

## Is alpha-Pinene a Substrate for Permeability-Glycoprotein in Wood Rats?

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**Abstract** alpha-Pinene is the dominant monoterpene in *Juniperus monosperma*. Wood rat species in the genus *Neotoma* that consume *J. monosperma* vary in their inclusion of it in their wild diet and in their tolerance of whole *J. monosperma* or alpha-pinene in laboratory feeding trials. A proposed mechanism for variable tolerance is a difference in absorption of alpha-pinene from the small intestine that is mediated by the intestinal transporter permeability glycoprotein (Pgp). To determine if alpha-pinene is a Pgp substrate, we tested whether it can competitively inhibit Pgp and thereby increase the accumulation of a known Pgp substrate (digoxin) in (1) everted sleeves of small intestine from *Neotoma stephensi*, a juniper specialist, *N. albigula*, a sympatric generalist that consumes juniper, *N. cinerea*, a more distantly related generalist, and Sprague-Dawley rats, and (2) in Caco-2 cells that over express Pgp. We also measured Pgp ATPase phosphate production in transfected insect membrane vesicles exposed to alpha-pinene. We found no significant increase in digoxin accumulation with competitive inhibition experiments, and no increase in phosphate production with transfected membranes, at any concentration of alpha-pinene up to 100  $\mu$ M. To test whether other compounds in juniper affect Pgp activity, we acclimated five *N. stephensi* to a juniper diet for 5 d, but found no significant effect

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compared to animals on control diet. Our data suggest that alpha-pinene is not a Pgp substrate.

**Keywords** P-glycoprotein · Wood rat · *Neotoma* · alpha-Pinene · Juniper · Small intestine · Everted sleeve · Monoterpenes · Rodents · Membrane bound transporter proteins · Biochemical defense mechanisms

## Introduction

Mammalian herbivores differ in their tolerance of defensive chemicals found in plant diets (Atsatt and Ingram, 1983; Mead et al., 1985; Mangione et al., 2000). Within the *Neotoma* genus of wood rats (Edwards and Bradley, 2002) are both dietary generalists and specialists that consume plant species high in defensive compounds (Atsatt and Ingram, 1983; Dial, 1988; Edwards et al., 2001). *Neotoma albigula* (Hartley), a generalist, and *N. stephensi* (Goldman), a juniper specialist, are sympatric, and in the field *N. stephensi* includes more *Juniperus monosperma* leaves in its diet (Dial, 1988). In captivity, *N. stephensi* voluntarily consumes more *J. monosperma* and maintains body mass on diets of higher concentration of alpha-pinene, the dominant monoterpene in *J. monosperma*, than *N. albigula* (Dearing et al., 2000).

Sorensen and Dearing (2003), using a pharmacokinetic approach, found that *N. stephensi* exhibits a lower plasma concentration of alpha-pinene than *N. albigula* after oral gavage. Additionally, Sorensen et al. (2004) found that *N. stephensi* excreted more unchanged alpha-pinene in the feces than *N. albigula* when both species were either fed a juniper diet or gavaged with a single dose of alpha-pinene. These data implicate a barrier to intestinal absorption.

The hypothesized mechanism is a difference in the activity of permeability glycoprotein (Pgp). This member of the ATP-binding cassette (ABC) superfamily, is a 170-kDa membrane bound protein that actively pumps out numerous potentially toxic endogenous and exogenous compounds as soon as they diffuse into the cell membrane (Ambudkar et al., 1999, 2003). Pgp has been implicated as an important factor in the ability of cells exposed to a single drug/xenobiotic to develop resistance to a broad range of structurally and functionally unrelated drugs/xenobiotics. This has been termed multidrug resistance (MDR) or multixenobiotic resistance (MXR) (Kurelec, 1992; Hunter and Hirst, 1997). Initially discovered in cancer cells, Pgp has since been found on the secretory side of normal cells associated with secretion, absorption, or barrier function such as blood–brain barrier, blood–testis barrier, placenta, small intestine, pancreas, colon, adrenal cortex, kidney, and liver in humans and rodents (Hunter and Hirst, 1997; Ambudkar et al., 1999), and with gills and eggs in aquatic species (Bard, 2000).

In our previous work, we found the activity of Pgp summed over the small intestine to be higher in *N. stephensi* than in *N. albigula* (Green et al., 2004), which is consistent with their feeding behavior in both the field and laboratory. Thus, we hypothesized that alpha-pinene is recognized by Pgp and we tested this hypothesis in three ways. First, we determined if racemic alpha-pinene can competitively inhibit Pgp as indicated by increased accumulation of a known Pgp substrate, digoxin (Sababi et al., 2001; Stephens et al., 2001), in everted sleeves of small intestine. We made these measures in *N. albigula* and *N. stephensi*, and included a

congeneric generalist not known to consume *J. monosperma*, *N. cinerea* (Ord), to broaden our phylogenetic comparison, and Sprague-Dawley rat, a species we have used extensively in everted sleeve experiments. The everted sleeves of small intestine from wood rats and Sprague-Dawley rats were previously shown to exhibit the predicted increased accumulation of digoxin when exposed to a known Pgp inhibitor, cyclosporin A (CsA), and maintained both structural and functional integrity (Green et al., 2004, 2005). Second, we used the same indicator of competitive inhibition in a human colon carcinoma cell line (Caco-2) that overexpresses Pgp and is commonly used in the investigation of Pgp substrates (i.e., Chiou et al., 2001). Third, we eliminated unknown variables associated with tissue sections or whole cells by determining the effect of racemic alpha-pinene on phosphate production from Pgp ATPase activity in Pgp transfected membranes (Sarkadi et al., 1992; Ambudkar et al., 1999).

