**In Vitro** Detection of Differential and Cell-Specific Hepatobiliary Toxicity Induced by Geldanamycin and 17-Allylaminogeldanamycin Using Dog Liver Slices

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Experiments on rat liver slices demonstrated the differential hepatobiliary toxic potency of two anticancer agents, geldanamycin (GEL) and 17-allylaminogeldanamycin (17-AAG), over a 5-day period. This report describes the pattern of toxicity of these agents in dog liver tissue, using the *in vitro* liver slice culture model. Liver slices (200 μm thick) from male beagle dogs were cultured for 5 days in chemically defined culture medium containing a range of GEL and 17-AAG concentrations (0.1–5 μM).

Tissues were evaluated using a panel of clinically relevant biomarkers and histological endpoints. GEL-induced reduction of alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) slice levels, indicators of biliary epithelial cell (BEC) viability, was supported by histological findings showing an increasing loss of BEC as higher concentrations were applied. At the highest concentrations studied, GEL caused both hepatocellular necrosis and BEC loss. Biomarker pattern results in the medium concurred with those from slice biochemistry measurements and histology. 17-AAG, a less potent compound *in vitro*, elicited more biomarker retention at higher concentrations than did GEL. Histological analysis revealed higher BEC viability and significant retention of BEC proliferation as compared with GEL. However, at the highest concentration, the toxic insult caused a marked decrease in BEC viability and proliferation. Comparison of responses with both compounds indicated that slices exposed to the same concentrations were more sensitive to GEL than to 17-AAG. Dog liver slices can thus be used to evaluate species-, compound-, and concentration-dependent differences in toxicity.

**Key Words:** geldanamycin; 17-AAG; dog; liver slices; toxicity.

Improved *in vitro* models are needed for studying systemic drug effects and developing assays to predict the potential for such effects in humans (ICCVAM/NTP, 2001; Pfaller *et al.*, 2004; Spielmann *et al.*, 1998; Tomaszewski, 2004). Adequate organotypic systems for studying toxic effects on the biliary tract have been lacking. For liver, interest has centered on investigating liver toxicity using hepatocytes (HPCs), but not biliary epithelial cell (BEC) cultures. New drugs undergoing development may adversely affect specific cell subpopulations such as BEC within target organs. Drug-induced injury (*e.g.*, that sustained during anticancer therapy) to the biliary tract and the surrounding tissue is associated with clinically significant injuries, including dose-limiting cirrhosis (Demetris, 1997; Grever and Grieshaber, 1993). A model system that uses only one organ cell type, such as HPC cultures, or that excludes cell types relevant to a particular organ gives an incomplete assessment of risk. In the case of liver toxicity, multicellular systems that retain both HPCs and BEC cells would enable researchers to identify cell-specific adverse effects. Coupling a model of this type with the detection of appropriate biochemical and histological changes that are mechanistically and clinically relevant would allow researchers to correlate these changes with known effects *in vivo*.

Precision-cut slices that retain biliary duct structures in their native environment are an attractive option, but they have been limited in value because these preparations deteriorate too rapidly in culture (Pfaller *et al.*, 2004). Advances in slice preparation and culture methods have resulted in more stable, long-lasting roller slice techniques (Behrsing *et al.*, 2003; Fisher and Vickers, 2003; Saulnier and Vickers, 2002).

