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Phenylacetylglycine, a putative biomarker of phospholipidosis: its origins and relevance to phospholipid accumulation using amiodarone treated rats as a model

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Amiodarone was given to male Sprague–Dawley rats at a dose of 150 mg kg^{-1} day^{-1} for 7 consecutive days to induce phospholipidosis in the lungs of treated rats. Amiodarone was given alone or concurrently with phenobarbitone. Animals given amiodarone had raised total phospholipid in serum, lung and lymphocytes, and elevated lyso(*bis*)phosphatidic acid (LBPA) in all tissues. Urinary and plasma phenylacetylglycine (PAG) and hepatic portal:aortal phenylacetate (PA) ratio were increased, whereas hepatic phenylalanine hydroxylase (PAH) activity and plasma phenylalanine:tyrosine ratio were not affected. Phenobarbitone treatment increased hepatic total P450 content and induced 7-pentoxyresorufin *O*-dealkylatian (PROD) activity, as expected, but had no effect on any other biochemical parameter. Plasma amiodarone concentration was reduced in rats co-administered both drugs and phospholipid accumulation in target tissues was attenuated compared with rats treated with amiodarone alone. However, phenobarbitone co-administration failed to alter the magnitude of response with regards to urinary PAG excretion and plasma concentration of its precursors after amiodarone treatment. Increased intestinal absorption of PAG precursors probably resulted in the raised urinary PAG after amiodarone treatment. Urinary PAG correlated weakly with serum, lymphocyte and lung phospholipids. However, urinary PAG excretion was similar in rats dosed solely with amiodarone or in combination with phenobarbitone, despite the fact that the degree of phospholipid accumulation was far less in rats given the combined treatment. Nevertheless, urinary PAG was raised only in animals exhibiting abnormal phospholipid accumulation in target tissues and may thus be useful as a surrogate biomarker for phospholipidosis.

Keywords: phenylacetylglycine, PAG, phospholipidosis, biomarker, amiodarone, validation.

Introduction

Many drugs currently being developed are cationic and amphiphilic in nature otherwise referred to as cationic amphiphilic compounds (CADs). The advantage of these drugs, which are diverse in pharmacology and therapeutic application, is that they readily translocate cell membranes. However, a side-effect of these drugs is that they ultimately cause abnormal phospholipid accumulation in target tissues of the host. Phospholipidosis, characterized by multilamellar inclusions of lysosomal origin in affected tissues/cells, is currently confirmed using electron microscopy (Hook 1991) and direct chemical analysis of phospholipids (Reasor *et al.* 1988, Mortuza *et al.* 2003). In recent years, high-resolution ^{1}H-NMR has been used as an

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investigative tool for screening urine collected from animals dosed with drugs from different chemical classes. This work resulted in the detection of a metabolite, identified as phenylacetylglycine (PAG), which appeared to be a biomarker of drug-induced phospholipidosis (PLD) (Nicholls *et al.* 2000). However, the authors were unable to make the biochemical link between phospholipid accumulation and the elevated levels of PAG. Other workers have also made similar observations using both amiodarone, chloroquine and other novel CAD-like compounds (DMP 777 a neutrophil elastase inhibitor) that cause phospholipid accumulation (Espina *et al.* 2001). To date there has been no investigation into the mechanism behind the raised urinary PAG concentration in treated animals nor has a direct link between PAG and phospholipidosis been made.

Whilst there are no known studies investigating urinary excretion of phenylacetylglutamine, the human equivalent of rodent PAG, in patients with genetic or drug-induced phospholipidosis, raised urinary phenylacetylglutamine, and its precursor phenylacetate (PA), has been reported in patients with impaired amino acid absorption (Van der Heiden *et al.* 1971a,b) and phenylketonuria (PKU) (Woolf 1951, Wadman *et al.* 1971, Michals and Matalon 1985). In the former excessive bacterial degradation of protein or amino acids in the gut is responsible (Seakins 1971) whereas in PKU, reduced activity of phenylalanine hydroxylase, the major degradative pathway for phenylalanine, is responsible (Erlandsen and Stevens 1999). In addition, urinary and plasma concentrations of phenylacetylglutamine and its precursors are raised in patients with uraemia or chronic renal failure (Zimmerman *et al.* 1989, Jankowski *et al.* 1998). The mechanism behind this perturbation of phenylalanine metabolism has not been elucidated in this particular case but may be related to: acyl-CoA:glutamine *N*-acyltransferase, a mitochondrial enzyme, which has been reported to constitute an alternative pathway for removal of excess ammonia (Asaoka 1991); abnormal colonization of bacterial populations in the small bowel (Simenhoff *et al.* 1974); or reduced hydroxylation of phenylalanine (Young and Parsons 1973). It is possible that these clinical manifestations of disrupted phenylalanine metabolism might give some insight into to the mechanism of action of CAD's with respect to PAG synthesis and subsequent excretion.

The rationale for investigating the origin of raised PAG precursors in response to amiodarone was based on the hypotheses discussed below. Repeated exposure to a CAD, via the oral route, might increase the absorption of PAG precursors, such as phenyl acetate from the gut either by altering the diffusion dynamics or by altering the rate of phenylalanine/phenol metabolism by gut flora, changing the dynamic of colonizing species of gut flora or damaging the intestinal brush border. Phenylacetate would be absorbed into the bloodstream, via portal blood, conjugated with glycine in the liver and kidney to yield PAG, and then excreted in urine. In addition, the CAD would also be absorbed from the gut and result in susceptible tissues developing PLD. An ideal comparator would be to give amiodarone intraperitoneally (i.p.) as this would not initially expose the gut to the drug but would induce PLD in the same target tissues. If gut bacteria were solely responsible for the generation of PAG precursors, the concentration of PAG in the urine would be less/similar to control in i.p. dosed rats but raised urinary PAG might indicate

perturbation of endogenous phenylalanine or phenol metabolism. Unfortunately, preliminary investigations showed that it was not possible to achieve equivalent responses by giving comparable doses of amiodarone orally and intraperitoneally and thus the validity of making direct comparisons was deemed questionable (data not shown). Co-administration of phenobarbitone with amiodarone ameliorates PLD because of increased metabolism and clearance of the latter (Reasor *et al.* 1988). Animals dosed with both of these drugs, phenobarbitone via the intraperitoneal route and amiodarone orally, should present with less severe PLD than animals receiving amiodarone alone but should have similarly raised urinary PAG if gut bacteria are responsible for the generation of PAG precursors. This outcome assumes that phenobarbitone does not influence gut flora in any way and that exposure of the gut to amiodarone is the same in these two groups of rats. In addition, this outcome would also suggest that urinary PAG does not directly correlate with PLD but is potentially acting as a surrogate marker of this condition.

The aim of the study was two-fold. First, to elucidate the mechanism behind raised urinary PAG by investigating possible drug-related perturbation of phenylalanine metabolism in the liver of amiodarone treated rats or to establish that the gut was the major contributor of PAG precursors. Second, to correlate urinary PAG with amiodarone concentration and blood and tissue lipids in order to ascertain whether there was a direct link between PAG and phospholipidosis.

Materials and methods

Animals

Seven-to-eight-week-old male Sprague–Dawley rats (Crl:CD(SD)IGS BR strain; Charles River UK Ltd, Margate, UK) were used after an acclimatization period of 5 days. Animals were initially housed five/cage in Techniplast (Type 4) cages and had unlimited access to food (Rat and Mouse 1, SDS, Manea, Cambridge, UK) and water. The local environment was maintained at 19–23°C ambient temperature and 45–70% humidity with a 12 h light/dark cycle.