Pgp can be induced by ingestion of Pgp substrates (Salphati and Benet, 1998; Sandstrom and Lennernas, 1999), and may recognize alpha-pinene or some other compound(s) in *J. monosperma*. To investigate the possible induction of Pgp activity by juniper ingestion, we acclimated one group of *N. stephensi* to a diet high in juniper and measured the same endpoints as we did in animals on a control diet.

## Methods and Materials

### Chemicals

We purchased [ $^3\text{H}$ ]digoxin and [ $^{14}\text{C}$ ]polyethylene glycol (PEG; MW = 4000) from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA), [ $^{14}\text{C}$ ]D-glucose and [ $^3\text{H}$ ]L-glucose from Moravsek (Brea, CA, USA), cyclosporin A from Qbiogene, Inc. (Carlsbad, CA, USA), and phloridzin and a (1:1) racemic mixture of alpha-pinene from Sigma Aldrich (St. Louis, MO, USA).

### Animals and Diets

We purchased Sprague-Dawley rats from Harlan Sprague-Dawley (Madison, WI, USA) and housed them in temperature-controlled facilities in the Department of Wildlife Ecology, University of Wisconsin–Madison with access to water and food *ad libitum*. Specialist (*N. stephensi*) (4 male, 6 female) and generalist (*N. albigula*) (4 male, 3 female) wood rats were trapped outside the south border of Wupatki National Park, 45 km NE of Flagstaff, AZ, USA (35°30'N, 111°27'W). The diets of these species were described in an extensive study by Dial (1988). We trapped *N. cinerea* (2 male, 3 female) at a private residence near Heber City, Summit Co., UT, USA. *N. cinerea* is a dietary generalist (Johnson and Hansen, 1979; Hauffer and Nagy, 1984; Frase and Sera, 1993), and the caches of *N. cinerea* used in this study suggested they were foraging on *Opuntia* clades, sagebrush (*Artemisia tridentata*) and various nonnative garden plants. Animals were transported to the University of Utah Animal Facility and were individually housed in shoe box cages (48 × 27 × 20 cm) with bedding and cotton batting at 20°C on a 12 L:12 D photoperiod. Animals were in captivity for 6–12 mos prior to the experiment. All animals on the control diet were fed Teklad ground rabbit chow (formula 2120) and water *ad libitum*. *N. stephensi* on the juniper diet were fed a rabbit chow based diet with

progressively increasing juniper (5, 25, 25, 70, and 70% juniper by dry mass, collected from Flagstaff, AZ, USA) over 5 d, a sufficient time to induce Pgp based on studies in cell lines (Fardel et al., 1996) and in rats (Salphati and Benet, 1998; Sandstrom and Lennernas, 1999), and which is a level of juniper on which *N. stephensi* are known to maintain body mass in the laboratory (M.D. Dearing, unpublished data). Based on weights prior to inclusion of juniper in the diet and immediately before the everted sleeve experiments, we found that *N. stephensi* maintained body mass over the 5-d dietary exposure (repeated-measures ANOVA,  $F_{1,3} = 3.944$ ;  $P = 0.141$ ).

All experiments on wood rats conformed to University of Utah IACUC protocol # 04-02012. All experiments on Sprague-Dawley rats conformed to University of Wisconsin–Madison IACUC protocol # A-07-6900-A00991-3-04-00.

### Measures of Pgp Activity Using Tissue Accumulation of Digoxin

For tissue preparation and mounting, we closely followed the procedures described in Karasov and Diamond (1983) and Karasov et al. (1985) and reported by Green et al. (2005). Briefly, animals were euthanized with CO<sub>2</sub>; then intestine from the stomach to the caecal attachment was quickly removed (4–6 min) and flushed with ice-cold Ringer solution (in mM: 50 mannitol, 100 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, and 20 NaHCO<sub>3</sub>, gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, 290 mOsm). After the intestine length was measured, the intestine was everted, and short sleeves (1.5 cm long) were cut and then mounted on stainless steel rods by tying the tissue down over grooves exactly 1 cm apart while kept in cold Ringer gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Limited intestinal length as well as concerns about experiment length precluded the use of all treatments in all animals. See Table 1 for allocation of samples among treatments.