Recently, two benzoquinone ansamycin anticancer compounds, GEL and its derivative 17-AAG, have received attention. This class of compounds is known to inhibit the function of a major molecular chaperone, Hsp90. Several key signaling molecules such as protein kinases and steroid hormone receptors require Hsp90 for their proper folding and activity (Miyata, 2005). Studies have shown that GEL fits into the ATP-binding pocket of Hsp90 and inhibits its ATP binding, ATPase, and molecular chaperone activity (Prodromou *et al.*, 1997). Thus, treatment of cells with geldanamycin results in inactivation, destabilization, and degradation of Hsp90 client proteins that play important roles in the regulation of the cell cycle, cell growth, cell survival, apoptosis, and oncogenesis (Miyata, 2005). Because documented *in vivo* toxicity demonstrated GEL-induced hepatic necrosis at efficacious dose
levels, the drug’s clinical development was halted (Page et al., 1997; Supko et al., 1995). However, 17-AAG, given its lower toxicity (Page et al., 1997), is currently being evaluated in Phase I clinical trials. In rat slices, GEL and 17-AAG produce generally similar results and in the same relative timescale as reported in vivo, indicating that this model system is useful as an assay of chemically induced hepatobiiliary injury (Behrsing et al., in press). In summary, results from examining clinical biomarker levels in slices and medium, coupled with histological analysis of tissue, have demonstrated differential, compound-specific and cell-specific injury in a concentration-dependent manner. Geldanamycin proved to be more potent than 17-AAG in causing initial biliary injury and, with higher doses, hepatocellular damage in rat slices. Given that the U.S. Food and Drug Administration (FDA) requires preclinical toxicology studies to be performed on two species, a rodent and a nonrodent, for all small molecules (Tomaszewski, 2004), testing was expanded from rat liver slices to another widely used higher species. This report describes GEL and 17-AAG slice experimentation with beagle dog liver and compares results with those for in vivo dog studies. Results obtained from Fischer-344 rat liver slice studies are also briefly compared.

Beagle dogs were selected for these experiments because the dog is the nonrodent species of choice for the preclinical toxicology studies in the Developmental Therapeutics Program (Tomaszewski, 2004; Tomaszewski and Smith, 1997). After culture of liver slices exposed for varying times to a range of GEL and 17-AAG concentrations, slices and medium were harvested and assessed for biochemical and histological biomarkers. Biochemical biomarkers examined include lactate dehydrogenase (LDH), alanine L-transferase (ALT), alkaline phosphatase (ALP), and 20 β-glutamyl transferase (GGT), as well as tissue protein content (to normalize data). Histological analysis includes HPC and BEC viability assessed with hematoxylin and eosin (H&E) staining and cell proliferation with 5-bromo-2-deoxyuridine (BrdU) immunohistochemistry. The results of our experimentation using dog liver slices show (1) the biochemical and histological cell-specific response of slices to GEL and 17-AAG, (2) the differential toxicity of the two compounds, (3) the time course of short-term exposure to GEL, (4) the concentration-dependency of the tissue response to both compounds, (5) the appearance of biochemical biomarkers in the medium after toxic insult, and (6) a species difference in toxic response when compared with rat data.

MATERIALS AND METHODS

Animals, supplies, and equipment. Glutathione was purchased from EM Science (Gibbstown, NJ). Bovine serum albumin (BSA) and sodium pyruvate were purchased from ICN Pharmaceuticals (Aurora, OH). Waymouth’s MB 752/1 basal medium (#078–5105EL), Ca l and Mg l-free phosphate-buffered saline (PBS), Glutamax I, and antibiotic-antimycotic solution were purchased from Invitrogen/Gibco (Carlsbad, CA). ViaSpan (Belzer—University of Wisconsin [UW] cold storage solution) was purchased through Fisher Scientific (Pittsburgh, PA). All other chemical supplies were purchased from Sigma (St Louis, MO). The tissue coring press and titanium inserts were purchased from Vitrion (Tucson, AZ). Hydroanalysis mixed cellulose ester Triton-free (HATF; 0.45-μm surfactant-free) filter paper used for slice placement was purchased from Millipore (Bedford, MA). The polytetrafluoroethylene (PTFE) membrane TF-200, 0.2-μm filters ( Pall Life Sciences, Port Washington, NY) were purchased from WVR International (West Chester, PA). Low-background, glass scintillation vials were purchased from Research Products International (Mt. Prospect, IL). The TC-8 roller drum unit was purchased from New Brunswick Scientific (Edison, NJ). Geldanamycin (99% purity) and 17-AAG were obtained from the NCI Repository (McKesson Biosciences, Rockville, MD).