Study design

Before commencement of the study, animals (56–63-days-old on day 0) were randomly assigned to one of four treatment groups ($n=5$ per group) and were housed individually in grid-bottomed urine collection cages (MET 1, 20 metabolism cage; Arrowmight Biosciences, Hereford, UK). The following treatments were made up as follows: phenobarbitone (sodium salt; Sigma, Gillingham, UK) in 0.9% saline (10 or 16 mg ml^{-1}; dosing volume 5 ml kg^{-1}); amiodarone (hydrochloride; Sigma) (15 mg ml^{-1}; dosing volume 10 ml kg^{-1}) in Polysorbate 80:95% ethanol:water (5:10:85). Treatment groups and dosing information are given in table 1. Phenobarbitone (Pb) was administered by intraperitoneal (i.p.) injection and amiodarone (amio) by gastric intubation (p.o.). Control animals (vehicle controls [VCon] and phenobarbitone controls [PbCon]) received the appropriate vehicle via a comparable route to its

Table 1. Treatment groups.

Group number	1 (Vcon)	2 (Amio)	3 (PbCon)	4 (Pb/Amio)
Treatment	Vehicle Control (p.o.)	Amiodarone (p.o.)	Phenobarbitone (i.p.)+vehicle (p.o.)	Phenobarbitone (i.p.)+Amiodarone (p.o.)
Predose (mg kg^{-1}) days −3 to −1	0	0	80	80
Dose (mg kg^{-1}) days 0–6	0	150	50+0	50+150

dosed counterparts. Dosing began on day 0 and continued for 7 consecutive days. Body weight and food and water intake were monitored daily throughout the dosing period. The study was terminated on day 7, 24 h after the last dose.

Sample collection

Urine was collected into sodium azide (1%; 1 ml; Sigma) from all animals between 08.00 and 16.00 h (8 h) and 16.00 and 08.00 h (16 h), starting from day −2 of the acclimatization period and for the remainder of the study. All collections were timed to start immediately after dosing (carried out at 08.00 h). Urine volumes were recorded and the samples retained at −80°C before analysis by HPLC-MS.

On day 6, blood samples were collected, via the tail vein, into heparinized containers from each animal, 5-h post-dose, for drug concentration analysis. Animals were euthanized on day 7 by exsanguination under isoflurane anaesthesia. Blood samples were collected as follows: via the aorta into heparinized tubes for determination of a battery of routine clinical chemistry parameters (routine methodologies for Hitachi 917 autoanalyser), amino acid concentrations (HPLC) and from aorta and hepatic portal vein, for determination of PAG and PA concentrations by HPLC-MS; via the aorta into tubes containing EDTA for purification of lymphocytes using Lymphoprep (Nycomed, Oslo, Norway).

Samples of lung, liver and mesenteric lymph nodes were retained and fixed in 10% (v/v) neutral buffered formalin for histological processing (3 μm paraffin wax-embedded sections stained with haematoxylin and eosin) or snap frozen in liquid nitrogen and stored at −80°C before analysis.

Biochemical analyses

Urinary PAG was separated by reversed-phase chromatography on an Agilent 1100 HPLC system. Peak separation was achieved at ambient temperature on a C18 ODS-3 column (50 × 2.1 mm i.d.; Capital HPLC Ltd, Broxburn, UK) with gradient elution in mobile phases: A, 0.1% formic acid; and B, 0.1% formic acid in 95% acetonitrile (Fischer Scientific UK Ltd, Loughborough, UK). The gradient profile was as follows: 0–25% mobile phase B between 0 and 3 min; 25–100% mobile phase B between 3 and 4 min; 0% mobile phase B between 4.1 and 5 min. The flow rate was 0.8 ml min^{-1} and run time 5 min. Typical injection volume was 10 μl. Before injection, each rat urine sample was diluted 100-fold with water and an aliquot of diluted urine or PAG calibration standard (100 μl; aqueous solutions of phenylacetyl(amino)acetic acid in the range 0.05–5 μg ml^{-1}; Bachem, St Helens, UK) mixed with an equal volume of internal standard, 4-benzoylbutyric acid (0.5 mg ml^{-1} stock in acetonitrile diluted to final concentration of 500 ng ml^{-1} with water; Sigma-Aldrich, Poole, UK). Identification and subsequent quantitation was performed by ionspray mass spectrometry (Micromass Quattro II) operated in negative 'selective ion-monitoring' mode.

Rat plasma contains high levels of endogenous phenylacetylglycine (PAG) and phenylacetate (PA). However, the levels of PAG in monkey plasma are significantly lower (approximately 100-fold) as compared with the levels in rat plasma. A bioanalytical method used to quantify PAG in rat plasma samples was validated using monkey plasma as the blank matrix to make PAG standards and quality control samples. A stable isotopically labelled compound ($^{13}C_3$-PAG; synthesized in-house) was used as the internal standard, and the validated calibration range for PAG (Bachem Biosciences, Bubendorf, Switzerland) was 50–5000 ng ml^{-1}. There were no sources of plasma that contained significantly lower amounts of PA as compared with levels of PA in rat plasma. In order to quantify PA in rat plasma, a stable isotopically labelled compound ($^{2}H_5$-PA; CND Isotopes, Quebec, Canada) was used to make standards and quality control samples in rat plasma. Here, the assumption was that the $^{2}H_5$-PA mass spectrometric response was the same as the mass spectrometric response of endogenous PA. The internal standard, 4-fluorophenyl acetate (Sigma-Aldrich), was used in the assay, and the calibration range for PA was 20–1000 ng ml^{-1}.

Stock solutions were prepared in methanol and dilutions were made as necessary. The calibration standards were prepared by spiking monkey plasma with PAG and rat plasma with $^{2}H_5$-PA to achieve the desired concentration ranges. Standards and quality control samples were prepared and added to the plate with the samples before extraction. Samples were extracted by adding internal standard (150 μl, 500 ng ml^{-1} $^{13}C_3$-PAG and 500 ng ml^{-1} 4-fluorophenyl acetate in acetonitrile) to each plasma sample (50 μl). The samples were mixed, centrifuged, and a 75-μl aliquot of the supernatant transferred to a 96-well plate containing 5 mM ammonium acetate and 0.1% formic acid solution (75 μl).

The samples were analysed by reversed-phase HPLC using a Shimadzu 10A vp HPLC system with a Cogent HPS C18 5 μm column (50 × 2.1 mm i.d.). An HPLC gradient, with a flow rate of 0.5 ml min^{-1} was used with the following mobile phases: A, 5 mM ammonium acetate and 0.1% formic acid; and B, methanol. Initial conditions were 85% A and 15% B for 0.3 min. B was increased to 85% from 0.3 to 1.3 min and held until 1.7 min. At 1.75 min, initial conditions were returned with an end run at 2.4 min. A 5-μl injection volume was used. Under these HPLC conditions the typical retention times for PAG, PA, $^{13}C_3$-PAG, $^{2}H_5$-PA, and 4-fluorophenyl acetate were: 1.3, 1.5, 1.3, 1.5 and 1.5 min, respectively. Detection of PAG and PA was performed using an Applied Biosystems API-4000 mass spectrometer in the positive-ion mode, with a TurboIon Spray source (−4500 V, 600°C), and multiple-ion reaction-

monitoring (MRM). The precursor to product ion transitions for PAG, PA, $^{13}C_3$-PAG, $^{2}H_5$-PA and 4-fluorophenyl acetate were *m*/z 192 →74, 135 →91, 195 →77, 140 →96 and 154 →110, respectively, with a dwell time of 100 ms for each transition. A calibration curve of analyte/internal standard peak area ratio versus concentration was constructed and a weighed $1/x^2$ linear regression applied to the data.

