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Identification of Phase-I Metabolites of the Kv1.3 Blocker PAP-1 (5-(4-Phenoxybutoxy)psoralen) in the Rat

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Abstract

PAP-1 (5-(4-phenoxybutoxy)psoralen), a potent small-molecule blocker of the voltage-gated potassium Kv1.3 channel, is currently in pre-clinical development for psoriasis. The present study was undertaken to identify the major phase-I metabolites of PAP-1 in rats. Following oral administration at 50 mg/kg, bile, plasma, urine and feces were collected, and separated by reversed-phase HPLC after sample preparation by solid-phase extraction. Five phase-I metabolites, i.e., 5-(oxybutyric-acid)psoralen (M1), 5-[4-(4-hydroxybutoxy)]psoralen (M2), 5-[4-(4-hydroxyphenoxy)]psoralen (M3), 5-[4-(3-hydroxyphenoxy)]psoralen (M4), 8-hydroxyl-5-(4-phenoxybutoxy)psoralen (M5), were isolated and identified by mass spectrometry and NMR spectroscopy. M3, M4 and M5 were hydroxylated products, while M1 and M2 were O-dealkylation products. Incubation of PAP-1 with rat liver microsomes rendered the same five major metabolites in a NADPH-dependent manner suggesting that cytochrome P450 (CYP) enzymes are involved in PAP-1 metabolism. Inhibitors of rat CYP1A1/2 (alpha-naphthoflavone) and CYP3A (ketoconazole) but not CYP2D6, CYP2E or CYP2C9 blocked the metabolism of PAP-1 in rat microsomes. Of the five metabolites M3, M4 and M5 were found to inhibit Kv1.3 currents with nanomolar IC₅₀s, while M1 and M2 were inactive. Our results identified the Kv1.3-inactive M1 as the major phase-I metabolite, and suggest that hydroxylation and O-dealkylation are the major pathways of PAP-1 metabolism principally via CYP1A1/2 and CYP3A. We further conducted a 6-months repeat-dose toxicity study with PAP-1 at 50 mg/kg in both male and female rats and did not observe any changes in hematology, blood chemistry, body weight, histology of any major organ, or speeding up of metabolism suggesting induction of its metabolizing enzymes (presumably CYPs).

Keywords

5-(4-phenoxybutoxy)psoralen (PAP-1); Kv1.3 blocker; metabolism

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Declaration of interest: B.H., Z.C., X.Z., Y.W. and P.I.Z. report no conflict of interest. H.W. is an inventor on the University of California patent claiming PAP-1 and related Kv1.3 blockers for immunosuppression. She is a scientific founder of Airmid, a start-up company that is aiming to develop Kv1.3 blockers as immunosuppressants. G.M. is the CEO and a full-time employee of Airmid. The authors alone are responsible for the content and writing of the paper.

Human T-lymphocytes primarily express two potassium channels, the voltage-gated potassium channel Kv1.3 and the calcium activated potassium channel KCa3.1 (Logsdon et al., 1997; Cahalan and Chandy, 2009). During T cell activation both channels play an important role in regulating membrane potential by promoting a countercurrent efflux of potassium to allow influx of extracellular calcium through calcium release-activated calcium channels (Chandy et al., 2004; Cahalan and Chandy, 2009). Blockade of Kv1.3 and KCa3.1 consequently leads to membrane depolarization, thus reducing Ca^{2+} influx and resulting in the prevention of T-cell activation (Chandy et al., 2004).

Based on their increased expression in activated CCR7⁻ effector memory T cells Kv1.3 channels in particular have been identified as a therapeutic target for the selective suppression of these T cells in autoimmune diseases such as type-1 diabetes, multiple sclerosis and psoriasis (Beeton et al., 2006; Wulff et al., 2009). The Kv1.3-blocking peptides kalitoxin (from scorpion venom), ShK (from the sea anemone *Stichodactyla helianthus*), and its more selective derivative ShK(L5) have been shown to prevent and reverse the symptoms of experimental autoimmune encephalomyelitis (EAE) (Beeton et al., 2001a, 2001b, 2005) and pristane induced arthritis (Beeton et al., 2006). However, from the point of clinical application, small molecular blockers of Kv1.3 for oral administration would be highly desirable. Currently, the most potent and selective available small molecule Kv1.3 blocker is PAP-1 (5-(4-phenoxybutoxy)psoralen), which blocks the channel with an IC_{50} of approximately 2 nM and exhibits 23-fold selectivity over Kv1.5 and 33- to 125-fold selectivity over other Kv1-family channels, respectively (Schmitz et al., 2005). PAP-1 has been demonstrated to suppress delayed-type hypersensitivity (Schmitz et al., 2005), prevent type-1 diabetes development in autoimmune diabetes prone rats (Beeton et al., 2006) and to inhibit oxazolone-induced allergic contact dermatitis (ACD), a simple rat model of psoriasis (Azam et al., 2007). Based on the fact that PAP-1 suppressed ACD not only after intraperitoneal and oral administration but also following topical application, the compound is currently in pre-clinical development for the treatment of psoriasis. Moreover, PAP-1 did not display any signs of acute toxicity *in vitro* or *in vivo* and did not prevent the development of flu vaccine responses in primates (Schmitz et al., 2005; Pereira et al., 2007).