Liver slice preparation. Beagle dog liver specimens were obtained during necropsy procedures from control animals in other ongoing studies. Dogs were purchased from Marshall Farms (Rose, NY); quarantined for 14 days following arrival before study began; and, following study completion, were necropsied after euthanasia with sodium pentobarbital (150 mg/kg iv). The liver tissue (consisting of one to two lobes) was removed immediately after the abdominal cavity was opened for liver slice studies. The lobes were immediately placed in ice-cold UW solution, supplemented with 3 mM glutathione, 2 mM Glutamax I, L-1× antibiotic-antimycotic solution, 30 μM L-ascorbic acid, 2 mM sodium pyruvate, 1 μM cortisol, and 100 nM insulin (human). The vessel containing the lobes was placed into a biosafety hood where all further work was done aseptically. The lobes were quickly cored into 8-mm-diameter cylinders and sliced (in supplemented UW solution) into ~200-μm-thick discs, using a Krundieck slicer (Alabama Research and Development, Munford AL). Only the most uniform-shaped slices were selected for experiments; each contained approximately 3 mg protein. The slices were placed onto precut, sterile, HATF paper inside the titanium inserts, and all manipulations were done in ice-cold supplemented UW solution.

Slices preincubation/culture. Slices on titanium inserts were placed in sterile scintillation vials, each containing 1.7 ml culture medium (Waymouth’s MB 752/1 basal medium containing, per milliliter: 2 mg BSA; 0.084 mg gentamicin sulfate; 5 μg oleic acid; 5 μg linoleic acid; 0.5 μg DL-α-tocopherol; 7.9 μg D-thyroxine; 5 μg insulin, 5 μg transferrin, and 5 ng selenium (ITS); 288 ng testosterone; 272 ng β-estradiol; 39.3 ng deoxanethasone; 30 ng glucagon; 0.02 U insulin; 0.2 μmol L-2-phosphate ascorbic acid; and 2 mM sodium pyruvate). The vials were then cpped with presterilized open-end caps containing PTFE membrane filters, each held in place by a hole-punched, Teflon liner to allow gas exchange with the external atmosphere. The capped vials were placed in the roller drum inside a humidified incubator at 37°C under a 75% O₂/5% CO₂/air atmosphere. The roller drum containing the vials was rotated at 6–7 rpm. After equilibration for 2.5–3.0 h, the zero time-point samples were harvested. The medium was aspirated from the remaining vials and then replaced with warmed media with or without GEL or 17-AAG at designated concentrations. The slice medium in each vial was replaced with fresh medium daily until the slices were harvested, and for the portion of the study examining medium biomarker content, the collected medium (1 ml/vial) was pooled for respective slices and stored at 4°C until later biochemical analysis. After medium change, the vials were repositioned on the roller system in the incubator, and the incubation was continued under the above conditions until all groups (four replicates/group) were harvested.

Slice harvest. At the indicated time points, slices plus filter paper were removed from titanium inserts with tweezers and briefly rinsed by submerging in a vessel containing ~20 ml PBS. The slices were then bisected so that one-half could be used for biomarker analysis and the other for histology. For bromo-deoxyuridine (BrdU) incorporation experiments, 20 μM BrdU was added to the medium and allowed to incubate with the slices for 18 h before their harvest.

Biomarker analyses. Each slice section designated for biochemical analysis was transferred into a 1.5-ml Eppendorf tube containing 0.5 ml PBS + 0.5% Triton X-100 on ice. The sections were then homogenized and briefly sonicated at ice-cold temperatures. The resulting lysates were
centrifuged in a Beckman Coulter Microfuge 18 centrifuge at 9000 × g for 5 min to remove particulate matter. The resulting supernatants were stored at 4°C until the experiment was complete and then sent on ice with the corresponding media by courier to Quality Clinical Labs (Mountain View, CA) for analysis on a Hitachi 911 clinical analyzer. Biomarker (aspartate aminotransferase [AST], ALT, ALP, GGT, and LDH) levels measured in slice lysates taken within the first 24 h and analyzed periodically thereafter were stable at 4°C for a minimum of 18 days (data not shown). A small fraction of the lysate was retained for protein analysis with the Pierce BCA protein assay kit (VWR International, West Chester, PA) and BSA standards in PBS + 0.5% Triton X-100. A chemiluminescent ALP-specific substrate purchased from Michigan Diagnostics (Troy, MI) was used to measure ALP activity in the medium. This was done by extrapolating activity in the control and treatment groups from an ALP standard curve. All medium ALP assays were performed in 96-well microtiter plates.