Everted sleeves were first preincubated in 37°C Ringer solution bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> for 2 min (Pgp assays) or 10 min (D-glucose uptake) that contained either test compounds or vehicle control to maximize tissue exposure to our treatments. The tissue was then incubated in 8 ml gassed Ringer at 37°C in a flat-bottomed water-jacketed test tube with spinning stir bar to reduce and control the unstirred layer thickness (Karasov and Diamond, 1983). Incubation solution included radiolabeled probe ([<sup>3</sup>H]digoxin (9.1 kBq/ml) for measuring Pgp activity; Sababi et al., 2001; Stephens et al., 2001), or [<sup>14</sup>C]D-glucose (1.1 kBq/ml) for measuring D-glucose uptake activity) and marker solutes to correct for both non-absorbed probe in adherent mucosal fluid and diffusive flux ([<sup>14</sup>C]polyethylene glycol (PEG), MW = 4000; 1.1 kBq/ml) for Pgp measures, or [<sup>3</sup>H]L-glucose (31.4 kBq/ml), for D-glucose measures) (Karasov and Diamond, 1983). After 12 min (Pgp assay) or 4 min (D-glucose uptake), tissues were removed and then either blotted (Pgp assay) or rinsed in stirred cold Ringer and then blotted (D-glucose uptake). The mounted tissue was removed, weighed, incubated in 1 ml of tissue solubilizer (Soluene-350; Packard, Meriden, CT, USA), and counted in 10 ml of scintillation cocktail (Ecolume; Packard) with 0.5% by volume acetic acid on a Beckman LS 5801 (Beckman Coulter, Inc.) with channels set to minimize spill (counts of the alternate isotope appearing in the same counting channel) and programmed with a standard curve to correct for background quench. Calculation of digoxin accumulation followed (Karasov and Diamond, 1983), and for simplicity of expression, we assumed a digoxin concentration of 1 fmol/μl incubation solution (it was actually approxi-

**Table 1** Allocation of everted sleeves to the different treatments in the proximal, mid, and distal sections of the small intestine of wood rats and Sprague-Dawley rats on control or juniper diets

Gut section	Proximal			Mid			Distal									
	Digoxin			Digoxin			Digoxin			D-Glucose						
	CsA	CsA	CsA	CsA	CsA	CsA	CsA	CON	10	100	CON	15	100	PHLOR		
Concentration (μM)	CON	10	CON	1	5	10	15	CON	10	100	CON	10	CON	15	100	1,000
<i>N. albigula</i>	7	7	7	5	4	7	6	7	7	7	7	7	7	6	5	5
<i>N. stephensi</i>	5	5	5	3	4	5	4	5	5	5	5	5	5	4	3	4
<i>N. stephensi</i> (juniper diet)	5	5	5	3	2	5	3	5	5	5	4	4	5	4	3	4
<i>N. cinerea</i>	5	5	5	3	4	5	4	5	4	5	4	4	5	4	3	4
Sprague-Dawley								4	4	4	4	4	4	4	4	4

mately 0.75 fmol/ $\mu$ l), yielding units of fmol digoxin accumulated per milligram tissue. Total intestine mass was estimated as the product of the summed mass of the sixteen 1-cm tissues and the ratio of total intestine length: 16 cm.

We have previously determined that wood rat and Sprague-Dawley rat intestinal sleeves maintain structural integrity during the experimental procedure (Green et al., 2004, 2005). To verify the functional integrity and to check for the nonspecific effects of known and putative inhibitors, we measured the ATP-dependent mediated D-glucose uptake over the same combined preincubation and incubation time in adjacent tissues exposed to alpha-pinene (100  $\mu$ M), vehicle control (0.15% ethanol), and phloridzin (1 mM), a known inhibitor of the intestinal glucose transporter SGLT-1, as previously described (Green et al., 2004, 2005).

Digoxin accumulation in the tissue is a balance between passive diffusion into and Pgp-mediated export out of the cells, so the desired measure of Pgp activity was the difference between digoxin accumulation in the presence of a maximal inhibitory concentration of a competitor of digoxin export, cyclosporin A (CsA) (Lan et al., 1996), and the accumulation in the absence of CsA (0.15% ethanol vehicle control), similar to Sababi et al. (2001). Pgp transport was maximally inhibited by 10  $\mu$ M CsA in the rodent species on control diet (Green et al., 2004), and we verified this with *N. stephensi* on juniper diet by conducting a dose response with CsA [1, 5, 10  $\mu$ M, and saturated ( $\sim$ 15  $\mu$ M)] in the midintestine. To estimate intestinal Pgp capacity, which is activity summed over the entire small intestine, we used 10  $\mu$ M CsA to determine Pgp activity in the proximal, mid, and distal intestinal sections. Because intestinal mass was not significantly different between the two dietary groups of *N. stephensi* (one-way ANOVA:  $F_{1,7} = 1.771$ ,  $P = 0.225$ ), any difference in Pgp capacity would be reflected in the Pgp activity along the intestinal tract.