Phenylalanine and tyrosine in rat plasma were measured by reverse-phase HPLC with precolumn derivatization and fluorescence detection using a modification of the AccQ Tag system for amino acid analysis (Waters Ltd, Elstree, UK). Amino acids were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) and separated on an Alliance 2690 HPLC system using a NovaPak C18 column (150 ×3.9 mm, 4 μm; Waters). Typical injection volume was 5 μl. AccQ-Tag Eluant A (AccQ-Fluor reagent kit WAT052880) was diluted 1:10 with water and pH adjusted to pHs 5.05 and 4.60 to give mobile phases A and B, respectively. Mobile phase C was HPLC grade (190 nm) acetonitrile (Fischer Scientific UK) and mobile phase D HPLC grade water. Separation was performed using the following gradient conditions: 0 min, 90% A, 10% B; 0.5 min, 90% A, 9% B, 1% C; 18 min, 86% A, 9.5% B, 4.5% C; 19 min, 91.5% B, 8.5% C; 20 min, 91% A, 9% C; 30.5 min, 85% A, 15% C; 37 min, 60% C, 40% D; 39 min, 90% A, 10% B. Column temperature was 50°C and flow rate was 1 ml min^{-1}. The eluate was monitored by fluorescence on a 474 variable wavelength fluorescence detector (Waters) at 250 nm excitation and 395 nm emission. Integration/quantitation of peaks was via Waters Millennium[32] Chromatography Data System.

Phenylalanine hydroxylase activity in rat liver cytosol was determined using a kinetic method (Ayling 1973) modified for use on an Advia 1650 autoanalyser. In brief, rat liver cytosols (30 μl) were diluted with saline (0.9%, 45 μl) and aliquots (25 μl) of the diluted cytosol injected into the reaction cells of the analyser. After addition of Reagent B (100 μl; 0.1 M Tris buffer, pH 7.4, containing 1.4 mM phenylalanine; Sigma) the reaction mixture was equilibrated to 37°C for 5 min. Reagent C (15 μl; 0.1 M Tris buffer, pH 7.4, containing 6.32 mM DMPH4 (6,7-dimethyl-5,6,7,8-tetrahydropterine; Sigma) was then added to initiate the reaction, which was monitored at 340 nm for 5 min. In order to account for the auto-oxidation of DMPH4, sample blanks were performed with reagent A (0.1 M Tris buffer, pH 7.4) substituting reagent B. A single assay was performed on each sample.

Amiodarone and its desethyl metabolite were determined in 0.2 ml rat plasma using a SPE/LLE-HPLC/UV method developed and validated at Aster-Cephac (Poitiers, France). Separation was performed at 30°C with a Kromasil C18 reversed-phase column (250 ×4.6 mm, 5 μm; Touzart et Matignon, France) using mobile phases A, 2 mM sodium acetate buffer (Merck Poole, UK) and 3.60 mM triethylamine (puriss for HPLC; Fluka) (pH 3.8) in acetonitrile (HPLC grade; SDS, Peypin, France); and B, 1.25 mM sodium acetate buffer and 2.24 mM triethylamine (pH 3.8) in acetonitrile delivered by a Waters 600 pump. Gradient conditions were as follows: 0–16.9 min, 100% A, 0.5 ml min^{-1}; 17.0–23.0 min, 100% B, 1.5 ml min^{-1}; 23.1 min, 100% A, 0.5 ml min^{-1}. Sample volumes of 20 μl were injected onto the column with a WISP 717plus autosampler (Waters) with sample carousel maintained at 4°C. The eluate was monitored at 242 nm with a UV/Vis 2487 detector (Waters). Total run time was 36 min and retention times of amiodarone (hydrochloride; Sigma), desethylamiodarone (hydrochloride; PDI-Research Laboratory, Inc., Laval, Canada) and internal standard (bepridil hydrochloride; Sigma) were 19, 15 and 11 min, respectively. The dynamic range of the method was 25–2000 ng ml^{-1} with precision of ±9%. All plasma samples (unknowns, quality controls, blanks, calibration standards) were centrifuged for 5 min at 4000 rpm and 4°C. An aliquot (0.2 ml) from each was added to a tube with bepridil (10 μl, 20 μg ml^{-1} in methanol; hypersolv for HPLC; BDH) and 1 M sodium acetate buffer (pH 5.5, 25 μl). After mixing, hexane (3.5 ml; 99% HPLC grade; SDS) was added and the samples were vortexed for 5 min and then centrifuged at 4000 rpm for 10 min at 4°C. An aliquot (3 ml) of the upper organic phase was evaporated to dryness under nitrogen at 30–40°C and the residues reconstituted with mobile phase A (250 μl). After thorough mixing, samples were transferred to autosampler vials. Quantitation was performed by referring to standard curves constructed from known amounts of amiodarone and desethylamiodarone (25–2000 ng ml^{-1}; dissolved in methanol) added to drug-free control rat plasma, using least-square weighted linear regression.

Mononuclear cells were isolated from whole blood taken at autopsy by gradient sedimentation in Lymphoprep™ tubes (Nycomed) according to manufacturer's instructions. Preparation of other tissues and subsequent analysis by HPLC-MS/MS to identify and semi-quantitate phospholipids are described by Mortuza *et al.* (2003). Total cytochrome P450 and pentoxyresorufin *o*-deethylase, to confirm hepatic enzyme induction after phenobarbitone treatment, were determined in liver homogenate using published methods (Omura and Sato 1964, Burke *et al.* 1994, respectively). Enzyme content or activity was normalized to protein, which was measured according to the bicinchoninic acid kit method (Product Number BCA-1; Sigma).

Statistical analyses

Data were analysed and graphed with commercial software (Graphpad Prism, version 3.0).

Unless otherwise stated, serum and tissue parameters were analysed using one-way ANOVA followed by a Newman–Keuls multiple comparison test. Data represented graphically are group means ±SEM.

Statistical significance is denoted as follows: $^{\star}p \leq 0.05$, $^{\star\star}p \leq 0.01$ and $^{\star\star\star}p \leq 0.001$; numbers in parentheses denote the groups against which statistical significance was attained.

Due to missing urine samples for urinary PAG and to obtain a value for 24 h excretion for each animal, the data was analysed as follows: 0–8 and 8–24 h collections were calculated as $\mu g\ kg^{-1}\ h^{-1}$ and log transformed to minimize the impact of inaccurate volume assessment (all volumes were rounded up to the nearest ml); log data were then adjusted to balance out the differences in excretion rate between 0–8 and 8–24 h collections; adjusted log data for 0–8 and 8–24 h collections were then meaned for each animal. Where a single value only was available, this could now be used to reflect more accurately the hourly excretion rate of PAG.

Individual clinical and biochemical parameters were then compared with body, liver and lung weights, urinary PAG excretion and plasma amiodarone concentration using Pearson's correlation test.