**Histology.** The faces of slice sections designated for morphological examination were covered by small squares of lens paper (pre-wet in 10% buffered formalin) and placed between two foam inserts. The entire “sandwich” was first placed in a histological cassette, fixed in 10% buffered formalin for 18–24 h, then transferred to 70% alcohol, and finally submitted to the Histology Lab (South San Francisco, CA) for paraffin embedding. Sections 4 µm thick were cut from paraffin blocks and used for H&E and BrdU immunostaining. A BrdU immunostaining kit (Zymed Labs, South San Francisco, CA) was used to label the proliferating cells. The immunostaining was carried out according to the protocol provided by the manufacturer.

**Semiquantitative histological analysis.** Hematoxylin and eosin (H&E)-stained slice sections were extensively examined under low (50×) and medium (100× and 200×) magnification and assessed for HPC and BEC viability. From this assessment, a viability percentage was assigned for both HPCs and BECs. In addition, glycogen deposition in HPCs was estimated, using a scale of 0–4 where 0 represents a finding of no glycogen, 1 = minimal, 2 = mild, 3 = moderate, and 4 = high. Biliary epithelial cell proliferation was evaluated on sections immunostained with BrdU. From each section, the five best portal tracts consisting of medium-sized bile ducts were selected and counted for BrdU-positive BEC using a Zeiss Axioskop 2 equipped with Axiocam digital camera and KS 300 interactive imaging software. A mean proliferating BEC count was then calculated for each slice.

**Statistics.** Replicates from control and treatment groups were consolidated between experiments and compared via t-test analysis using an Excel spreadsheet. Values were considered significantly different if \( p < 0.05 \). Treatment group averages and standard deviations were calculated. Data presented as percentage of control had treatment groups divided by their respective control values and multiplied by 100 to obtain percent values. For percent of control values, standard deviation was calculated as a percentage of the ratio between the treated group and control.

### RESULTS

#### Slice Culture

Experiments assessing the toxicity of GEL and 17-AAG were continued for 5 days. At designated time points (days 0, 1, 3, and 5) slices and medium were harvested and analyzed biochemically and histologically. Data indicated well-maintained viability over the culture period (Table 1, Fig. 1 and Fig. 2). Although AST and ALT values declined over the culture period, LDH values gradually increased, GGT values doubled, and ALP values increased 20-fold over initial values. Histological data indicated an initial HPC and BEC viability of 86% and 94%, respectively, and by day 5 those values had not diminished (88% and 95%, respectively). Dog liver slice HPC accumulated glycogen and BEC proliferated in culture; these phenomena were also observed in rat liver slices (Behrsing et al., 2003). Overall, histological observations showed excellent tissue integrity, and biochemistry indicated good metabolic function.

#### GEL and 17-AAG Toxicity in Slices

Exposure of slices to GEL resulted in a more potent effect when compared to 17-AAG at the same concentration. When evaluating cell viability, an overlap of morphological features characteristic of two major forms of cell death (i.e., necrosis and apoptosis) was observed. However, a necrotic pattern was predominant, especially in HPC. Features consistent with apoptosis were mostly seen in BEC exposed to lower concentration of both drugs. By day 3, a moderate loss of BEC viability was seen histologically when 0.5 µM GEL was included in the medium. A tenfold higher concentration (5 µM)
of 17-AAG was required to cause a similar loss of viability at the same time point (Table 2). Higher concentrations of GEL also caused a loss of HPC viability on day 3. By day 5, the loss of BEC viability with 0.5 \( \mu \text{M} \) GEL was even higher, and HPC toxicity also became apparent. Hepatocyte viability was markedly reduced as higher concentrations of GEL were included in the medium. On day 5, only one-third of the HPC were viable as compared with control when 5 \( \mu \text{M} \) GEL was applied (Table 3). A reduced capacity for glycogen accumulation appeared to precede a loss of viability, as assessed histologically. Biochemical results indicate a reduction of slice ALP and LDH content with increasing GEL concentrations at day 3, but at day 5, slice GGT values (along with ALP and LDH) were also lower. However, slice biomarkers for HPC were not reduced with increasing GEL (or 17-AAG) concentrations. On both days 3 (Table 2) and 5 (Table 3, Fig. 2 and Fig. 3) the histological and biochemical findings indicate that BEC and HPC were more sensitive to GEL than to 17-AAG.