Although the pharmacokinetics of PAP-1 have been reported in rats and rhesus macaques (Beeton et al., 2006; Azam et al., 2007; Pereira et al., 2007), the metabolism of PAP-1 is still unclear and none of its metabolites have been identified. In particular it is not clear whether the potentially “toxicophoric” psoralen group of PAP-1 exhibits toxicity *in vivo*. In addition to possibly exhibiting phototoxicity, which has already been demonstrated to not be the case for PAP-1 (Schmitz et al., 2005), the metabolites resulting from initial oxidative metabolism of the furan ring of the psoralen moiety are also potentially toxic and have been shown to be suicide inhibitors of CYP (cytochrome P450) 1A1 and CYP2B1 for some psoralens (Koenigs and Trager, 1998a, 1998b; Baumgart et al., 2005). This is because the furan ring, an important site of metabolic alteration in psoralen derivatives such as 8-methoxypsoralen and 5-methoxypsoralen, can generate an extremely reactive carbonyl via an epoxide. This metabolite is then capable of binding covalently to macromolecules including the metabolizing P450 enzyme and has been suggested to be responsible for the carcinogenicity, mutagenicity and hepatotoxicity observed for some psoralens (da Silva et al., 2009). To gain deeper insight into PAP-1's metabolic fate, we examined the biotransformation of the compound after oral administration to rats. Our preliminary experiments identified more metabolites in bile than in other matrixes such as plasma, urine or feces. The aims of this study were undertaken to 1) isolate and identify major phase-I metabolites of PAP-1 in rat's plasma, urine, feces and particularly in bile; 2) test identified metabolites of PAP-1 on kv1.3 activity; and 3) postulate the metabolic pathways of PAP-1 based on *ex vivo* and *in vitro* liver micorsome incubation results.

Materials and Methods

Chemicals and reagents

PAP-1 was synthesized at the Department of Pharmacology at the University of California Davis (Davis, CA, USA) as previously described (Schmitz et al., 2005). Glucose 6-phosphate, NADP, glucose 6-phosphate dehydrogenase, alpha-naphthoflavone, diethyldithiocarbamate, quinidine and sulfaphenazole were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ketoconazole was provided by Nanjing Baijingu Pharmaceutical Factory (Nanjing, China). Other chemicals were of analytical grade or HPLC grade.

Apparatus

HPLC was carried out on a LC-2010CHT integrated system (Shimadzu, Kyoto, Japan) with a UV-VIS detector. PAP-1 and its metabolites were separated with a reversed-phase column (C₁₈, 4.6 mm × 250 mm I.D., 5 μm, Unimicro, China). The mobile phase consisted of three solvents, A (0.1% formic acid in water), B (acetonitrile) and C (methanol), with the following linear gradients: 0 min, 55% A; 20 min, 5% A; 25 min, 5% A; 26 min, 55% A; 35 min, 55% A; C was maintained at 15% at all times. The flow rate was 0.8 ml/min, the column temperature was set to 35 °C and the absorbance was monitored at 311 nm.

Mass spectrometric analyses were performed using an LCMS-IT-TOF (Shimadzu, Kyoto, Japan) with an ESI source under the following conditions: 0.1% formic acid in water : acetonitrile = 20 : 80; flow rate: 0.1 ml/min; ESI in positive scan mode; nebulizing gas: 1.5 l/min; CDL temperature: 200 °C; interface temperature: 200 °C; ion accumulation time: 30 ms; MS scan range: 100 – 500 m/z; detector voltage: 1.7 kV. Data acquisition and processing were carried out with the LCMSsolution software (version 3.50).