Lacking radiolabeled alpha-pinene, we tested whether racemic alpha-pinene competitively inhibits the exclusion of our known Pgp substrate, [ $^3$ H]digoxin. We used racemic alpha-pinene because we lacked data on which enantiomer is found in *J. monosperma* and wished to account for both possibilities. We incubated intestinal sleeves of the midgut with alpha-pinene at concentrations that approximated 10 and 100  $\mu$ M made by adding the appropriate amount of a 1000 $\times$  solution in ethanol directly to the stirring incubation solution. These chosen concentrations bracket the concentration estimated to reach the intestine after gavage of 34.32  $\mu$ g alpha-pinene/ml peanut oil used in a previous experiment, and corresponds to the amount in a full meal of *J. monosperma* (Sorensen and Dearing, 2003). Because of the volatility of alpha-pinene, we measured the loss from incubation solution over time by subsampling incubation solution every 2 min after an initial dose at the target concentration of alpha-pinene (i.e., 100  $\mu$ M) and measured alpha-pinene concentration by using gas chromatography as described by Sorensen and Dearing (2003). We determined that adding half the initial amount every 2 min during both preincubation and incubation kept levels near to the desired dose (range approximately: 5–10  $\mu$ M for the “10  $\mu$ M” dose, and 50–100  $\mu$ M for the “100  $\mu$ M” dose). Vehicle control matched these additions with ethanol.

### Caco-2 Cell Experiments

[ $^3$ H]Digoxin accumulation was measured in Caco-2 cell monolayers (passage 3–10; ATCC, Manassas, VA, USA) exposed to 1, 3, 10, 30, and 100  $\mu$ M alpha-pinene made by diluting a 1000 $\times$  solution in ethanol with cell media. First, cells were

washed twice with serum-free, phenol red-free Dulbecco's modified Eagles media (DMEM; Sigma Aldrich, St. Louis, MO, USA) and media replaced with phenol red-free DMEM containing 1% fetal bovine serum (FBS) and vehicle control (5% ethanol), or alpha-pinene (1, 3, 10, 30, 100  $\mu\text{M}$  final concentration) for 30 min ( $N = 4$  wells/treatment). Then, digoxin uptake was measured by adding [ $^3\text{H}$ ]digoxin (37 kBq/ml) in base media with 1% FBS 45 min before the end of the trial. Subsamples (50  $\mu\text{l}$ ) of media were removed for specific activity, and the remainder of solution was aspirated and cells washed twice with 1 l ice-cold phosphate-buffered saline (PBS). Cells were solubilized in 0.5 ml 0.5 N NaOH and 200- $\mu\text{l}$  aliquots were mixed with 200  $\mu\text{l}$  0.5 M HCl and counted on a Beckman LS 3800 (Beckman Coulter, Inc.) scintillation counter. Decays per minute were scaled to protein level in the wells, determined by spectrophotometrically comparing a 5- $\mu\text{l}$  aliquot of solubilized cells from each well to bovine serum albumin standards.

### Phosphate Release Assay with alpha-Pinene

Verapamil-stimulated, vanadate-sensitive ATPase activity associated with Pgp was assayed as described by the manufacturer (BD Biosciences, Woburn, MA, USA). Briefly, insect cell membranes containing human Pgp (BD Gentest Recombinant MDR1 microsomes; BD Biosciences, Woburn MA, USA) were used to assess Pgp ATPase activity in the presence and absence of vanadate and vehicle control (1% ethanol), verapamil (60  $\mu\text{M}$ ), a known Pgp substrate and positive control, or alpha-pinene at the same concentrations used in the everted sleeve experiments (10, 100  $\mu\text{M}$ ). Pgp membranes were added to reaction media (50 mM Tris-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol, 5 mM sodium azide, 5 mM MgATP) with and without 100  $\mu\text{M}$  sodium orthovanadate and the test compound. The ATPase activity measured in the presence of orthovanadate represents non-Pgp ATPase activity and is subtracted from activity measured in the absence of orthovanadate. The reactants were incubated at 37°C for 20 min and terminated by the addition of 10% SDS and Antifoam A. Hydrolysis of ATP was measured on a spectrophotometric microplate reader (Dynatech MR-5000) as the release of inorganic phosphate (absorbance at 800 nm).

### Statistical Analyses

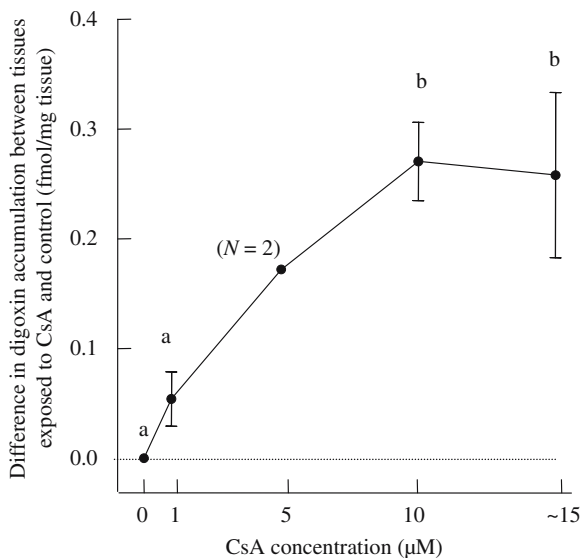
When possible, we analyzed treatment effect in the everted sleeve experiments with repeated-measures ANOVA and examined interactions between treatment and species or diet. In experiments with Caco-2 cells, phosphate release assay, and everted sleeves when all treatments were not represented in every animal, we used one-way ANOVA. Effect of treatments on mediated D-glucose uptake was tested with a general linear model with treatment as a factor, and species and diet as interactions with treatment. In everted sleeves, the experimental unit was the individual rodent, while in the Caco-2 cell experiment and phosphate release assay the experimental unit was the well. We used Fisher's LSD for pairwise comparisons when global tests resulted in  $P < 0.05$ . A value of  $P < 0.05$  was considered significant and  $0.05 < P < 0.10$  a trend. All data are reported as mean  $\pm$  SE,  $N$  = sample size.