Results

Plasma amiodarone levels

Plasma amiodarone levels were significantly higher in the animals given amiodarone alone than those which had been co-administered phenobarbitone. This supports the hypothesis that the rate of amiodarone clearance had been increased following the induction of P450 enzymes. There was an apparent contamination of the control samples with amiodarone, amounting to a maximum of 3%. It is likely that this was due to an endogenous metabolite interfering at the same run time, or 'carry-over' in the system. The plasma levels of the main metabolite of amiodarone, desethylamiodarone, were not significantly different in the amiodarone treated and the animals treated with amiodarone and phenobarbitone (425 ng $ml^{-1} \pm$SEM 44 verses 470 ng $ml^{-1} \pm$SEM 40, respectively). No desethylamiodarone was found in the control samples.

Clinical observations, body and liver weights

Oral administration of vehicle or amiodarone had no adverse clinical effects. However, as expected, the major clinical signs displayed by animals receiving phenobarbitone were those consistent with sedation. After the first dose of amiodarone (day 0) all animals showed a loss of body weight on day 1; however, body weight gain mirrored the relevant controls in subsequent days. Phenobarbitone had no effect on body weight compared with control animals (figure 1).

Amiodarone did not have any effect on absolute liver weight (VCon: 11.4 ± 0.49; Amio: 10.1 ± 0.49; mean $\pm$SEM), whereas animals receiving phenobarbitone, as expected, had significantly enlarged livers (PbCon: 13.6 ± 0.3 and Pb/Amio: 13.6 ± 0.5 compared with VCon; mean $\pm$SEM, $p < 0.001$).

Hepatic effects of treatment

Serum clinical chemistry did not indicate overt liver damage with any of the treatments (data not shown); however, alkaline phosphatase activity was significantly raised, in isolation, by amiodarone, given alone or in combination with phenobarbitone (VCon (group 1): 563 ± 66, Amio (group 2): $977 \pm 72^{\star(1)}$, PbCon (group 3): 599 ± 99, Pb/Amio (group 4): $1145 \pm 291^{\star(3)}$ IU l^{-1}; mean $\pm$SEM, $^{\star}p \leq 0.05$). Phenobarbitone had no effect on serum clinical chemistry but did raise hepatic total P450 content and PROD activity (table 2). Sole administration of amiodarone did not alter total P450 content and PROD activity but markedly

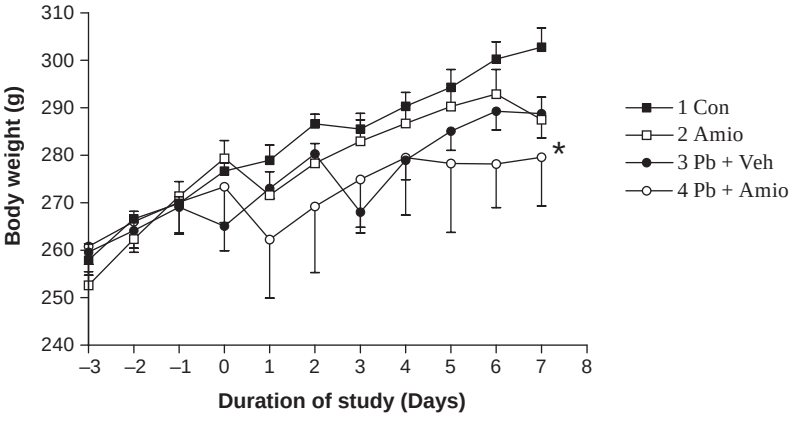

Figure 1. Effect of amiodarone, with or without phenobarbitone, on body weight. Values are means ± SEM; $n = 5$ for groups 1, 2 and 4, and $n = 4$ for group 3, $^{*}p \leq 0.05$, significantly different from comparator group by pairwise comparison using a two-tailed t-test.

reduced enzyme induction when given concurrently with phenobarbitone (table 2). In addition, co-administration of phenobarbitone with amiodarone reduced the concentration of amiodarone measured in plasma taken 5 h after the last dose compared with that seen after amiodarone administration alone (table 2). Plasma desethylamiodarone concentration was similar in all animals given amiodarone (Amio: 425 ± 44, Pb/Amio: 470 ± 40 ng ml^{-1}; mean ± SEM).

Serum lipids

As cellular lipids are predominantly synthesized in the liver the following data are presented as a ratio with liver weight in order to abolish any effect related directly to liver size, which was increased after treatment with phenobarbitone.

Total cholesterol and phospholipids were raised after administration of amiodarone compared with that measured in vehicle controls (175 and 90% increase, respectively) (table 3). Phenobarbitone, when given alone, was shown to have no effect on these parameters, but significantly diminished the response to amiodarone when co-administered with the drug (42 and 23% increase, respectively compared with PbCon). Triglycerides were reduced to a similar extent by sole

Table 2. Effect of treatment on hepatic cytochrome P450 induction and plasma drug concentration.

Group	Total P450 content (nmol mg^{-1} protein)	PROD Activity (pmol min^{-1} mg^{-1} protein)	Plasma amiodarone (ng ml^{-1})
1 (VCon)	0.92 ± 0.10	31.54 ± 3.65	128 ± 71
2 (Amio)	0.74 ± 0.07	34.44 ± 3.08	3888 ± 246***(1, 3, 4)
3 (PbCon)	2.15 ± 0.08***(1, 2) *(4)	1155.50 ± 78.80***(1, 2, 4)	78 ± 28
4 (Pb/Amio)	1.82 ± 0.08 ***(1, 2) *(3)	448.60 ± 75.05***(1, 2, 3)	2144 ± 273 ***(1, 2, 3)

Group 1 = vehicle control (p.o.); group 2 = amiodarone (p.o.); group 3 = phenobarbitone control (i.p.); group 4 = phenobarbitone (i.p.) plus amiodarone (p.o.). Values are means ± SEM; $n = 4$ in group 3, $n = 5$ in groups 1, 2 and 4. Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls multiple comparison test; significance denoted as $^{*}p \leq 0.05$, $^{***}p \leq 0.001$; numbers in parentheses denote the group against which statistical significance was attained.

Table 3. Effect of treatment on serum lipids assessed using standard clinical chemistry methods.

Group	Total phospholipid	Total cholesterol	HDL	Triglycerides
1 (VCon)	11.7 ± 0.5	12.9 ± 0.8	10.7 ± 0.6	6.2 ± 0.4
2 (Amio)	22.3 ± 1.3 ***(1, 3, 4)	35.5 ± 2.3 ***(1, 3, 4)	31.9 ± 2.1 ***(1, 3, 4)	3.8 ± 0.4 **(1)
3 (PbCon)	12.1 ± 0.35	15.3 ± 1.0	12.6 ± 0.8	5.0 ± 0.3
4 (Pb/Amio)	14.9 ± 0.4 *(1, 3), ***(2)	21.8 ± 0.8 **(3), ***(1, 2)	18.7 ± 1.1 **(1, 3), ***(4)	3.4 ± 0.4 *(3), ***(1)

Group 1 = vehicle control (p.o.); group 2 = amiodarone (p.o.); group 3 = phenobarbitone control (i.p.); group 4 = phenobarbitone (i.p.) plus amiodarone (p.o.). Values are means ± SEM are mmol l^{-1} expressed as a percentage relative to liver weight. Group 3, $n = 4$; groups 1, 2 and 4, $n = 5$. Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls multiple comparison test; significance denoted as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$; numbers in parentheses denote the group against which statistical significance was attained.

administration of amiodarone compared with vehicle controls or by combination treatment with phenobarbitone (39% Amio versus VCon and 32% Pb/Amio versus PbCon). Serum high-density lipoprotein (HDL) was raised after amiodarone treatment (98%, Amio versus VCon), in concert with elevated cholesterol. Phenobarbitone alone had no effect but significantly diminished amiodarone-induced elevation of HDL (48%, Pb/Amio versus PbCon) (table 3).