**BEC Proliferation**

The application of GEL or 17-AAG to the dog liver slice incubations resulted in concentration– and time-exposure–dependent changes in BEC proliferation (Fig. 1, Table 2, and Table 3). Overall BrdU incorporation into BEC decreased in a concentration-dependent manner; thus, by day 5, only...
occasional proliferating BEC were observed at 1.5 μM and higher concentrations of GEL (Table 3). With lower concentrations of 17-AAG (0.1 μM and 0.5 μM), a slight increase in BEC proliferation occurred, given the greater number of BrdU-positive BEC. However, the proliferation response was significantly attenuated when slices were exposed to higher (1.5 μM and 5 μM) 17-AAG concentrations, as indicated by the decrease in the number of BrdU-positive BEC (0.4x or 40% of control) with 5 μM on day 5.

**Effect of GEL and 17-AAG on Medium Biomarkers**

Plasma levels of clinical biomarkers as an indicator of toxicity are routinely examined in *in vivo* studies. Several experiments were also conducted to measure clinical chemistry parameters (AST, ALT, LDH, and ALP) in medium after slice treatment with GEL or 17-AAG. Medium biomarker values represent cumulative levels between slice harvests (*e.g.*, day 3 medium = pooled medium from days 2 and 3, multiplied by the number of days collected; 2 in this example). To increase sensitivity, a luminescence assay was used to measure ALP in medium. Control value averages indicated that AST and ALT activity levels remained relatively steady over the 5-day incubation period (Table 4). Interestingly, ALP and LDH activities measured in the medium were found to increase over time.

Results from average medium biomarker values for days 3 (data not shown) and 5 (Fig. 3) indicated that treatment altered HPC and BEC biomarker activities in a concentration-dependent manner. Although slice levels of HPC biomarkers were not reduced when histological loss of HPC viability occurred, medium HPC biomarkers were found to increase with increasing GEL concentrations. Comparing medium activity values on day 5, GEL induced a larger increase (~3–4 fold) in AST, ALT, and LDH than did 17-AAG at all concentrations greater than 0.1 μM (Fig. 3). The addition of GEL and 17-AAG diminished the levels of ALP in the medium in a concentration- and compound-dependent manner—an effect opposite that which they had on HPC markers. For example, GEL application (1.5 μM) resulted in a reduction of medium ALP activity to 47% of control, whereas 17-AAG caused an increase to 136% of control. Only the highest concentration of 17-AAG applied (5 μM) resulted in a lower medium ALP activity measurement.

**Latent GEL Toxicity**

Prior experiments using rat tissue indicated that a delayed toxic effect occurs in slices after a short exposure to GEL. To examine whether dog liver tissue responded similarly, the study was repeated using dog liver slices. Medium containing 1.5 μM
GEL was applied to slices for 24 h, followed by 48 h of medium alone before harvest on day 3. Results were compared statistically with the 72 h constant exposure groups and with the constant exposure group harvested at 24 h. The values were listed as percent of day 3 control (Table 2) for comparison with the biomarker values of the 3-day constant exposure group. A latent toxic effect was demonstrated after a limited (24 h) exposure of GEL to liver slices. Although slices were exposed for only 24 h, results from biomarker patterns were similar to 72 h of continuous exposure. Slices with only 24 h exposure did not display changes in HPC markers compared with day 1 control (data not shown), but BEC biomarkers (ALP, GGT, and viability) were significantly lower. Lactate dehydrogenase activity also diminished. The overall toxic response appears to be more severe than that elicited for slices harvested on day 1, but less severe than that for slices exposed to 1.5 µM GEL continuously for 72 h.