Plots of residuals were visually inspected to verify a random distribution. We used Systat 10 (SPSS Inc., Chicago, IL, USA) for analyses.

## Results

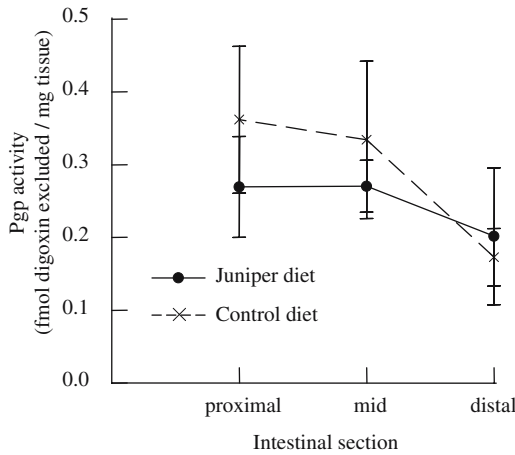
Neither of our test compounds, CsA (15  $\mu\text{M}$ ) or alpha-pinene (100  $\mu\text{M}$ ), affected mediated [ $^{14}\text{C}$ ]D-glucose uptake in any rodent species on control diet, or *N. stephensi* on the juniper diet, whereas uptake was significantly inhibited by phloridzin (1 mM) ( $P = 0.005$ ), the specific inhibitor of the SGLT-1 Na/glucose cotransporter (general linear model,  $F_{3,71} = 3.9$ ;  $P = 0.013$ , followed by pairwise comparisons, interactions not significant). One *N. cinerea* and one *N. stephensi* on juniper diet were removed from the study due to loss of functional integrity in intestinal sleeves as indicated by uniformly low [ $^{14}\text{C}$ ]D-glucose uptake.

Consistent with our previous results in rodents on control diet (Green et al., 2004, 2005), net accumulation of [ $^3\text{H}$ ]digoxin, corrected for adherent label, increased sublinearly with increasing concentration of CsA in the solution for intestinal sleeves of *N. stephensi* on the juniper diet, reaching an asymptote by 10  $\mu\text{M}$  (Fig. 1; one-way ANOVA,  $F_{4,11} = 11.4$ ;  $P = 0.001$ , followed by pairwise comparisons). There was no significant variation in Pgp activity along the intestinal tract for *N. stephensi* on either diet (Fig. 2; repeated-measures ANOVA, control,  $F_{2,8} = 3.3$ ;  $P = 0.091$ ; juniper,  $F_{2,6} = 0.31$ ;  $P = 0.748$ ), and there was no significant difference in Pgp activity/mg tissue between *N. stephensi* on different diets in proximal (one-way ANOVA,

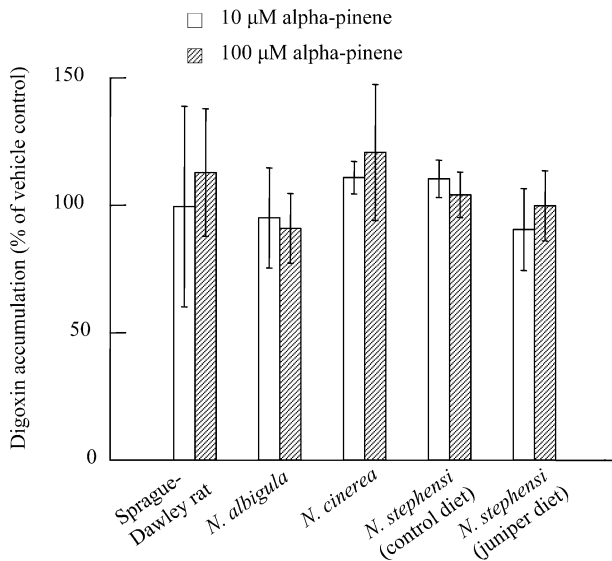


**Fig. 1** Difference in digoxin accumulation (fmol/mg tissue) between midintestinal sleeves exposed to cyclosporin A (1, 5, 10,  $\sim 15$   $\mu\text{M}$ ) and vehicle control (0.15% ethanol) during 2-min preincubations and 12-min incubations in *Neotoma stephensi* fed a diet containing *Juniperus monosperma* ( $N = 2$ –5 sleeves/concentration). The difference shows a sublinear increase that reaches an asymptote by 10  $\mu\text{M}$  (one-way ANOVA,  $F_{3,8} = 5.736$ ;  $P = 0.022$ , followed by pairwise comparisons). Different letters (a, b) indicate significant differences between concentrations ( $P < 0.01$ ). Points are mean  $\pm$  SE