Examination of tissues by light microscopy showed a treatment-related increase in foamy macrophage accumulation in the lungs and mesenteric lymph nodes, a finding consistent with phospholipid accumulation, in animals dosed orally with amiodarone. The magnitude of response was similar with or without phenobarbitone co-administration (data not shown). Different results were obtained when tissue and serum lipids were assessed using HPLC-MS/MS.

HPLC analysis of tissue phospholipids

Peripheral lymphocytes. Neither cell counts nor protein assays were performed on the lymphocyte samples used for phospholipid extraction and thus no absolute values for lipids could be accurately determined. However, comparison of relative changes showed an increase in several molecular species of a particularly interesting phospholipid, identified as lyso(*bis*)phosphatidic acid (LBPA: 18:2–16:0 plus two species with uncharacterized acyl chains). LBPA (18:2–16:0) was virtually absent in control animals (vehicle and phenobarbitone) but was raised to approximately 4% of total phospholipid in rats treated with amiodarone compared with 1% of total phospholipid in rats co-administered phenobarbitone and amiodarone (table 4). There was also a significant increase in LBPA in animals treated with phenobarbitone alone. However, the amount was less than 4% of that seen in lymphocytes taken from animals given amiodarone alone. In addition phosphatidylcholine (PC: 16:0–20:4) was also raised after amiodarone treatment accompanied by reductions in other PCs (18:2–18:1 and 18:0–18:1) and phosphatidylinositol (PI: 16:0–20:4). After combined treatment with phenobarbitone/amiodarone only LBPA (18:2–16:0) was raised and PC (18:0–18:1) and PI

Table 4. Phospholipid accumulation in circulating lymphocytes and liver, assessed by HPLC-MS/MS.

Group	Tissue	Total phospholipid (total chromatographic peak area/liver weight)	LBPA (peak area/total chromatographic peak area, %)
1 (VCon)	Lymphocytes	n/a	0
	liver	808 ± 93	0
2 (Amio)	Lymphocytes	n/a	3.66 ± 0.53 ***(1, 3, 4)
	liver	785 ± 64	0.59 ± 0.13 ***(1, 3, 4)
3 (PbCon)	Lymphocytes	n/a	0.14 ± 0.07 ***(2, 4)
	liver	1131 ± 08	0 ***(2)
4 (Pb/Amio)	Lymphocytes	n/a	1.24 ± 0.14 *(1),***(2, 3)
	liver	1145 ± 144	0.09 ± 0.06 ***(2)

Group 1 = vehicle control (p.o.); group 2 = amiodarone (p.o.); group 3 = phenobarbitone control (i.p.); group 4 = phenobarbitone (i.p.) plus amiodarone (p.o.). Values are means ± SEM. For liver data: groups 1 and 2, $n = 5$; group 3 and 4, $n = 4$; lymphocyte data: groups 1, 2 and 4, $n = 5$; group 3, $n = 4$. Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls multiple comparison test; significance denoted as *$p \leq 0.05$, ***$p \leq 0.001$; numbers in parentheses denote the group against which statistical significance was attained. n/a, Not applicable.

(16:0–20:4) reduced. Some of these data have been reported by Mortuza *et al.* (2003).

Liver. There were no quantitative differences in the amount of total hepatic phospholipids with any of the treatments given. However, amiodarone administration increased LBPA (18:2–16:0) approximately four-fold compared with vehicle control (table 4) and decreased PC (18:2–18:1) and another unidentified lipid, also likely to be a PC (data not shown). Phenobarbitone ameliorated the amiodarone-induced elevation of LBPA (18:1–16:0) (table 4).

Lung. Phenobarbitone itself did not alter the total amount of phospholipid in the lungs but it did serve to attenuate the influence of amiodarone. Amiodarone, when given alone, increased total phospholipids by 104% compared with vehicle control whereas in combination with phenobarbitone the increase was only 68% greater than with phenobarbitone alone (figure 2). LBPA (18:2–16:0) seemed to be a sensitive indicator of phospholipidosis in the lung as the increase was 10-fold higher than the vehicle control after amiodarone treatment in contrast to a one-fold increase in total phospholipids (figure 2).

Serum. Phenobarbitone had no effect on total serum phospholipids compared with vehicle controls (figure 3). Amiodarone raised serum phospholipids by 218% compared with vehicle control whereas amiodarone/phenobarbitone combined raised them only 43% above the phenobarbitone controls (figure 3).

The relative changes in serum lipids were similar after treatment with amiodarone or amiodarone in combination with phenobarbitone. In general, molecular species of LBPA (figure 3) and PC containing unsaturated fatty acids were raised, particularly those containing 18:1, 18:2 and 20:4 (data not shown). There was also a compensatory decrease in other 18:1 and 20:4 containing PC and PI species (Mortuza *et al.* 2003).

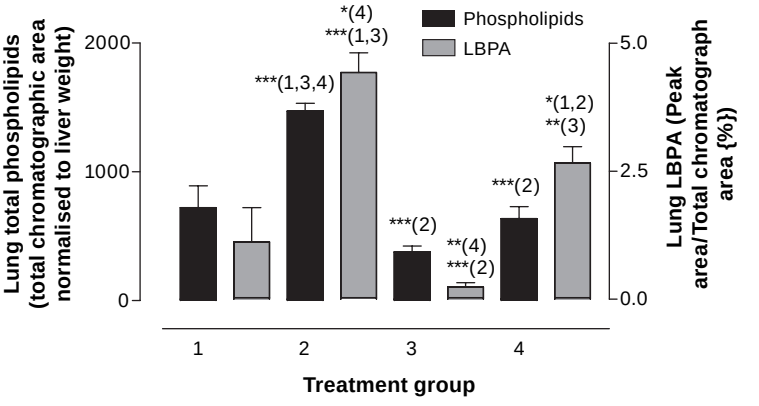

Figure 2. Effect of amiodarone, with or without phenobarbitone, on lung phospholipids: assessment using HPLC-MS/MS. Values are means ± SEM; $n = 5$ for groups 1 and 4, and $n = 4$ for groups 2 and 3; Group 1 = control (vehicle p.o.); group 2 = amiodarone (p.o.); group 3 = phenobarbitone (i.p.); group 4 = phenobarbitone (i.p.) plus amiodarone (p.o.). Values are means ± SEM; total phospholipids, LBPA. Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls multiple comparison test; significance denoted as $^{\star}p \leq 0.05$, $^{\star\star}p \leq 0.01$, $^{\star\star\star}p \leq 0.001$; numbers in parentheses denote the group against which statistical significance was attained.

Urinary excretion of PAG

Amiodarone, with or without phenobarbitone, raised the excretion of urinary PAG (day 6) three- to four-fold compared with the relevant control group. Phenobarbitone alone did not influence the excretion of PAG. The total amount of PAG excreted in the urine (calculated from days 0 to 6) was also similar in animals treated with amiodarone, alone or in combination with phenobarbitone but higher than their respective controls (figure 4).