**DISCUSSION**

The toxicity of the anticancer compounds GEL and 17-AAG in the dog has been studied (Sausville et al., 2003; Supko et al., 1995). Although acute toxic effects have made GEL's

### TABLE 2

Concentration-Dependent Changes in Slices: Comparison of Geldanamycin and 17-Allylamino geldanamycin on Day 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>GGT</th>
<th>LDH</th>
<th>Viability</th>
<th>BEC</th>
<th>Glycogen</th>
<th>BrdU</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM GEL</td>
<td>105 ± 6</td>
<td>106 ± 6</td>
<td>113 ± 9</td>
<td>106 ± 12</td>
<td>98 ± 5</td>
<td>101 ± 2</td>
<td>96 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90 ± 10</td>
<td>0.7x&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1 µM 17-AAG</td>
<td>105 ± 7</td>
<td>91 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95 ± 9</td>
<td>104 ± 10</td>
<td>93 ± 4</td>
<td>99 ± 3</td>
<td>98 ± 1</td>
<td>84 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1x&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5 µM GEL</td>
<td>136 ± 5</td>
<td>106 ± 6</td>
<td>95 ± 7</td>
<td>115 ± 10</td>
<td>98 ± 4</td>
<td>97 ± 2</td>
<td>87 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94 ± 7</td>
<td>0.3x&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5 µM 17-AAG</td>
<td>109 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>93 ± 9</td>
<td>106 ± 11</td>
<td>89 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96 ± 3</td>
<td>97 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90 ± 7</td>
<td>1.4x&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>1.5 µM GEL</td>
<td>137 ± 6</td>
<td>104 ± 4</td>
<td>66 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98 ± 8</td>
<td>86 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0x&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5 µM GEL (24h)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>117 ± 6</td>
<td>98 ± 12</td>
<td>72 ± 12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>97 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85 ± 10</td>
<td>90 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>75 ± 3</td>
<td>0.2x</td>
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<tr>
<td>1.5 µM 17-AAG</td>
<td>105 ± 9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>103 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>111 ± 11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>91 ± 6</td>
<td>97 ± 2</td>
<td>95 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>5 µM GEL</td>
<td>138 ± 7</td>
<td>107 ± 6</td>
<td>56 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95 ± 10</td>
<td>81 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0x&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>5 µM 17-AAG</td>
<td>123 ± 6</td>
<td>102 ± 6</td>
<td>79 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>113 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>87 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>92 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>84 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3x&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup>All results (n = 8, except 24 h 1.5 µM GEL pulse application and BrdU scores, n = 4) are expressed as a percent of control ± SD of the ratio, except BrdU (fold increase over control).

<sup>b</sup>Indicates 17-AAG treatment significantly different (p < 0.05) than control values.

<sup>c</sup>Indicates 17-AAG treatment significantly different (p < 0.05) from the GEL-treatment group.

<sup>d</sup>Slices were exposed to 24 h of GEL followed by 48 h of medium and harvested on day 3 (n = 4). Results are listed as % of day 3 control.

<sup>e</sup>24 h treatment is significantly different (two-tailed, equal variance, p < 0.05) from day 1 treatment of 1.5 µM GEL (data not shown).