**Fig. 2** Pgp activity (fmol digoxin excluded/mg tissue) along the intestinal tract in *N. stephensi* on control ( $N = 5$ ) and juniper diet ( $N = 4$ ) as determined by the difference in digoxin accumulation in intestinal sleeves exposed to ethanol vehicle control or 10  $\mu\text{M}$  CsA during 2-min preincubations and 12-min incubations. There was no significant difference along the tract in either diet group (repeated-measures ANOVA, intestinal section:  $F_{2,14} = 2.486$ ;  $P = 0.119$ , intestinal \* section diet:  $F_{2,14} = 0.500$ ;  $P = 0.617$ ) and no difference in Pgp activity between diet groups in the proximal (one-way ANOVA,  $F_{1,7} = 0.678$ ;  $P = 0.437$ ), mid ( $F_{1,7} = 0.332$ ;  $P = 0.582$ ), or distal ( $F_{1,7} = 0.130$ ;  $P = 0.729$ ) sections. Points are mean  $\pm$  SE

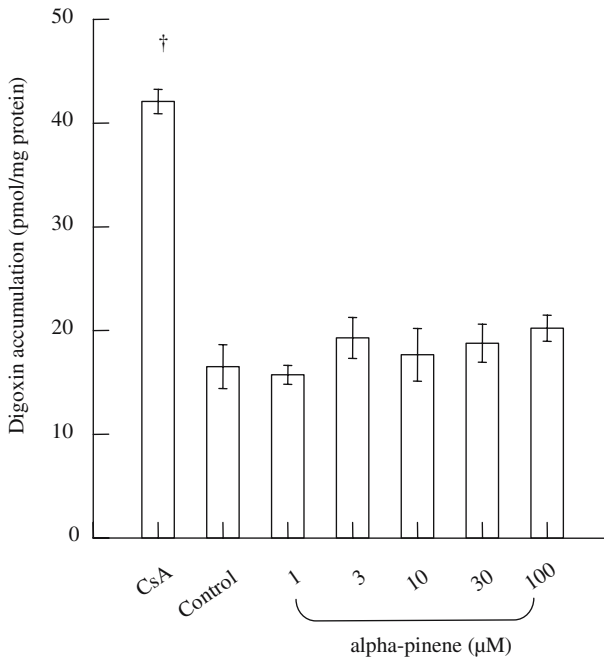


**Fig. 3** Digoxin accumulation as percent of ethanol vehicle control in midintestinal sleeves exposed to alpha-pinene (10, 100  $\mu\text{M}$ ) during 2-min preincubations and 12-min incubations in Sprague-Dawley rats ( $N = 4$ ), *Neotoma albigula* ( $N = 7$ ), *N. cinerea* ( $N = 3$ ), and *N. stephensi* on control ( $N = 5$ ) and juniper diet ( $N = 4$ ). There was no significant difference in digoxin accumulation in any group (repeated-measures ANOVA,  $F_{2,36} = 0.150$ ;  $P = 0.861$ ; interactions were not significant). Values are mean  $\pm$  SE

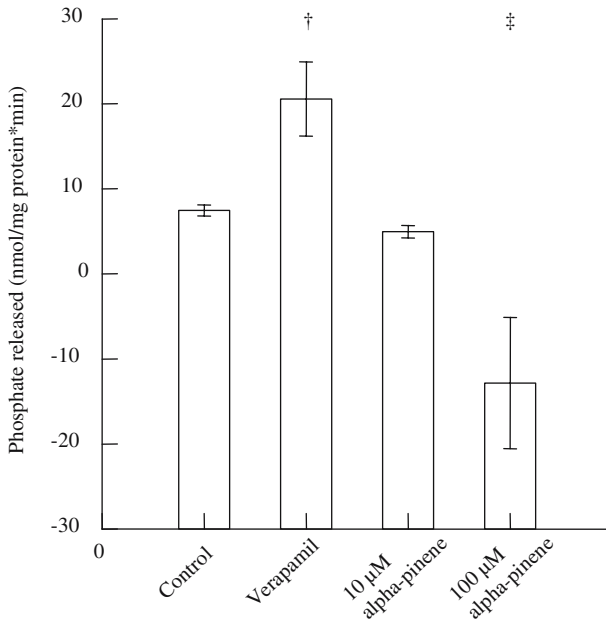
$F_{1,7} = 0.68$ ;  $P = 0.437$ ), mid ( $F_{1,7} = 0.33$ ;  $P = 0.582$ ), or distal ( $F_{1,7} = 0.13$ ;  $P = 0.729$ ) sections (Fig. 2). Several tissue sections from one *N. stephensi* on juniper diet were damaged during the procedure eliminating this animal from the comparison with control diet and some samples from the dose–response curve. With no difference in intestinal mass (one-way ANOVA,  $F_{1,7} = 1.77$ ;  $P = 0.225$ ), we conclude that acclimation to a juniper diet over 5 d does not induce intestinal Pgp activity. The mean Pgp activity in proximal and midintestine of *N. stephensi* on juniper diet was lower than *N. stephensi* on the control diet, opposite of what would be expected if induction occurred, suggesting that a larger sample size would not affect this conclusion.

