncoproteins such as $p185^{erbB2}$, mutant p53, and Raf-1 [2, 3, 4, 6, 7, 8, 15, 16, 18, 22, 23, 24, 25, 27, 28, 29, 31, 32, 34, 37, 38, 39, 41, 42].

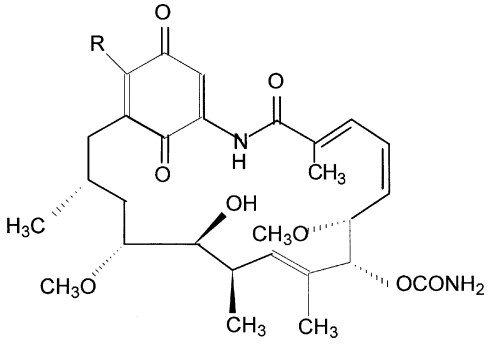
As part of an effort to develop novel, potent, and selective heat-shock protein 90-interactive compounds that might be useful antitumor agents, a number of geldanamycin derivatives have been synthesized and characterized biologically to varying degrees [10, 17, 19, 20, 21, 31, 32, 33, 44]. One of these derivatives, 17-(allylamino)-17-demethoxygeldanamycin (17AAG, NSC 330507) (Fig. 1), is currently undergoing clinical testing [1, 5, 9, 26, 43]. As part of the preclinical evaluation of 17AAG, its metabolism by hepatic preparations has been characterized [12] as have several metabolites observed in urine and plasma of mice treated with 17AAG (Fig. 1) [13]. Each of the 17AAG metabolites characterized has involved modifications to the allylamino moiety attached to the 17-position. In subsequent studies the biliary excretion by rats of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17DMAG, NSC 707545) (Fig. 1), another geldanamycin analogue undergoing preclinical evaluation in preparation for subsequent clinical trials, has been evaluated [14, 36]. These studies have demonstrated a number of likely 17DMAG metabolites that involve modifications to sites not previously shown to be modified on 17AAG [14]. In view of the lack of information about the biliary excretion of 17AAG and in an attempt to describe 17AAG metabolites analogous to those produced from 17DMAG, we studied the biliary excretion of 17AAG by rats.

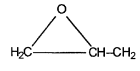
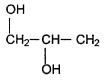
Materials and methods

Reagents

Triethylamine and formic acid (99.9% pure) were obtained from Sigma Chemical Company (St. Louis, Mo.). Sodium phosphate

Fig. 1 Structures of geldanamycin, 17AAG, 17AG, 17DMAG, and previously known metabolites of 17AAG



Compound		R	Molecular Weight
Geldanamycin		CH_30	560
17-(allylamino)-17-demethoxygeldanamycin	**17AAG**	CH_2=CH-CH_2-NH	585
17-(amino)-17-demethoxygeldanamycin	**17AG**	NH_2	545
Epoxide Metabolite			601
Diol Metabolite			619
17-(dimethylaminoethylamino)-17-demethoxygeldanamycin	**17DMAG**	$(CH_3)_2$NH-CH-CH_2-NH	617

(monobasic, certified A.C.S.), *o*-phosphoric acid (certified A.C.S.), ethyl acetate (certified A.C.S.) and acetonitrile (Optima grade) were obtained from Fischer Scientific (Fair Lawn, N.J.).

Drug

17AAG and 17-(amino)-17-demethoxygeldanamycin (17AG, NSC 255109) (Fig. 1) were obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, Md.). 17AAG was supplied by the National Cancer Institute as the clinical formulation of 25 mg/ml in DMSO, which was diluted in EPL diluent (NSC 704057) immediately before use.