¹H-NMR spectra were obtained in deuteromethanol (CD₃OD) with an Avance III NMR spectrometer (Bruker BioSpin Corp., Billerica, MA, USA) at 400 MHz. The spectrometer was controlled using TopSpin (Bruker BioSpin, v 2.1 pl 5). Each sample was dissolved in 0.5 ml CD₃OD and placed in the magnet at the same depth.

Bile collection

Male Sprague-Dawley rats weighing 200 to 250 g were purchased from Shanghai SLAC Laboratory Animal CO., Ltd. (Shanghai, China). They were acclimatized for at least 1 week with a standard diet *ad libitum* and kept in a controlled 12 h light/dark cycle at 20 ± 2 °C (room temperature) and 60 ± 5% (relative humidity). PAP-1 (50 mg/kg in peanut oil) was orally administered after rats were fasted for 12 hours with access to water. Bile ducts were cannulated (PE-50 tubing) in 10 rats after anesthetization with ether and bile samples were continuously collected for 24 hours. Three rats were placed in metabolic cages to collect samples of urine and feces continuously for 24 hours. 0.2 ml of blood was also collected in 3 anesthetized rats at 6 h after PAP-1 administration from the mesenteric vein and abdominal artery and plasma was obtained by centrifugation. All samples stored at –20 °C before HPLC analysis. The metabolism study was conducted with approval of the Experimental Animal Care and Use Committee of the School of Pharmacy, Shanghai Jiao Tong University, while the toxicity study (see below) was approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Sample treatment

Bile samples (0.5 ml) were mixed with 100 μl of phosphoric acid (0.1 M), 3 ml of acetic ether and 1 ml of n-hexane. After 3 minutes of vortexing the mixture was centrifuged at 12,500 g for 10 minutes at room temperature. The organic phase was removed by

evaporation to dryness under N₂ at 50 °C. The residue was reconstituted in 100 µl methanol and 20 µl of the solution was injected into the HPLC system. Plasma, urine and feces samples were also treated as above except that feces samples were prior extracted with methanol.

Preparation of metabolites by SPE and HPLC

A multiple-step elution procedure was developed by using C18 solid-phase extraction (SPE) columns that would allow us to isolate the metabolites of PAP-1 from bile according to their different lipophilicity (see Fig. 1 for scheme). The SPE procedures were briefly described below. Rat bile samples were thawed in a water bath at room temperature and then centrifuged at 2,000 *g* for 10 minutes. 15 ml aliquots of the resultant supernatant were loaded onto LC-18 cartridges (3 ml/500 mg, SUPELCO, Bellefonte, PA, USA) previously conditioned with 6 ml of methanol and 6 ml of deionized water. Fractions (F-1, F-2, and F-3) were collected after elution with 2 ml of 10%, 50%, and 80% methanol, respectively. The first eluant fraction (F-1) samples were combined, acidified to pH 2.0 with 0.1 M HCl and then applied to a second LC-18 cartridge, which was subsequently washed with 2 ml of 40% methanol prior to elution of the final fraction (F-4) with 2 ml of 60% methanol. F-2 was discarded and both F-3 and F-4 were dried under N₂ at 50 °C, redissolved in methanol and separated by HPLC. The eluants corresponding to each metabolite were collected and combined, then evaporated to dryness and subjected to LCMS-IT-TOF and ¹H-NMR analysis.

Preparation of liver microsomes and incubation with metabolites

Rat liver microsomes were prepared as described previously (Hill, 2003). Briefly, the liver was removed rapidly and perfused to be pale brown with ice-cold homogenizing buffer (0.1 M Tris-HCl, pH 7.4, 10 mM EDTA and 150 mM KCl), cut into pieces, and homogenized in ice-cold homogenizing buffer. Microsomal protein was separated by differential ultracentrifugation. The resulting pellet was resuspended in ice-cold microsome buffer (0.05 M Tris-HCl, pH 7.5, 10 mM EDTA and 20% glycerol) and stored at -80 °C. The content of microsomal protein was determined using a Bradford protein assay kit purchased from Tiangen Biotech Co., Ltd. (Beijing, China).