Accumulation of digoxin in mid-intestinal sleeves was not affected by alpha-pinene at 10 or 100  $\mu\text{M}$  in any of the rodent species on control diet, or in *N. stephensi* on the juniper diet (Fig. 3; repeated-measures ANOVA,  $F_{2,36} = 0.15$ ;  $P = 0.861$ ; interactions not significant). This indicates that alpha-pinene does not competitively inhibit exclusion of digoxin by Pgp in wood rats or Sprague-Dawley rats over the range of concentrations tested.

We also measured digoxin accumulation in Caco-2 cells with and without alpha-pinene (1, 3, 10, 30, 100  $\mu\text{M}$ ) to test the ability of alpha-pinene to competitively inhibit Pgp in a commonly used and sensitive human cell line. alpha-Pinene, at 1  $\mu\text{M}$ , yielded one extreme outlier, which was removed from analysis. We ob-



**Fig. 4** Digoxin accumulation in Caco-2 cells exposed to cyclosporin A (5  $\mu\text{M}$ ), vehicle control (5% ethanol), or racemic alpha-pinene (1, 3, 10, 30, and 100  $\mu\text{M}$ ) for 75 min with [ $^3\text{H}$ ]digoxin added 45 min before the end of the trial. Values are means  $\pm$  SE of 4 wells/treatment, except at 1  $\mu\text{M}$  alpha-pinene where  $N = 3$  wells. Decays per minute in cells were scaled to total protein in the wells. Significant differences from control in pairwise comparisons after one-way ANOVA ( $F_{6,20} = 37.1$ ;  $P < 0.001$ ) are indicated by ( $\dagger P < 0.001$ )



**Fig. 5** Phosphate production from Pgp ATPase activity in Pgp transfected insect membranes exposed to verapamil (60  $\mu\text{M}$ ), racemic alpha-pinene (10, 100  $\mu\text{M}$ ), and vehicle control (1% ethanol). Values are means  $\pm$  SE of three replicates per treatment. Significant differences in pairwise comparisons after one-way ANOVA ( $F_{3,8} = 16.607$ ;  $P = 0.001$ ) are indicated by ( $\dagger P < 0.05$ ) and ( $\ddagger P < 0.005$ )

served the expected increased accumulation of digoxin with the known Pgp substrate, cyclosporin A (5  $\mu\text{M}$ ), but no difference in digoxin accumulation between control and alpha-pinene at any concentration (Fig. 4; one-way ANOVA,  $F_{6,20} = 37.1$ ;  $P < 0.001$ , followed by pairwise comparisons).

#### Phosphate Release Assay with alpha-Pinene

We observed the expected increase in phosphate release with our positive control, verapamil (60  $\mu\text{M}$ ), a known Pgp substrate, while alpha-pinene had no effect at 10  $\mu\text{M}$ , and actually resulted in a decrease in phosphate release at 100  $\mu\text{M}$  (Fig. 5; one-way ANOVA,  $F_{3,8} = 16.61$ ;  $P = 0.001$ , followed by pairwise comparisons). These data, along with the Caco-2 cell-line results, indicate that alpha-pinene is not a substrate for human Pgp. The decrease in phosphate production by 100  $\mu\text{M}$  alpha-pinene implies inhibition of ATPase activity.

#### Discussion

Although our previous results indicated a correlation between intestinal Pgp capacity and the tolerance of a *J. monopserma*-dominated diet (Green et al., 2004), we failed to establish a link between Pgp and alpha-pinene, the dominant

monoterpene in *J. monosperma*. Digoxin accumulation in wood rat intestine, Sprague-Dawley rat intestine, and Caco-2 cells was not affected by exposure to alpha-pinene. Phosphate released from ATPase activity did not show an increase in the presence of alpha-pinene at 10  $\mu\text{M}$ , and 100  $\mu\text{M}$  alpha-pinene actually decreased phosphate production, indicating an inhibition of ATPase activity. The possibility remains that wood rat Pgp may recognize alpha-pinene, but that digoxin has higher affinity for Pgp preventing inhibition by alpha-pinene in the everted sleeve and Caco-2 cell-line experiments. This would also require that the Pgp assay with human Pgp is not a valid surrogate for investigating wood rat Pgp. The best way to address this possibility would be to use radiolabeled alpha-pinene as the probe and CsA as the inhibitor, or to develop another method that measures alpha pinene concentration in tissue (i.e., gas chromatography). Another potential method would be to measure the accumulation of a probe of lower affinity than digoxin (i.e., propranolol; Lan et al., 1996) in everted wood rat intestinal sleeves with alpha-pinene as the potential inhibitor. A negative result, however, would still leave the question of relative affinities.