Rats

Male Fischer 344 rats (7–8 weeks of age and weighing 220–234 g) were purchased from Hilltop Lab Animals (Scottsdale, Pa.). Rats were allowed to acclimate to the University of Pittsburgh Animal Facility for at least 1 week before studies were initiated. To minimize exogenous infection, rats were maintained in microisolator cages in separate rooms and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Ventilation and air flow in the animal facility were set to 12 changes per hour. Room temperatures were regulated at 72 ± 2°F, and the rooms were kept on automatic 12-h light/dark cycles. Rats received Prolab ISOPRO RMH 3000 Irradiated Lab Diet (PMI Nutrition International, Brentwood, Mo.) and water ad libitum except on the evening prior to dosing, when all food was removed. Sentinel rats (Sprague-Dawley rats in cages with bedding that contained 20% bedding removed from study rat cages at cage change) were maintained in the room housing the study rats and assayed at monthly intervals for specific murine pathogens by murine antibody profile testing (Charles River, Boston, Mass.). Sentinel rats remained free of specific pathogens throughout the study period, indicating that the study rats were free of specific pathogens.

Biliary excretion studies

Biliary excretion of 17AAG was studied in three rats with surgically implanted bile duct cannula. This was accomplished by anesthetizing each animal with 40 mg/kg i.p. pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill.), isolating its bile duct through a midline abdominal incision, and cannulating the bile duct with a 28-g L-Cath Peel Away polyurethane catheter (Luther Medical Products, Tustin, Calif.). After the cannula was secured proximally and distally with 2-0 silk sutures and allowed to drain under gravity, the abdominal wound was closed with Michel wound clips. 17AAG was administered as a bolus dose of 10 mg/kg through a tail vein, and during the subsequent 4 h, bile was collected as 15-min timed fractions in preweighed cryogenic vials (Corning, Corning, N.Y.). Anesthesia was maintained with additional 10 mg/kg i.p. doses of pentobarbital as needed, and at the conclusion of the 4-h bile collection, rats were killed with 100 mg/kg i.v. pentobarbital. The accuracy of each dosing solution was confirmed with the HPLC system described below.

Bile and dosing solutions were stored at −70°C until analyzed.

In vitro studies

Human CYP3A4 + P450 reductase + cytochrome b5 Supersomes and human microsomal epoxide hydrolase microsomes were purchased from Gentest (Woburn, Mass.) and incubated, alone or in combination, with 17AAG and an NADPH regenerating system (Solutions A and B, Gentest) according to the manufacturer's instructions. Reaction mixtures (0.5 ml), containing 20 μg/ml 17AAG, were incubated at 37°C for 15 min, at which point they were snap-frozen in liquid N_2 and stored at −70°C until analyzed.

Analysis of in vivo samples

Biliary concentrations of 17AAG were determined with HPLC, using a modification of a previously published method [15, 16]. In anticipation of 17AG being a potential metabolite of 17AAG, standard curves were prepared for both 17AAG and 17AG.

Extraction procedure

To a 200-μl sample of bile was added 5 μl 50 μg/ml α-naphthoflavone (internal standard) in acetonitrile and mixed. Each sample was extracted with 1 ml ethyl acetate by mixing for 10 min on a Vortex Genie 2 (Model G-560, Scientific Industries, Bohemia, N.Y.) set at 4. The samples were subsequently centrifuged at 14,000 *g* for 5 min, and the resulting organic layers were removed and transferred to 12×75 mm glass culture tubes. Each sample was extracted with an additional 1 ml ethyl acetate, vortexed, centrifuged, and the second organic layers were combined wit the first. The organic layers were evaporated to dryness under a stream of nitrogen (medical grade, Praxair, Pittsburgh, Pa.), and the dried residues were resuspended in 200 μl of the initial mobile phase described below. These samples were transferred to microcentrifuge tubes and centrifuged at 14,000 *g* for 2 min. The resulting supernatants were placed into glass, microvial inserts, and 175 μl was injected by autosampler into the HPLC system.