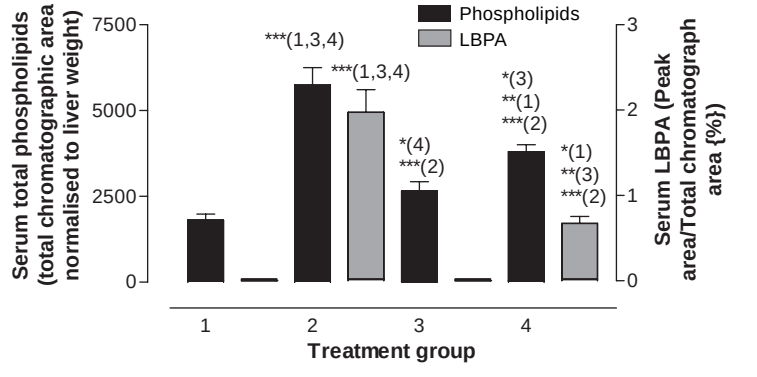

Figure 3. Effect of amiodarone, with or without phenobarbitone, on serum phospholipids: assessment using HPLC-MS/MS. Values are means ± SEM; $n = 5$ for groups 1, 2 and 4, and $n = 4$ for group 3; Group 1 = control (vehicle p.o.); group 2 = amiodarone (p.o.); group 3 = phenobarbitone (i.p.); group 4 = phenobarbitone (i.p.) plus amiodarone (p.o.). Values are means ± SEM; total phospholipids, LBPA. Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls multiple comparison test; significance denoted as $^{\star}p \leq 0.05$, $^{\star\star}p \leq 0.01$, $^{\star\star\star}p \leq 0.001$; numbers in parentheses denote the group against which statistical significance was attained.

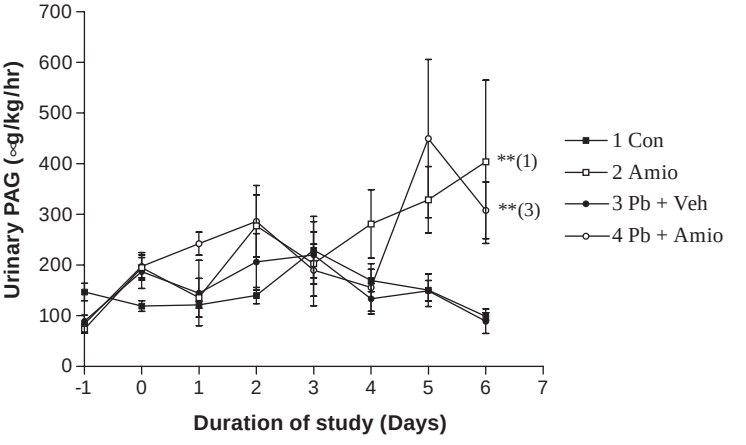

Figure 4. Effect of amiodarone, with or without phenobarbitone, on urinary PAG excretion. Values are means ± SEM; $n = 5$ for groups 1, 2 and 4, and $n = 4$ for group 3; statistical analysis was performed using a series of two-sided linear contrasts, comparing several group pairs of interest, and the p values modified, using a Sidak multiplicity adjustment, $^{**}p \leq 0.01$ significantly different from comparator group ( ).

Phenylalanine metabolism

Amiodarone, alone or in combination with phenobarbitone, did not influence the activity of phenylalanine hydroxylase (VCon, 5.0 ± 2.2; Amio, 5.3 ± 1.2; PbCon, 4.0 ± 0.1, Pb/Amio, 3.6 ± 0.5 nmol min^{-1} mg^{-1} protein) or the serum tyrosine:phenylalanine ratio (VCon, 1.4 ± 0.1; Amio, 1.3 ± 0.1; PbCon, 1.4 ± 0.2, Pb/Amio, 1.3 ± 0.1), although amiodarone, when given as a single treatment significantly raised plasma phenylalanine concentration to 122% of control (VVon, 24.0 ± 3.1; Amio, 29.4 ± 1.4 ng ml^{-1}, $^{*}p \leq 0.05$) but when combined with phenobarbitone had no significant effect (PbCon, 23.0 ± 2.4, Pb/Amio, 26.7 ± 3.2 ng ml^{-1}), although there was a small increase to 116% of the relevant control. Phenobarbitone treatment did not effect any of the above.

Phenylacetylglycine (PAG) and Phenylacetate (PA) in portal and peripheral blood

Portal and aortal PAG and PA. The ratio of PAG in portal:aortal blood was approximately 1 in all animals, irrespective of treatment. Phenobarbitone treatment did not influence the concentration of PAG in aortal or portal blood; however, amiodarone, whether given with vehicle or phenobarbitone, increased PAG concentration approximately six-fold (table 5).

Unlike PAG, PA concentration was approximately double the concentration in portal blood compared with aortal blood in rats given vehicle or phenobarbitone, respectively (table 5). Amiodarone, when given alone or in combination with phenobarbitone, raised mean aortal PA by 2.8- and 5.6-fold, respectively, but raised mean portal PA by 4.4- and 7.1-fold, respectively (table 5). This greater rise in portal versus aortal PA concentration after amiodarone treatment was reflected in increased portal:aortal PA ratio which was significantly raised following amiodarone treatment (figure 5). Phenobarbitone itself had no significant effect

Table 5. Effect of treatment on plasma PAG and its precursor, PA: distribution in hepatic portal and aortal blood. Samples were collected, at autopsy, from the hepatic portal vein and the aorta.

Group	Portal PAG	Aortal PAG	Portal PA	Aortal PA
1 (VCon)	97.12±20.29	103.90±23.08	44.92±7.63	27.82±5.52
2 (Amio)	500.68±143.80 ***(1, 3)	568.13±186.93 **(1, 3)	195.74±54.81 **(3)	77.66±27.02
3 (PbCon)	98.26±14.31	103.29±15.30 **(1, 3)	32.47±9.29	15.74±1.63
4 (Pb/Amio)	612.60±148.83 ***(1, 3)	648.20±193.48	226.09±76.28 *(1) **(1, 3)	90.44±20.08 **(1, 3)

Group 1 = vehicle control (p.o.); group 2 = amiodarone (p.o.); group 3 = phenobarbitone control (i.p.); group 4 = phenobarbitone (i.p.) plus amiodarone (p.o.). Values are means ± SEM are for ng ml^{-1} plasma. Group 3, $n = 4$; groups 1, 2 and 4, $n = 5$. Statistical analysis was performed using one-way ANOVA followed by a Newman–Keuls multiple comparison test; significance denoted as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$; numbers in parentheses denote the group against which statistical significance was attained.

on blood PA concentration although lower group mean values tended to exaggerate the fold change in portal and aortal PA induced by amiodarone.

Correlation analysis

Correlations were carried out on all parameters measured with body weight, liver weight, lung weight, day 6 urinary PAG and plasma amiodarone concentration. Of the statistically significant correlations obtained only those with an $r^2 \geq 0.5$ are discussed.

Body weight and lung weight did not correlate strongly with any parameter measured. Liver weight, as expected, correlated with total P450 ($r^2 = 0.713$). Urinary PAG and amiodarone correlations are shown in table 6. Of particular relevance were positive correlations between plasma amiodarone concentration with lung, lymphocyte and serum lipids and lack of correlation with urinary PAG ($r^2 = 0.109$; plasma amiodarone versus day 6 urinary PAG — log μg kg^{-1} h^{-1}). Urinary PAG concentration correlated with serum and lymphocyte lipids and, as expected, plasma PAG and PA.