The highly lipophilic alpha-pinene molecule ( $\text{C}_{10}\text{H}_{16}$ ,  $\log K_{\text{ow}} = 4.83$ ; Li et al., 1998) is not ideal for recognition by Pgp. Although Pgp does recognize a number of compounds characterized as lipophilic, the majority appears to be amphipathic with polar oxygen or nitrogen groups attached (Ford and Hait, 1990; Hunter and Hirst, 1997; Ambudkar et al., 1999). Some diterpenes have been found to interact with Pgp (Hohmann et al., 2002; Appendino et al., 2003), but they have polar groups attached to the ring structure. It may be that one or more of the phase I metabolites of alpha-pinene are recognized by Pgp and are pumped back into the intestinal lumen. We would expect, however, that this would still result in competitive inhibition of Pgp and an increased accumulation of digoxin in both the everted sleeve and Caco-2 cell experiments. Furthermore, microbial conversion of a metabolite back to the parent compound is unlikely and, therefore, would not explain the difference in parent compound found in the feces of the two wood rat species (Sorensen et al., 2004).

The larger question of tolerance to a diet high in *J. monosperma* may involve compounds other than alpha-pinene. *N. albigula* decreased food intake and exhibited decreased urine pH after acclimating over 9 d to a diet containing alpha-pinene at a level corresponding to a 100% juniper diet (Dearing et al., 2000). Despite a decreased food intake, *N. albigula* did not lose significant body mass. In contrast, *N. albigula* and, to a lesser extent, *N. stephensi* lost significant body mass on a diet consisting of artificial diet (15% of maintenance levels) and *J. monosperma* leaves *ad libitum*. Both artificial diets with and without alpha-pinene matched nutrient levels of juniper.

In another plant herbivore system, Lawler et al. (1999) found that the feeding deterrence of common ringtail possums (*Pseudocheirus peregrinus*) and common brushtail possums (*Trichosurus vulpecula*) by the Eucalyptus terpene, 1,8-cineole, is a conditioned flavor aversion due to its association with the postingestive effects caused by diformylphloroglucinol compounds, such as jensenone. Could alpha-pinene serve a similar purpose in juniper as cineole does in Eucalyptus?

Because there may be other compounds in juniper that interact with Pgp, we also measured Pgp activity in *N. stephensi* acclimated to a juniper diet. We did not observe induction in Pgp activity, which suggests at least three possible explanations. (1) Although 5 d are sufficient to induce Pgp in laboratory rat studies using known Pgp substrates (Salphati and Benet, 1998; Sandstrom and Lennernas, 1999),

possibly the level of Pgp substrate(s) in juniper are not sufficient to induce Pgp on the same time scale. (2) *N. stephensi* maintains a high constitutive level of Pgp that is not inducible. Our previous results indicate that *N. stephensi* showed higher Pgp capacity than *N. albigula* when on a control diet (Green et al., 2004) in support of a high constitutive level of Pgp. Of interest would be whether the generalist, *N. albigula*, exhibits induced levels of Pgp when acclimated to a juniper diet. We lacked sufficient individuals to test this possibility. (3) Possibly Pgp does not recognize any component of *J. monosperma*, and our previous results on intestinal Pgp capacity are purely correlational. We consider this unlikely due to the long list of Pgp substrates and modulators, but a test would be to use extracts of *J. monosperma* in everted sleeve, Caco-2 cell line, and/or phosphate release experiments.

In addition to the possibility that other compounds besides alpha-pinene may be involved in juniper tolerance is the potential that other mechanisms besides Pgp may be involved. The difference in intestinal Pgp capacity between *N. stephensi* and *N. albigula* is only one difference in the arsenal of biochemical defense mechanisms of wood rats. Although Sorensen and Dearing (2003) did not see any difference in elimination rate of alpha-pinene in these two species, other results indicate they may detoxify compounds differently (S. Haley, unpublished data; Dearing et al., 2000, 2002). Could these species biotransform juniper toxins differently producing metabolites that differ in toxicity? Recently, more membrane-bound transport proteins related to Pgp have been described, and as many as 10 have been implicated in a cell's ability to defend against toxins (Taipalensuu et al., 2001; Scotto, 2003). If wood rats vary in Pgp activity and activity of phase I and II detoxifying enzymes, then they may vary in activity of other transporters or defense mechanisms that could result in the observed variability in juniper tolerance. Adding to the variation in the function of these mechanisms is a possible variation in the regulation of mechanisms that may be coordinated at the gene level (Scotto, 2003). A full understanding of the interplay between ingested toxins and the biochemical defense mechanisms in mammalian herbivores may require techniques that can simultaneously measure the activity of a myriad of proteins, or the corresponding genes. Efforts using DNA microarray analyses are currently underway. The difficulty in finding an explanation for the observed differences in alpha-pinene absorption and *J. monosperma* tolerance between *N. stephensi* and *N. albigula* highlights the challenges we face.

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