HPLC

The HPLC system consisted of a Hewlett-Packard 1090L HPLC (Hewlett-Packard, Palo Alto, Calif.) fitted with a Waters Novapak C18 guard column and a Waters Novapak C18 column (5 μm, 3.9×150 mm; Waters Associates, Milford, Mass.). The initial mobile phase, consisting of acetonitrile/25 m*M* sodium phosphate, pH 3.00 (35:65, v/v) with 10 m*M* triethylamine, was pumped at 1 ml/min for 6 min. Between 6 and 14 min, the mobile phase was changed with a linear gradient to acetonitrile/25 m*M* sodium phosphate, pH 3.00 (50:50, v/v) with 10 m*M* triethylamine, which was pumped isocratically until 18 min. Between 18 and 22 min, the mobile phase was returned to the initial conditions, which were maintained for an additional 10 min before injection of the next sample. The column eluate was monitored at 313 nm with a Waters 440 absorbance detector fitted with a 313-nm filter and slit. Under these conditions, the retention times of 17AG, 17AAG, and internal standard were approximately 6.2, 14.8, and 19.4 min, respectively. Standard curves, prepared in control bile, were performed in duplicate and included 17AG and 17AAG concentrations of 0.05, 0.1, 0.3, 1, 3, 10, and 30 μg/ml. There were no endogenous materials in bile or dosing vehicle that interfered with the determination of 17AG, 17AAG, or internal standard. The lower limit of quantitation [35] in bile was 0.1 μg/ml, and the coefficients of variation in bile at a low mid-range concentration (0.5 μg/ml) and a high mid-range concentration (7.5 μg/ml) were 9% and 3%, respectively. The standard curves of 17AAG and 17AG in bile were linear between 0.1 and 30 μg/ml. Samples containing concentrations above the upper limits of each standard curve were reassayed after being diluted in phosphate-buffered saline (pH 7.4), to a degree calculated to produce concentrations within the linear range.

Metabolite characterization and identification

Absorbance spectra of 17AAG and proposed metabolites in bile were obtained using an isocratic mobile phase of acetonitrile/25 m*M* sodium phosphate, pH 3.00 (30:70, v/v) with 10 m*M* triethylamine, pumped at 1 ml/min, and the same columns described above. The column eluate was monitored for absorbance between 200 and 600 nm with a Hewlett-Packard 1050 diode-array detector and a Hewlett-Packard Chemstation operating under Microsoft Windows 95-based software.

LC/MS and LC/MS/MS analyses used an Agilent (Palo Alto, Calif.) model 1100 autosampler and pump that provided linear gradients and a constant flow rate of 200 µl/min. All chromatography was performed on a Phenomenex (Torrance, Calif.) Luna C18 (2) column (2×150 mm) packed with 3-µm particles. A gradient elution program was used for the separation of metabolites. The pH of the mobile phase was maintained with 0.1% formic acid throughout the elution program. Initial conditions consisted of acetonitrile/water (1:99, v/v), which were maintained for 2 min after sample injection. Following these initial isocratic conditions, the mobile phase was immediately changed to acetonitrile/water (20:80, v/v) and from that point was increased linearly over 60 min to a final composition of acetonitrile/water (60:40, v/v). Mass spectrometry was performed on a Micromass (Manchester, UK) QTOF-II mass spectrometer. Materials absorbing at 330 nm were detected in-line with an Agilent model 1100 photodiode array detector prior to entry into the mass spectrometer. Mass spectra were acquired in the negative-ion mode at a rate of one scan per second and over a mass range of 42–700 Da. MS/MS mass spectra were acquired with a resolution of 10,000, a collision cell pressure of 1 mTorr, and a collision energy of 30 V.

Results

By 15 min after i.v. delivery of 17AAG, the bile contained at least 15 other materials with absorbance spectra similar to that of 17AAG (Fig. 2). Of these, metabolites eluting at 2.7, 2.9, and 8.6 min were present in sufficient concentrations to be quantified, although the lack of authentic standards resulted in their being expressed as 17AAG equivalents. Within the first 4 h after drug delivery, biliary excretion of quantifiable metabolites accounted for $28.9 \pm 6.1\%$ of the 10-mg/kg 17AAG dose (Fig. 3). 17AAG and 17AG accounted for $4.1 \pm 1.0\%$ of the delivered dose, with 17AAG accounting for $2.0 \pm 0.5\%$ and 17AG accounting for $2.1 \pm 0.5\%$ (Fig. 3). The metabolites eluting at 2.7, 2.9, and 8.6 min accounted for $10.6 \pm 2.0\%$, $9.8 \pm 1.2\%$, and $1.0 \pm 0.2\%$, respectively, of the administered dose (Fig. 3).