PAP-1 and its metabolites obtained from the HPLC separation were incubated with an NADPH-regenerating system (3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 1 mM NADP, 3 mM MgCl₂) containing rat liver microsomes (0.2 mg protein/ml) in a final incubation volume of 500 µl. Reactions were initiated by the addition of NADPH-regenerating system after the 5-min preincubation period and were continued at 37 °C for 15 minutes in an Eppendorf Thermomixer Compact. Incubations were terminated by the addition of 100 µl of 0.1 M phosphoric acid and 2 ml of ethyl acetate. Following vortex and centrifugation at 1,250 *g* for 5 minutes, 1.5 ml of the organic layer was dried under a stream of nitrogen gas. The residue was reconstituted in 100 µl of methanol and analyzed by HPLC to identify PAP-1 metabolites.

Effects of CYP isoenzymes on the metabolism of PAP-1 were also evaluated by using five selective inhibitors, including alpha-naphthoflavone for rat CYP1A1/2, quinidine for rat CYP2D6, diethyldithiocarbamate for rat CYP2E, sulfaphenazole for rat CYP2C9 and ketoconazole for rat CYP3A (Daniel et al., 2002; Martignoni and de Kanter, 2006; Erickson et al., 2007). Various concentrations (up to 100 µM) of inhibitors were preincubated with rat liver microsomes for 5 minutes at 37 °C, followed by the addition of PAP-1 (10 µM). The incubation mixtures were prewarmed for 5 minutes before addition of the NADPH regenerating system, incubated for 30 minutes and then terminated as described above. The metabolites in the incubation mixture were determined by using the HPLC system.

Electrophysiology

The effectiveness of the PAP-1 metabolites in blocking Kv1.3 was assayed on L929 cells stably expressing mKv1.3 (Grissmer et al., 1994). Experiments were controlled with a HEKA EPC-10 amplifier and Pulse software (HEKA, Bellmore, NY and Lambrecht, Germany). Patch pipettes were pulled from soda lime glass (2 – 3 M Ω resistance when submerged in the bath solution). All experiments were conducted in the whole-cell configuration of the patch-clamp technique with a holding potential of –90 mV. Currents were recorded in normal Ringer solution (160 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.4, 290 – 310 mOsm) with an internal pipette solution containing 160 mM KF, 2 mM MgCl₂, 10 mM HEPES, 10 mM EGTA (pH 7.2, 290 – 310 mOsm). If currents exceeded 2 nA 60–80% series resistance compensation was used. Depolarizing pulses to 40 mV were applied every 45 s for 500 ms. IC₅₀ values were determined by fitting the reduction of area under the current curve to the Hill equation. Data analysis, fitting and plotting were performed with IGB-Pro (WaveMetrics, Lake Oswego, OR, USA) or Origin 6 software (OriginLab, Northampton, MA, USA).

6-Months repeat-dose toxicity study

Female and male 9–11 weeks old Lewis rats were purchased from Charles River and gavaged daily for 6 months with either 50 mg/kg of PAP-1 (n = 12, 6 male and 6 female rats) or with the vehicle peanut oil (3 ml per kg body weight, n = 12, 6 male and 6 female rats). At the end of the trial, rats were sacrificed and complete blood chemistry, hematology and necropsy performed by the Comparative Pathology Laboratory of University of California, Davis. Briefly, whole animal weight (to mg) was determined using an Ohaus analytical scale. The rats were then euthanized with CO₂, and blood collected via cardiac puncture. A 100 ml sample was placed into an EDTA coated samplette tube for determination of the hematological profile, while the rest of the blood was placed in a serum separator samplette tube and separated by centrifugation at 3,500 rpm for 10 minutes. The hematological profile was performed with a Drew Mascot 850 automated cell counter and included: RBC count, mean corpuscular Hb, WBC count, mean corpuscular volume, platelet count, reticulocyte count, hematocrit, WBC differential and Hb concentration. Blood cell morphology was evaluated manually by microscopy. Serum chemistry was performed on a Cobas Mira Plus chemistry analyzer and included alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, calcium, phosphorus, blood urea nitrogen, creatinine, glucose, total protein, albumin, total bilirubin, cholesterol, and triglycerides. For necropsies spleen, thymus, liver, and each kidney were dissected and weighed (to mg) using an Ohaus analytical scale. Pelage and cecal/intestinal contents were sampled and examined microscopically for parasites. Cultures were taken from the nasopharynx and the cecal wall for presence of aerobic bacteria including potential opportunistic pathogens. Liver, both kidneys, spleen, GI tract, pancreas, lung, heart, brain, bone marrow, proximal and distal lymph nodes, thymus, and gonads were collected and fixed in 10% buffered formalin. Tissues were sectioned and processed to paraffin blocks using routine methods. Hematoxylin and eosin stained sections from each block were examined for histologic changes by a trained pathologist. Serology was performed by ELISA testing for the following infectious agents: Rat Corona Virus, Sialodacryoadenitis Virus, Sendai Virus, Pneumonia Virus of Mice, Kilham Rat Virus, H1 Virus, Rat Parvovirus, Mycoplasma pulmonis, Reovirus-3, Lymphocytic Choriomeningitis Virus, Theiler's Virus (GDVII), CAR-bacillus, and Hantaan virus.