### TABLE 3

Concentration-Dependent Changes in Slices: Comparison of Geldanamycin and 17-Allylamino geldanamycin on Day 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>GGT</th>
<th>LDH</th>
<th>Viability</th>
<th>BEC</th>
<th>Glycogen</th>
<th>BrdU</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 µM GEL</td>
<td>102 ± 6</td>
<td>102 ± 6</td>
<td>125 ± 8</td>
<td>115 ± 9</td>
<td>101 ± 8</td>
<td>96 ± 2</td>
<td>94 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>88 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0x</td>
</tr>
<tr>
<td>0.1 µM 17-AAG</td>
<td>99 ± 5</td>
<td>97 ± 7</td>
<td>108 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>94 ± 10</td>
<td>107 ± 17</td>
<td>101 ± 2</td>
<td>97 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>88 ± 7</td>
<td>1.4x</td>
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<tr>
<td>0.5 µM GEL</td>
<td>123 ± 8</td>
<td>102 ± 9</td>
<td>105 ± 9</td>
<td>94 ± 8</td>
<td>92 ± 15</td>
<td>81 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75 ± 7</td>
<td>0.7x</td>
</tr>
<tr>
<td>0.5 µM 17-AAG</td>
<td>98 ± 7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>82 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>101 ± 9</td>
<td>111 ± 10</td>
<td>92 ± 14</td>
<td>96 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>95 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>88 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3x&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5 µM GEL</td>
<td>151 ± 12</td>
<td>111 ± 16</td>
<td>77 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77 ± 11</td>
<td>45 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1x&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.5 µM 17-AAG</td>
<td>114 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108 ± 9</td>
<td>139 ± 9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>135 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108 ± 16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>94 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>91 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7x&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 µM GEL</td>
<td>136 ± 12</td>
<td>89 ± 14</td>
<td>59 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60 ± 44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0x&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 µM 17-AAG</td>
<td>133 ± 10</td>
<td>110 ± 8</td>
<td>95 ± 9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86 ± 13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5x&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>All results (n = 8) are expressed as a percent of control ± SD of the ratio, except BrdU (fold increase over control).

<sup>b</sup>Bold = significantly lower (p < 0.05) than control values.

<sup>c</sup>Indicates 17-AAG treatment significantly different (p < 0.05) from the GEL-treatment group.
suitability for further development questionable (Supko et al., 1995), 17-AAG is now undergoing Phase I clinical trials (Adams and Elliott, 2000; Sausville et al., 2003). The liver was found to be the principal target organ for acute GEL toxicity, and 17-AAG is markedly less hepatotoxic than GEL. Geldanamycin treatment elevated hepatic serum enzyme levels and, histologically, was shown to cause hepatobiliary necrosis.

The current study demonstrated differential hepatobiliary toxicity induced by GEL and 17-AAG using the dog liver slice model and a panel of clinically relevant biomarkers measured in slice lysates and medium, coupled with histological analysis. The results obtained indicate that (1) GEL clearly elicited a greater hepatobiliary toxic response in dog liver slices than did 17-AAG at the same time points and the same concentrations; (2) the response to both compounds was concentration-dependent and cell-specific, as indicated by biochemistry readings and concurrent histological analysis; (3) compound-induced reduction of slice biomarker levels and parenchymal cell viability (biochemical and/or histological) was concomitant with the appearance of AST, ALT, and LDH in the medium; in contrast, ALP in the medium for control slices increased with time in culture, and as toxicity to BEC increased by exposure to GEL or 17-AAG, both slice and medium activities of ALP were reduced in a concentration-dependent manner; (4) the lower 17-AAG concentrations (0.1 and 0.5 μM) appeared to induce BEC proliferation, as evidenced by elevated BrdU incorporation on days 3 and 5, an in vitro observation that correlates with the bile duct hyperplasia seen in vivo; and (5) with regard to cell viability, GEL was approximately tenfold more potent than 17-AAG in producing hepatobiliary cell necrosis.

### FIG. 3
Mean biomarker levels in medium (day 5) after GEL or 17-AAG treatment. Biomarker values are expressed as a percentage of control values ± SD of the ratio. *Significant difference (p < 0.05) from control groups. †Significant difference (p < 0.05) from GEL groups.