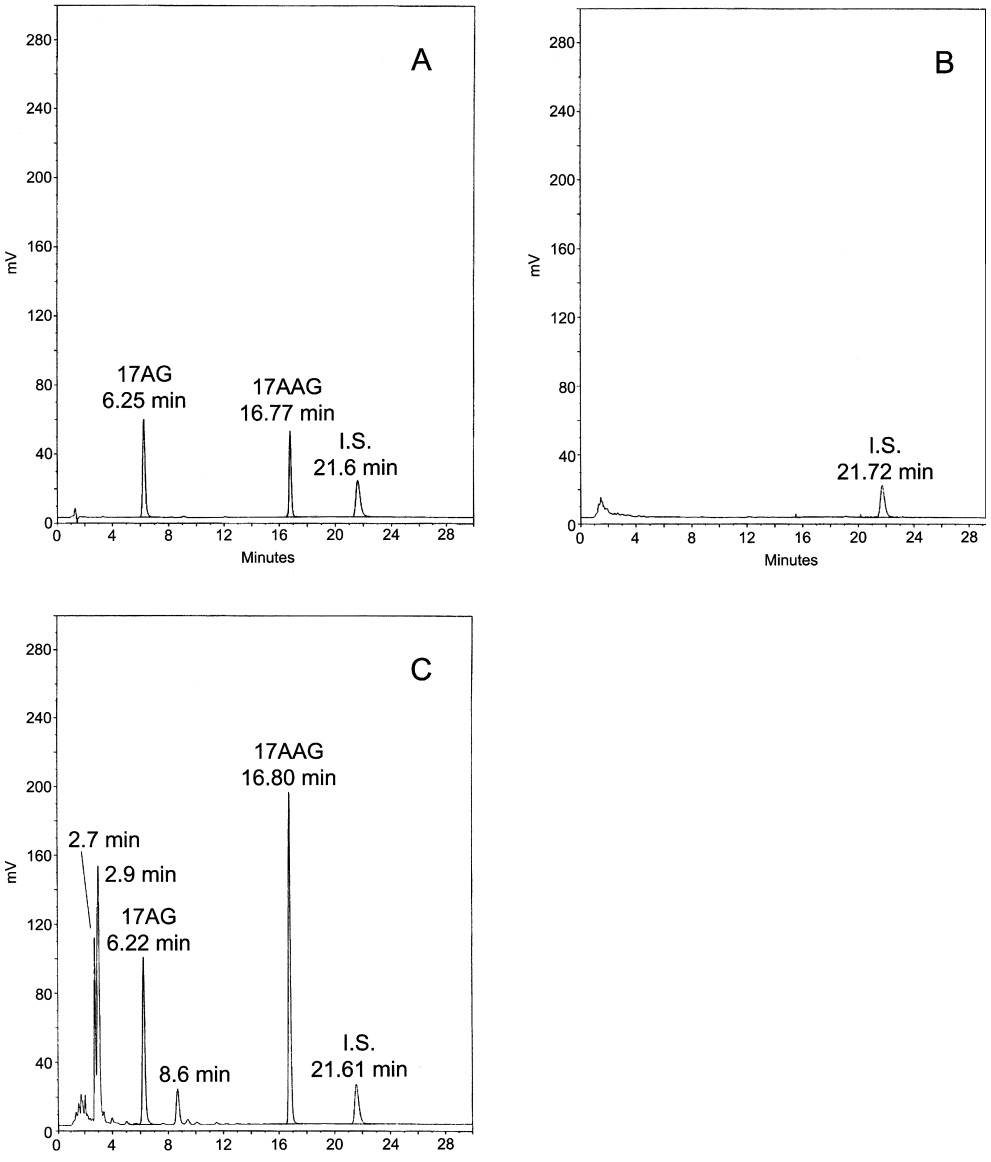


Fig. 2A–C HPLC chromatograms of (**A**) control bile to which 17AG (3 µg/ml), 17AAG (3 µg/ml), and internal standard were added, (**B**) pretreatment bile from a rat, and (**C**) 1:20 dilution of bile collected from the same rat 0–15 min after i.v. administration of 10 mg/kg of 17AAG