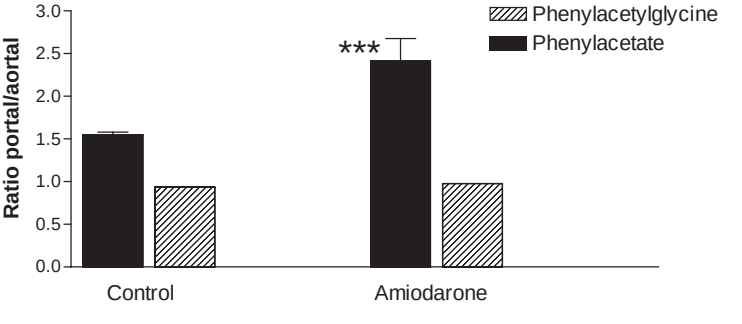

Figure 5. Ratio of PAG in portal and aortal blood and PA in portal and aortal blood in control animals and those treated with amiodarone. Values are means ± SEM. SEM bars are too small to be seen on the PAG ratio bars. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's test to compare selected pairs of data, $^{***}p \leq 0.001$ from control group.

Table 6. Correlation analysis of urinary PAG and plasma amiodarone concentration with blood and tissue lipids and other biochemical parameters. Data were analysed using Pearson's correlation test and achieved $p \leq 0.05$ and $r^2 \geq 0.5$. PAG correlations used data from all study animals whereas amiodarone correlations include data from Amio- and Pb/Amio-treated rats only.

PAG (log μg kg^{-1} h^{-1}, day 6)		Amiodarone concentration (ng ml^{-1})	
Parameter	r^2	Parameter	r^2
Serum total phospholipid	0.544	Serum total phospholipid	0.670
Serum cholesterol	0.639	Serum cholesterol	0.728
Serum HDL	0.656	Serum HDL	0.742
Serum LBPA	0.594	Serum LBPA	0.701
Lymphocyte LBPA	0.569	Lymphocyte LBPA	0.744
Portal PAG	0.770	Lung total phospholipid	0.763
Aortal PAG	0.770	Lung LBPA	0.905
Portal PA	0.831	Total P450 content	0.745
Aortal PA	0.796	PROD activity	0.752
Plasma amiodarone	0.678		
Plasma desethylamiodarone	0.701		

Discussion

There were two major questions this study was set up to answer: (1) Was the origin of the precursor for PAG, the gut or the liver?, and (2) Was urinary PAG a valid biomarker for phospholipidosis measured as the degree of correlation with phospholipid accumulation?

Phenylacetylglycine is the end product of phenylalanine metabolism in rodents and most other animal species except humans and primates, which excrete phenylacetylglutamine (Moldave and Meister 1957, James *et al.* 1972). These metabolites are synthesized in the liver and kidney (Webster *et al.* 1976, James and Bend 1978, Asaoka 1991). Phenylalanine hydroxylase (PAH), which is also predominantly expressed in liver and kidney (Petruschka *et al.* 1990), represents the major pathway for conversion of phenylalanine to tyrosine (Waters *et al.* 1998). In the disease state phenylketonuria (PKU), PAH protein is deficient (Erlandsen and Stevens 1999) and reduced PAH activity results in excessive phenylalanine and reduced tyrosine concentrations in blood (Hanley *et al.* 2000, Schulpis *et al.* 2002) in concert with elevated urinary excretion of phenylalanine metabolites, such as phenylacetate, phenylpyruvate, phenyllactate, phenylethylamine and phenylacetylglutamine (Woolf 1951, Michals and Matalon 1985, Alvarez *et al.* 1992).

In the present study, amiodarone treatment raised urinary PAG excretion (figure 4) but had no effect on hepatic PAH activity. Lack of effect on PAH activity was reflected in the plasma phenylalanine:tyrosine ratio, which was comparable in treated and control rats, despite a small but significant increase in plasma phenylalanine concentration. This data indicated that an endogenous hepatic lesion is probably not a major contributory factor in the generation of PAG and its precursors after amiodarone treatment.

To investigate further the factors involved in amiodarone-induced elevation of urinary PAG, blood was collected from the hepatic portal vein and the aorta at autopsy. Metabolites absorbed from the intestinal mucosa are initially transported in portal blood and enter the peripheral circulation after first pass in the liver. Any

metabolite that is in a higher concentration in portal blood compared with aortal blood, especially in fed animals, is therefore likely to be of intestinal origin (Remesy and Demigne 1976, Cummings *et al.* 1987, Cummings 1995).

Detection of higher concentration of PA, the immediate precursor to PAG, in hepatic portal blood compared with that in arterial peripheral blood in control animals (table 5) suggested that the gut was providing the majority of the precursor for PAG production. This is in agreement with published data, which suggests that the bulk of PA in humans originates from bacterial degradation of protein in the gut (Seakins 1971). Also, germ-free rats excrete very little PAG but urinary concentration increases over time when these animals are housed in a non-sterile environment during which time urinary PAG concentration becomes comparable to that measured in 'normal' untreated rats (Nicholls *et al.* 2003). The increased portal:aortal PA ratio in amiodarone treated rats compared with controls (figure 5) was indicative of a treatment-related increase in PA absorption from the gut. Although the concentration of PAG increased in portal and peripheral plasma in amiodarone treated rats, in proportion with elevated portal PA concentration, the portal:aortal PAG ratio remained the same (table 5 and figure 5). This suggested that PAG synthesis in the liver, and possibly the kidney, equilibrated in the blood and that there was little contribution from the gut. This is apparently the case as phenylalanine degradation by anaerobic bacteria from human colon and rumen intestine has been reported to terminate with phenylacetate (Smith and MacFarlane 1996, 1997, Khan *et al.* 1999).

The increased PA absorbed from the gut into portal blood after amiodarone treatment may have been due to altered bacterial metabolism of phenylalanine, as is the case in patients with amino acid malabsorption (Van der Heiden *et al.* 1971a, b) and possibly uraemia (Simenhoff *et al.* 1974). As absorption of orally administered amiodarone proceeds for many hours, suggesting that this process occurs along the length of the gastrointestinal tract (Holt *et al.* 1983), and substantial metabolism of amiodarone occurs in the gut lumen or intestinal mucosa (Berdeaux *et al.* 1984) it is not inconceivable that gut flora might be effected. Reselection or redistribution of colonizing species of gut bacteria is known to occur with dietary alteration (Phipps *et al.* 1998) and may result after addition of amiodarone to the local environment. Low concentrations of amiodarone (1–3 μM) have been shown to inhibit bacterial growth in controlled laboratory experiments (Rosa *et al.* 2000) and if this were to happen *in vivo* in treated rats, reduced PA synthesis might be expected. In the present study, maximum plasma drug concentration of approximately 4000 ng ml^{-1} was observed in amiodarone-treated rats (table 2), which is equivalent to 5.8 μM. As the gut would initially be exposed to far greater concentrations of amiodarone, the elevation of portal PA in treated animals compared with controls is unlikely to be due to inhibition of bacterial metabolism.

Another reason for increased PA absorption from the gut may be disruption of the intestinal mucosa. Orally administered doses of 175 mg kg^{-1} day^{-1} of aqueous amiodarone suspensions to rats have been shown to reduce intestinal ALP (alkaline phosphatase) activity between 7 and 14 days of treatment followed by reduced villus and crypt size of intestinal mucosa and reduced cellularity of the lamina propria (Sirajudeen *et al.* 2000). A similar dose of 150 mg kg^{-1} day^{-1} amiodarone was

employed in this study and administered for 7 consecutive days. In the present study, serum ALP activity was raised in amiodarone treated rats and enzyme activity correlated with urinary PAG concentration ($r^2 = 0.540$). ALP is a ubiquitous enzyme and serum ALP in the healthy individual originates predominantly from liver and bone but in disease states intestinal and placental isoforms can also be present. Damage to the kidney would result in raised urinary alkaline phosphatase activity (Moss *et al.* 1987). The intestine is the most likely source of the raised plasma ALP as there was no clinical evidence of renal or hepatic damage (data not shown), the placenta is not relevant as a source of ALP in this case (male animals were used), and there is no published evidence that amiodarone has bone effects. Thus there may have been a direct damaging effect of amiodarone on the intestinal lining or the use of a surfactant to solubilize amiodarone might have promoted intestinal damage in this study. Surfactants can themselves disrupt the intestinal mucosa (Martin-Algarra *et al.* 1994, 1995) and may thus be a contributory factor. Further investigation is required to clarify this.