Statistics

Data are expressed as means $\pm$ standard deviation and statistical significance is determined as $P < 0.05$ by using unpaired and two-tailed student t-test.

Results

HPLC analysis of PAP-1 and its metabolites in bile, plasma, urine and feces samples

Bile samples were continuously collected for 24 hours following oral administration of PAP-1 (50 mg/kg), extracted with organic solvents as described in the Methods and analyzed by HPLC. PAP-1 and five phase-I metabolites (M1–M5) were found in bile (Fig. 2A). PAP-1 eluted at 25.6 min, while the more polar metabolites eluted at 10.7 min (M1), 11.1 min (M2), 18.5 min (M3), 20.0 min (M4) and 20.7 min (M5), respectively. PAP-1 and all five metabolites were also present in feces (Fig. 2B), but in contrast to bile, where the major metabolite was M1, the most abundant metabolite in feces was M3. On the other hand, only PAP-1 and M1 could be detected in abdominal arterial plasma or mesenteric venous plasma and M1 alone was detectable in urine samples (Fig. 2C, 2D).

Preparation and identification of biliary metabolites of PAP-1

We next wanted to determine the structures of the five metabolites from SPE procedures (see Fig. 1). We found that the pH of the sample solutions significantly affected the retention of M1 on SPE cartridges. Under the basic conditions of the original bile samples, the carboxylic acid function of the M1 molecule would exist almost exclusively in the ionized form, while the lipophilicity of the psoralen group still ensured that M1 was retained on the SPE cartridge and then was easily eluted with 10% methanol. When the elution was acidified to pH 2.5, M1 was eluted by 60% methanol due to its stronger retention. Thus with the help of SPE M1 could be isolated from bile with a purity of above 90% yielding 15 mg from approximately 350 ml of bile. The other four PAP-1 metabolites in bile were also prepared by using SPE and HPLC as described in the Method: M2 (8 mg), M3 (5 mg), M4 (2 mg), and M5 (3 mg). M1, M2, M3 and M4 were amorphous white powders while M5 was an amorphous grayish-white powder.

We then further determined structures of the isolated metabolites by mass spectrometry and $^1\text{H-NMR}$. The $^1\text{H-NMR}$ of PAP-1 exhibits very characteristic proton signals from the 5-substituted psoralen moiety: two coupled protons from the coumarin ring double bond at δ 8.30 (d, 1H, $^3J = 9.8$ Hz, 3-H) and 6.29 (d, 1H, $^3J = 9.8$ Hz, 4-H), an aromatic singlet at 7.18 (s, 1H, 8-H), and two coupled protons from the furan ring at δ 7.80 (d, 1H, $^3J = 2.4$ Hz, 5'-H) and 7.20 (d, 1H, $^3J = 2.4$ Hz, 4'-H)] (Table 1).

Metabolite M1, 5-(oxybutyric-acid)psoralen—The mass spectrum of M1 exhibited a pseudomolecular ion $[\text{M}+\text{H}]^+$ at m/z 289.0706, compatible with the molecular formula $\text{C}_{15}\text{H}_{12}\text{O}_6$ (calculated molecular weight: 289.0707) (Fig. 3A). In the MS^2 spectrum, the major product ion at m/z 203.0335 presented 5-hydroxypsoralen formed by the cleavage of the ether bond between the psoralen ring and the butyric acid side chain. The $^1\text{H-NMR}$ of M1 exhibited the same characteristic proton signals for a psoralen system as PAP-1 and a set of protons on the butanoic acid chain [δ 4.59 (t, 2H, $^3J = 6.16$ Hz, 5- $\text{OCH}_2\text{CH}_2\text{CH}_2\text{COOH}$), 2.60 (t, 2H, $^3J = 7.16$ Hz, 5- $\text{OCH}_2\text{CH}_2\text{CH}_2\text{COOH}$), 2.18 (m, 2H, 5- $\text{OCH}_2\text{CH}_2\text{CH}_2\text{COOH}$)] (Table 1). Comparison of the $^1\text{H-NMR}$ data of M1 with those of PAP-1 further showed that M1 was missing the side-chain phenyl ring. On the basis of the above evidence, the structure of M1 was elucidated to be 4-[(7-oxo-7H-furo[3, 2-g]chromen-4-yl)oxy]butanoic acid, a compound that had been previously reported and synthesized as an intermediate product (Ropp et al., 2004).