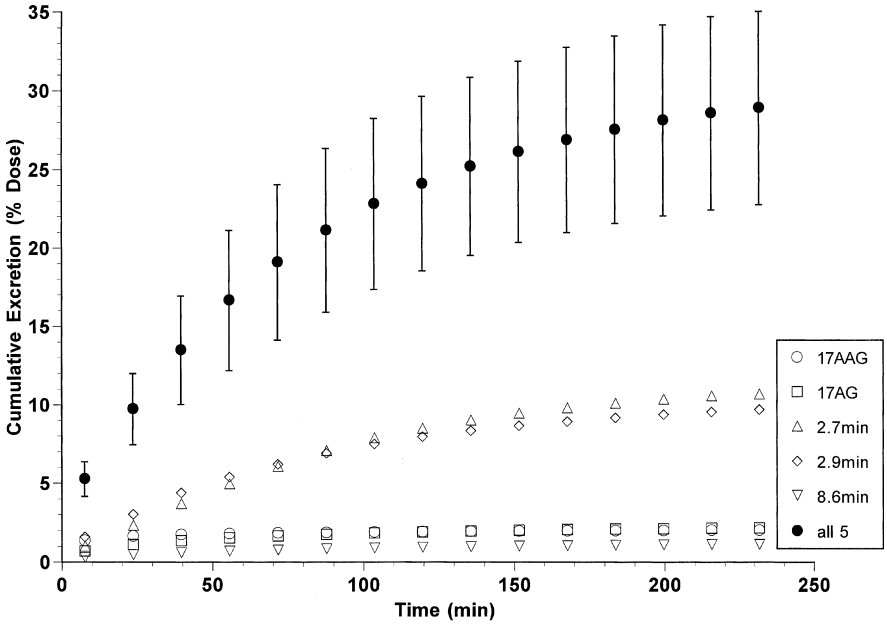


Fig. 3 Cumulative biliary excretion of 17AAG and metabolites by three rats given i.v. 17AAG doses of 10 mg/kg. Symbols represent the means of three rats, and error bars represent one SD

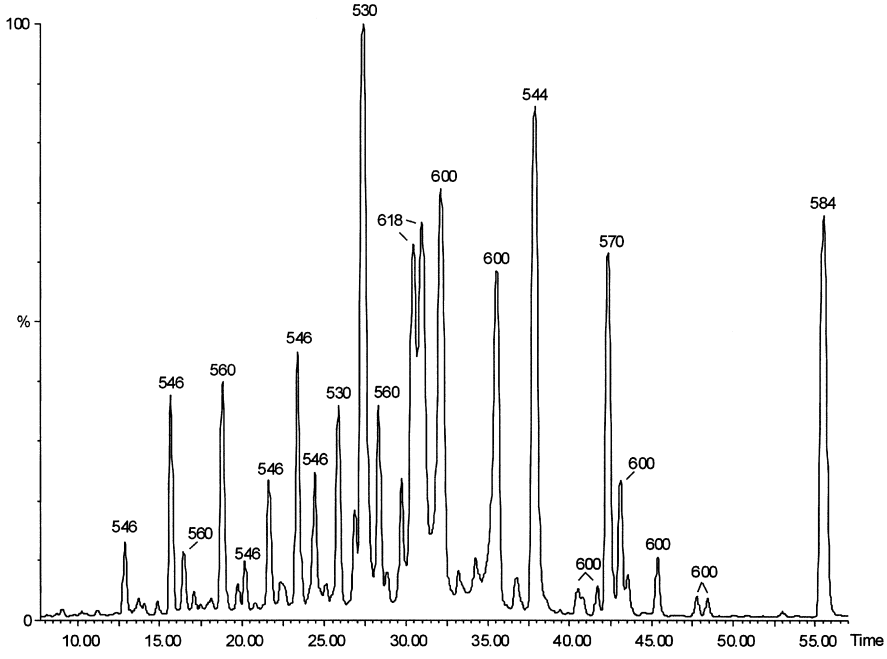


Fig. 4 Summed ion chromatogram of significant biliary metabolites of 17AAG. Peak numbers indicate the observed molecular anion $[M–H]^-$ for each peak