### TABLE 4
Control Biomarkers in Medium

<table>
<thead>
<tr>
<th>Day</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>415 ± 215</td>
<td>140 ± 70</td>
<td>12 ± 5</td>
<td>40 ± 16</td>
</tr>
<tr>
<td>3</td>
<td>296 ± 92</td>
<td>114 ± 33</td>
<td>60 ± 23</td>
<td>62 ± 12</td>
</tr>
<tr>
<td>5</td>
<td>541 ± 246</td>
<td>164 ± 58</td>
<td>152 ± 58</td>
<td>166 ± 70</td>
</tr>
</tbody>
</table>

*Expressed as U/l/mg slice protein ± SD.
incubation with 0.5, 1.5, and 5 μM GEL, on both days 3 and 5. Although this result may seem paradoxical, in fact, such an effect on the protein-regulation processes is not surprising, given that GEL’s toxic effects may have widespread implications for HPCs. The addition of GEL to cells containing heat shock protein 90 (Hsp90) has been reported to lead to the upregulation of Hsp70 (Elo et al., 2005). It is also known that Hsp70 is involved in the processing of precursory mitochondrial AST (Artigues et al., 1998). Thus exposure to GEL may increase Hsp70, which then facilitates the production of a greater amount of active mitochondrial AST.

When slice toxicity was evaluated, the levels of biomarkers detected in the medium did not make up the difference between the treated groups and control slice values, although concentration dependency was clearly observed. Because the biomarker enzymes are released over the entire incubation period between medium collections, they are probably subject to degradation by the proteolytic enzymes that are also released from dead cells. Thus, an accurate stoichiometric correlation between slice and media biomarker levels cannot be made.

During the culture period, BEC in both rat and dog liver slices proliferated. We hypothesize that this is a normal response to the slice preparation process. From the results presented, Figure 4 depicts the proliferative BEC response in slice culture. The proliferative response seen in control cultures can be augmented based on treatment of slices. For example, increased proliferation has been noted with low concentrations of 17-AAG or other compounds such as 2-naphthylisothiocyanate (unpublished results). Higher concentrations of 17-AAG or other more potent hepatobiliary toxicants such as GEL have been shown to inhibit the proliferative response (Fig. 4). Interestingly, ALP levels in the medium of control slices also increased over time, although no overt toxicity was found. More investigation is needed to explain this phenomenon.

As drug development costs continue to rise, early elimination of nonviable drug candidates is required. Major obstacles to using in vitro models for predictive toxicology have been the lack of detection of lesions associated with multicellular interactions (ICCVAM/NTP, 2001) and the lack of long-term cultures for the detection of chronic effects (Pfaller et al., 2004). In vitro systems that retain the endogenous compliment of liver cell types include slices and 3D/co-culture systems of isolated HPCs and BEC. Previous reports on HPC and BEC co-culture systems have indicated some success in recreating in vivo-like functionality (Auth et al., 1998, 2001; LeCluyse et al., 2000), but, to our knowledge, no reports have been published on the application of such cultures for detecting differential and cell-specific hepatobiliary toxicity. A distinct advantage of using liver slices instead of traditional dissociated liver cell culture systems is that the slices retain the tissue architecture seen in vivo.

Our recent findings using rat liver slices (Behrsing et al., in press) indicate that it is possible to detect compound-, concentration-, and cell-specific toxicities in response to GEL and 17-AAG application. The current study demonstrates that dog liver slices can also be used for such studies, and that species-related differences in toxicity seen in vivo are replicated in this in vitro system using GEL and 17-AAG. Results from these experiments indicate that dog liver slices differentiate the toxic and cell-specific effects of GEL and 17-AAG as well or better than do rat liver slices. In comparing toxic responses from rat and dog tissues, it appears that rat liver (in regard to HPC and BEC) is more susceptible to GEL.
toxicity than is dog liver (e.g., greater sensitivity of rat BEC [LC$_{50}$ < 0.5 µM] to GEL than dog BEC [LC$_{50}$ = 1.5–5 µM] on day 5 when assessed histologically).

The availability of both rat and dog liver slices that can be maintained in long-term culture (e.g., rat liver slices survive for at least 21 days with 70% viability [unpublished results]) and that are capable of discriminating compound-, concentration-, cell-, and species-specific toxicities suggests that such models could serve as valuable approaches for early drug evaluations. Future work will be directed toward further optimizing and extending the usefulness of the liver slice systems for the culture of human liver slices so that the benefits now afforded by the use of rat and dog liver slices can be realized using this more relevant tissue.

SUPPLEMENTARY DATA

Supplementary data are available online at www.toxsci.oxfordjournals.org.

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REFERENCES