Metabolite M2, 5-[4-(4-hydroxybutoxy)]psoralen—The positive mass spectrum showed a pseudomolecular ion peaks $[\text{M}+\text{H}]^+$ at m/z 275.0917, compatible with the molecular formula $\text{C}_{15}\text{H}_{14}\text{O}_5$ (calculated molecular weight: 275.0914) (Fig. 3B). The molecular ion of M2 could lead to a main MS^2 ion at m/z 203.0329. $^1\text{H-NMR}$: δ [ppm]

=8.30 (d, 1H, $^3J = 9.8$ Hz, 3-H), 7.80 (d, 1H, $^3J = 2.4$ Hz, 5'-H), 7.19 (d, 1H, $^3J = 2.4$ Hz, 4'-H), 7.18 (s, 1H, 8-H), 6.28 (d, 1H, $^3J = 9.8$ Hz, 4-H), 4.57 (t, 2H, $^3J = 6.50$ Hz, 5-OCH₂CH₂CH₂CH₂OH), 3.65 (t, 2H, $^3J = 6.44$ Hz, 5-OCH₂CH₂CH₂CH₂OH), 2.18 (m, 4H, 5-OCH₂CH₂CH₂CH₂OH) (Table 1). Comparison of the ¹H-NMR data of M2 with those of M1 suggested that they were identical except that M2 had a downfield shift (δ 3.65), which represented the 4''-H of the hydroxybutoxy group. Based on analysis of the ¹H-NMR spectral data, the structure of M2 was deduced as 4-(4-hydroxybutoxy)-7*H*-furo[3,2-*g*]chromen-7-one. This compound was not reported previously.

Metabolite M3, 5-[4-(4-hydroxyphenoxy)]psoralen—The MS¹ spectrum of M3 showed a pseudomolecular ion [M+H]⁺ at *m/z* 367.1176, compatible with the molecular formula C₂₁H₁₈O₆ (calculated molecular weight: 367.1176) (Fig. 3C). M3 had 15.9949 mass units more than PAP-1, suggesting that it was a hydroxylated product of PAP-1. In the MS² spectrum, fragment ions appeared at *m/z* 203.0335 and 165.0915, which indicated a hydroxy group located on the side chain (149 + 16). ¹H-NMR: δ [ppm] = 8.23 (d, 1H, $^3J = 9.7$ Hz, 3-H), 7.76 (d, 1H, $^3J = 2.3$ Hz, 5'-H), 7.18 (d, 1H, $^3J = 2.3$ Hz, 4'-H), 7.16 (s, 1H, 8-H), 6.70 (m, 4H, $^3J = 8.1$ Hz, 5-OCH₂CH₂CH₂CH₂OC₆H₄OH), 6.23 (d, 1H, $J = 9.8$ Hz, 4-H), 4.62 (t, 2H, $^3J = 5.99$ Hz, 5-OCH₂CH₂CH₂CH₂OC₆H₄OH), 4.01 (t, 2H, $^3J = 6.00$ Hz, 5-OCH₂CH₂CH₂CH₂OC₆H₄OH), 2.18 (m, 4H, 5-OCH₂CH₂CH₂CH₂OC₆H₄OH) (Table 1). A symmetrical quartet at δ 6.70 in the ¹H-NMR spectrum of M3 suggested that the hydrogen atom in para-position of the phenyl ring had been replaced by a hydroxyl group. Thus, the structure of compound M3 was elucidated to be 4-[4-(4-hydroxyphenoxy)butoxy]-7*H*-furo[3,2-*g*]chromen-7-one). This compound was not reported previously.