LC/MS/MS analysis of bile showed that extensive metabolism of 17AAG had occurred (Fig. 4). More than 40 individual components, consistent with metabolites of 17AAG, were found in the bile extract. Although previously described metabolites corresponding to 17AG and a diol were among the major metabolites formed, numerous other significant metabolites were observed. Among these, were a series of ten proposed metabolites that had a mass (601) and MS/MS spectrum consistent with mono-oxygenated 17AAG. In vitro experiments confirmed the identity of the epoxide metabolite, which was detected at 32 min (Fig. 5). MS/MS spectra (data not shown) of this metabolite placed the epoxide on the allyl side-chain of 17AAG. Another significant mono-oxygenated metabolite, detected at 36 min, had an MS/MS spectrum identical to that observed for the epoxide metabolite, implying that oxidation had also occurred on the allyl side-chain of 17AAG. However, this metabolite was not formed in vitro in large amounts by CYP3A4. The other proposed mono-oxygenated metabolites were found at much lower levels, with MS/MS spectra indicating that oxidation had occurred at various locations on the ansamycin ring. Two other proposed metabolites had a mass and MS/MS spectrum consistent with mono-demethylated 17AAG metabolites (m.w. 571). However, the proposed mono-demethylated metabolite eluting at 42.5 min was approximately 20 times greater than the mono-demethylated

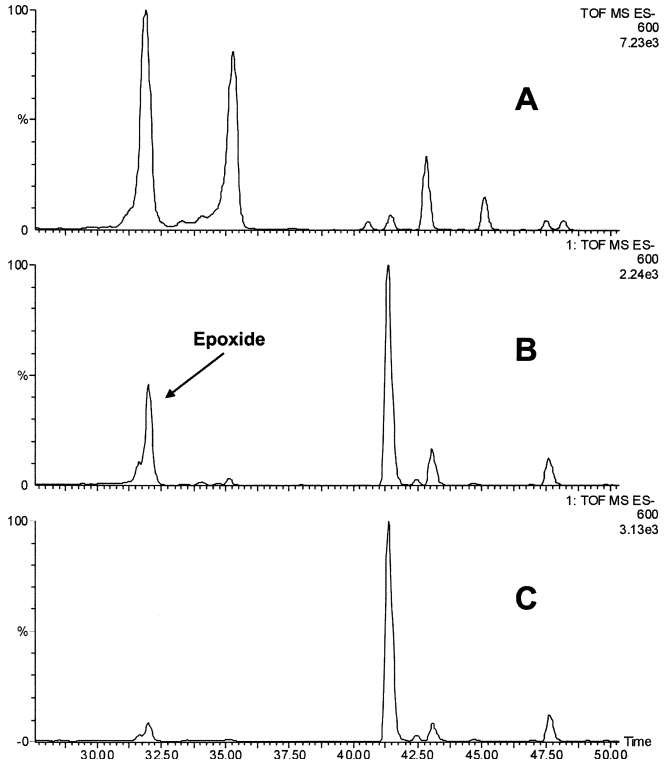


Fig. 5A–C Selected ion chromatograms for mono-oxygenated metabolites (m/z 600) of 17AAG. **A** Total metabolites observed in rat bile; **B** mono-oxygenated metabolites formed with recombinant human CYP3A4 without epoxide hydrolase; **C** mono-oxygenated metabolites formed with recombinant human CYP3A4 in the presence of epoxide hydrolase

metabolite occurring at 41 min, indicating a clear preference for removal of one of the methoxy-methyl groups on the ansamycin ring. A di-demethylated 17AAG metabolite (m.w. 557) was also observed, but at very low abundance. Finally, because removal of the allyl side-chain of 17AAG to produce 17AG (m.w. 545) was a major metabolic pathway, a series of mono-oxygenated and demethylated metabolites were observed for this 17AG that were similar to those observed for 17AAG, with the notable exceptions of the mono-oxygenated metabolites that occurred on the allyl side-chain of 17AAG.