To answer the second question, phenobarbitone was administered concurrently with amiodarone and the data compared with that obtained from animals receiving amiodarone alone.

Phenobarbitone effectively increased the amount of total hepatic cytochrome-P450 activity and also the activity of pentoxyresorufin *o*-deethylase, the marker enzyme indicating induction of cytochrome P4502B1 in rats. It was also noted that amiodarone diminished the induction of cytochrome P450 by phenobarbitone but was not inhibitory when given alone (table 2). The inhibitory effect of amiodarone on phenobarbitone-induced cytochrome P450 activity was likely to be due to drug–drug interaction. Such interactions between amiodarone and a number of therapeutic agents have been well publicized (Marcus 1983, Chitwood *et al.* 1993, Ha *et al.* 1996). Phenobarbitone induces CYP3A, CYP2B and CYP2C in various species including rat, rabbit and non-human primates (Jones *et al.* 1992, Burke *et al.* 1994, Weaver *et al.* 1994, Schulz *et al.* 2001). CYP3A is the major isoenzyme metabolizing amiodarone to its *N*-desethyl metabolite (Trivier *et al.* 1993, Libersa *et al.* 2000) and further metabolism of *N*-desethylamiodarone to 3-hydroxy-*N*-desethylamiodarone (Ha *et al.* 2001, Kozlic *et al.* 2001). Liver is the major organ for amiodarone metabolism but there is a relatively small contribution from the gut. Induction of cytochromes P450 with phenobarbitone enhances hepatic, but not gut, metabolism of amiodarone and additionally induces extrahepatic metabolism in the lung and kidney (Young and Mehendale 1987). This would explain the reduced levels of amiodarone in plasma (table 2) and tissues (Fruncillo *et al.* 1985) of rats receiving concurrent treatment with both drugs and the resultant reduction in the degree of phospholipid accumulation in these tissues (figure 2), which is proportional to concentration of amiodarone/metabolite accumulated in the same tissue and the duration of exposure (Reasor *et al.* 1988).

Amiodarone raised total and specific phospholipids in blood and tissues of dosed animals (tables 3 and 4, and figures 2 and 3). The lungs (alveolar macrophages), mesenteric lymph nodes and circulating lymphocytes are the primary target tissues in the rat (Mazue *et al.* 1984, Dake *et al.* 1985, Reasor *et al.* 1988, Kodovanti and Mehendale 1990) and all had accumulated phospholipid

in the present study. Total lung phospholipids were doubled in treated animals whereas LBPA was increased 10-fold. The slight increase in LBPA levels in lymphocytes seen following phenobarbitone treatment may reflect an increase in cell growth or turnover. LBPA is a lipid of late endosomal and/or lysosomal origin, which has been shown to be raised in patients and animals with genetic or drug-induced lipid storage diseases (Yamamoto *et al.* 1971, Tjiong *et al.* 1978, Fredman *et al.* 1982, Nakashima *et al.* 1984, Bargal and Bach 1988, Elleder 1989). Due to its accessibility in peripheral blood (Mortuza *et al.* 2003) and its very long half life of 90 h, which relates to its unique stereo-configuration (Cochran *et al.* 1987), this lipid may prove to be a useful biomarker of phospholipidosis in its own right. Indeed studies are currently being undertaken to investigate the sensitivity and specificity of LBPA in relation to phospholipidosis and to compare the response with that of urinary PAG.

Phenobarbitone attenuated the phospholipid accumulation in the lungs, lymphocytes and serum of amiodarone treated rats (tables 3 and 4, and figures 2 and 3). This supports the assumption that tissue amiodarone concentrations were reduced in animals receiving the dual dosing regimen. The above conclusions were supported by significant correlations between plasma amiodarone concentration and serum total phospholipid, cholesterol and HDL, total lung phospholipid content and lung, serum and lymphocyte LBPA content (table 6). Plasma drug concentration, and likely tissue drug concentration, was directly related to the degree of phospholipidosis observed. This is consistent with other published work relating to co-administration of cationic amphiphilic drugs with phenobarbitone (Reasor and Davis 1985, Reasor *et al.* 1988, Pakuts *et al.* 1990).

However, unlike lipid parameters, urinary PAG excretion was not altered as a function of plasma amiodarone concentration (figure 4). This argument was strengthened by the lack of correlation between these parameters when data obtained exclusively from amiodarone treated rats were used ($r^2 = 0.109$; plasma amiodarone versus day 6 urinary PAG — log $\mu g\, kg^{-1}\, h^{-1}$). Although urinary PAG did correlate with serum and lymphocyte lipids, these were less significant than those obtained with amiodarone. Also there was no correlation between urinary PAG and lung phospholipids. As the lung is the primary target tissue after amiodarone treatment, this is perhaps the most compelling evidence suggesting that PAG does not directly relate to drug-induced phospholipid accumulation. As might be expected, urinary PAG correlated with concentrations of its precursor, PA, and PAG in both portal and aortal plasma. Almost all PA is converted to PAG and little is excreted into the urine of normal animals (James *et al.* 1972). Increases of similar magnitude of plasma and urinary PAG with plasma PA reflect this (table 5).

Conclusions

Although raised urinary PAG has been identified consistently as being raised in animals treated with compounds that cause phospholipid accumulation (Nicholls *et al.* 2000, and others), the biochemical link has not been made between phospholipid accumulation and increased levels of PAG. Amiodarone increased

total phospholipid concentration in serum, lung and lymphocytes but not in the liver. Urinary and plasma PAG and portal:aortal PA ratio were increased. Hepatic PAH activity and plasma phenylalanine and tyrosine concentrations were not affected.

Phenobarbitone treatment itself had no effect on any clinical parameter measured but increased hepatic total P450 content and induced PROD activity, as expected. As a result plasma amiodarone concentration was reduced in rats co-administered both drugs and this served to attenuate the phospholipid accumulation in target tissues of these rats compared with those treated only with amiodarone. However, phenobarbitone co-administration failed to alter the magnitude of response with regards to urinary PAG excretion and plasma concentration of its precursors after amiodarone treatment.

Collectively these data imply the following:

- Raised urinary PAG, after treatment with a cationic amphiphilic drug such as amiodarone, was probably not due to disturbed hepatic phenylalanine metabolism but may be related to changes in gut flora or the integrity of the intestinal brush border. The weight of evidence suggests that PAG probably arises from an increased uptake of precursors from the gut following the oral administration of amiodarone and possibly other CADs that raise PAG levels and cause phospholipids to accumulate.
- Drug concentration was positively correlated with the degree of phospholipid accumulation but not with urinary and plasma PAG or its plasma precursors.
- Although urinary PAG positively correlated with serum and lymphocyte lipid parameters, it appears that the degree of phospholipid and other lipid changes may not be directly/mechanistically related to an increase in PAG. Urinary PAG may, however, be useful as a surrogate biomarker for phospholipidosis.

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