Metabolite M4, 5-[4-(3-hydroxyphenoxy)]psoralen—The MS¹ spectrum showed a pseudomolecular ion [M+H]⁺ at *m/z* 367.1173, compatible with the molecular formula C₂₁H₁₈O₆ (calculated molecular weight: 367.1176) (Fig. 3D). Both MS¹ and MS² spectra were the same as those of M3, suggesting that M4 was also a hydroxylated product of PAP-1. ¹H-NMR: δ [ppm] = 8.30 (d, 1H, $^3J = 9.7$ Hz, 3-H), 7.80 (d, 1H, $^3J = 2.2$ Hz, 5'-H), 7.19 (d, 1H, $^3J = 2.2$ Hz, 4'-H), 7.16 (s, 1H, 8-H), 6.91 (m, 1H, 5''-H), 6.76 (m, 3H, 2'', 4'', 6''-H), 6.22 (d, 1H, $^3J = 9.7$ Hz, 4-H), 4.63 (t, 2H, $^3J = 5.98$ Hz, 5-OCH₂CH₂CH₂CH₂OC₆H₄OH), 4.14 (t, 2H, $^3J = 5.98$ Hz, 5-OCH₂CH₂CH₂CH₂OC₆H₄OH), 2.18 (m, 4H, 5-OCH₂CH₂CH₂CH₂OC₆H₄OH) (Table 1). The ¹H-NMR data of M4 was similar to M3, except for the multiplet at δ 6.76 (3H, 2'', 4'', 6''-H), which showed that the hydroxyl group was linked in the meta position of the phenyl ring. Based on the spectral evidence, the structure of compound M4 was elucidated as 4-[4-(3-hydroxyphenoxy)butoxy]-7*H*-furo[3,2-*g*]chromen-7-one, which was not reported previously.

Metabolite M5, 8-hydroxyl-5-(4-phenoxybutoxy)psoralen—Its spectrum presented a pseudomolecular ion [M+H]⁺ at *m/z* 367.1175, compatible with the molecular formula C₂₁H₁₈O₆ (calculated molecular weight: 367.1176) (Fig. 3E). The molecular formula of M5 was 15.9948 mass units higher than that of PAP-1, suggesting that M5 was a hydroxylated product of PAP-1. In the MS² spectrum, two fragment ions appeared at *m/z* 219.0283 and 149.0980, which indicated that hydroxylation had occurred on the psoralen ring (203+16). ¹H-NMR showed: δ [ppm] = 8.23 (d, 1H, $^3J = 9.8$ Hz, 3-H), 7.78 (d, 1H, $^3J = 2.3$ Hz, 5'-H), 7.23 (m, 2H, 3'', 5''-H), 7.09 (d, 1H, $^3J = 2.3$ Hz, 4'-H), 6.89 (m, 3H, 2'', 4'', 6''-H), 6.24 (d, 1H, $^3J = 9.8$ Hz, 4-H), 4.41 (t, 2H, $^3J = 5.60$ Hz, 5-OCH₂CH₂CH₂CH₂OC₆H₅), 4.08 (t, 2H, $^3J = 6.00$ Hz, 5-OCH₂CH₂CH₂CH₂OC₆H₅), 2.18 (m, 4H, 5-OCH₂CH₂CH₂CH₂OC₆H₅) (Table 1). The ¹H-NMR data of M5 were similar to PAP-1, except for the absence of 8-H on the psoralen ring. From spectral evidence, the structure of

compound M5 was elucidated to be 9-hydroxy-4-(4-phenoxybutoxy)-7*H*-furo[3,2-*g*]chromen-7-one. This compound was not reported previously.

Identification of PAP-1 metabolites in the rat microsomes

Since incubation with liver microsomes could potentially offer additional valuable information to interpret the relationship among the above metabolites and reveal the metabolic pathway of PAP-1, we incubated PAP-1 with rat liver microsomes in the presence of NADPH. PAP-1 was rapidly metabolized and the above-described five metabolites (M1-M5) were detected with HPLC in contrast to control incubations without NADPH. Under the same incubation conditions with rat microsomes, M1 was not metabolized but M2, M3, M4 and M5 were further metabolized. While only one metabolite (M1) was detected following the incubation of M2 with rat liver microsomes, two metabolites (M1 and M2) appeared in the incubation of both M3 and M4. M5 was then probably metabolized to an unknown product, which was not detected in bile samples due to its low abundance. These results are shown in Fig. 4.