Discussion

The heat-shock protein-interactive drug, 17AAG, has entered clinical trials [1, 5, 9, 26, 43], and a number of aspects of its pharmacology have been described [2, 3, 4, 6, 7, 8, 15, 16, 18, 22, 23, 24, 25, 27, 28, 29, 31, 32, 34, 37, 38, 39, 41, 42]. While 17AAG is undergoing clinical evaluation, there is an ongoing effort to develop other heat-shock protein-interactive agents that might not have the theoretical drawback of metabolism to potentially toxic metabolites and the practical problem of complex formulation that are associated with 17AAG. One such agent is 17DMAG (NSC 707545) [14, 36], for which we have previously characterized the biliary excretion [14]. After performing those studies, it was recognized that the quantitative aspects of 17AAG biliary excretion were uncharacterized and that a number of the metabolites produced from 17DMAG involved changes on the molecule for which analogous 17AAG metabolites had not been described. The studies described here addressed these deficiencies by providing data on the biliary excretion and metabolites of 17AAG similar to those previously developed for 17DMAG [14].

The significant biliary excretion of 17AAG is consistent with the knowledge that 17AAG undergoes extensive metabolism by CYP3A [12, 13], and urinary excretion of 17AAG accounts for only a small percentage of the delivered dose [13]. Furthermore, the fact that most of the biliary excretion of 17AAG is in the form of metabolites, rather than parent compound, is consistent with the previously mentioned extensive metabolism of 17AAG [12]. From a clinical perspective, these biliary excretion data argue strongly for the need to use and evaluate 17AAG carefully in patients with altered hepatic excretory or metabolic function.

Although 17AAG is known to undergo extensive metabolism by CYP3A [12, 13], a number of the metabolites observed in the bile of rats treated with 17AAG have not been described previously. However, their presence in bile was not entirely unexpected because of previously published studies with 17DMAG [13]. Although the fraction of 17DMAG that is metabolized is much less than that of 17AAG [13], the number of potential 17DMAG metabolites observed with LC/MS is much larger than the number of metabolites that had been demonstrated for 17AAG [13], and the sites of 17DMAG metabolism are at positions not previously noted for metabolites of 17AAG. The presence of ten potential 17AAG metabolites with a mass of 601 implies that oxidation can occur at numerous positions on 17AAG. However, the most significant location of oxidation is on the allyl side-chain. The presence of two potential metabolites with a mass of 571 implies removal of two different methyl groups, and the metabolite with a mass of 557 presumably represents the end result of di-demethylation. These metabolites are likely the result of the loss of methyl groups from the two methoxy moieties on the ansamycin ring. Finally, the presence of analogous 17AG metabolites reflecting mono-oxygenation and demethylation indicates that the metabolic processes affecting sites on the ansamycin benzoquinone portion of 17AAG can also affect the metabolite resulting from CYP3A removal of the allyl group. Although it is unclear whether these 17AG metabolites reflect metabolism of 17AG after it is produced from 17AAG, or CYP3A-mediated de-allylation of one of the previously mentioned metabolites of 17AAG, the relative amounts of 17AG and its metabolites argue for the former rather than the latter pathway. Although we have not yet definitively identified the structures of these presumed 17AAG metabolites, they are not likely to involve alterations to the benzoquinone moiety because their absorbance spectra are not altered when compared

to that of parent compound. Future studies will be directed at complete characterization of the structures of these materials and the enzymes responsible for their production.

In summary, we have characterized the biliary excretion and biliary metabolites of 17AAG after i.v. administration to rats. The data regarding the degree of hepatic metabolism and biliary excretion of 17AAG and its metabolites have obvious relevance to the potential use of this 17AAG in patients with hepatic dysfunction in whom hepatic metabolism and biliary excretion would be impaired. Less obvious, but possibly of equal importance, would be the potential relevance of these data to understanding the dose-limiting hepatotoxicity that occurs in patients given 17AAG on a daily ×5 schedule [26, 43].

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