Thus we here identified five major phase-I metabolites (M1-M5) from the novel experimental immunosuppressant PAP-1, which were generated in a NADPH-dependent manner suggesting that CYP enzymes play a major role in PAP-1 metabolism. Three of the metabolites, 5-[4-(4-hydroxyphenoxy)]psoralen (M3), 5-[4-(3-hydroxyphenoxy)]psoralen (M4) and 8-hydroxyl-5-(4-phenoxybutoxy)psoralen (M5) were hydroxylated and had the same molecular mass. However, no 5-hydroxypsoralen from dealkylation of the complete side-chain was detected in any samples as no peaks were found in the extracted ion chromatograms of LCMS-IT-TOF with $[M+H]^+$ at m/z 203.0339 for 5-hydroxypsoralen, m/z 167.1067 for 4-phenoxybutan-1-ol, or m/z 183.1016 for 3-(4-hydroxybutoxy)phenol.

Involvement of CYP isoenzymes in the metabolism of PAP-1

In order to identify the CYP isoenzymes principally responsible for the metabolism of PAP-1 in rat liver microsomal preparations we used five selective inhibitors of rat CYP1A1/2, 2D6, 2E, 2C9 and 3A as specific probes (Daniel et al., 2002; Martignoni M, 2006; Erickson et al., 2007) since CYP1A1/2, CYP2E, 2D6 and 3A have been reported to be important CYP isoenzymes in rats (Elsherbiny et al., 2008). Following incubation of PAP-1 (10 μ M) with liver microsomes (quadruplicates) we determined the concentrations of the five metabolites (M1 - M5) and plotted them as percentage of corresponding control (area under peak). The CYP1A1/2 inhibitor α -naphthoflavone (10 – 100 μ M) concentration-dependently decreased productions of M1 (with maximum inhibition of 92%), M4 (90%), M2 (60%) and M5 (48%), respectively but not M3 (Fig. 5A), while the CYP3A inhibitor ketoconazole (0.5 – 100 μ M) also concentration-dependently decreased productions of M1 (96%), M2 (82%), M3 (69%) and M5 (83%), respectively but not M4 (Fig. 5B). On the other hand, the CYP2D6 inhibitor quinidine (1 – 50 μ M), the CYP2E inhibitor diethyldithiocarbamate (1 – 50 μ M) and the CYP2C9 inhibitor sulphaphenazole (5 – 100 μ M) failed to significantly affect the metabolism of PAP-1 (Fig. 5C – 5E). These results suggest that CYP1A1/2 and CYP3A but not CYP2D6, CYP2E or CYP2C9 are principally involved in hydroxylation and O-dealkylation of PAP-1.

Activity of the metabolites on Kv1.3

In order to determine if any of the identified PAP-1 metabolites are still active on Kv1.3 we next tested their effect on Kv1.3 currents by whole-cell patch-clamp (Fig. 6). While the O-dealkylated metabolites M1 and M2 exhibited no effect (M1) (Fig. 6A) or only showed weak blocking activity (M2, 40% block, data not shown) at concentrations of 25 μ M, the hydroxylated metabolites M3, M4 and M5 potently blocked Kv1.3 in a reversible fashion (Fig. 6B), with IC_{50} s of 6.5 nM, 139 nM and 159 nM (Fig. 6C). Similar to the parent

compound PAP-1 the active metabolites exhibited Hill coefficients of 2 suggesting that 2 or more molecules interact with one Kv1.3 channel (Schmitz et al., 2005; Zimin et al., 2010).

PAP-1 exhibits no toxicity in a 6-months repeat dose study

Since treating autoimmune disease often requires long-term to life-long treatment we here also evaluated the long-term toxicity profile of Kv1.3 inhibition with PAP-1 at a therapeutic dose, which had previously been shown to prevent type-1 diabetes development (Beeton et al., 2006) and to suppress allergic contact dermatitis in rats (Azam et al., 2007). Following administration of PAP-1 for 6 months at 50 mg/kg by daily gavage to 6 male and 6 female Lewis rats no significant changes in hematology or blood chemistry were observed (Table 2), except a small increase in cholesterol in PAP-1 treated females (from 103 ± 10 mg/dl to 123 ± 16 mg/dl), and a small decrease of blood glucose in PAP-1 treated males (from 175 ± 19 mg/dl to 147 ± 19 mg/dl), which probably was rather due to the slightly different timing of sacrifice after the last food consumption than drug related. With respect to potential liver toxicity, it is noteworthy that long-term PAP-1 treatment did not result in elevations of aspartate aminotransferase, alanine aminotransferase or alkaline phosphatase.

PAP-1 treatment also did not result in any changes in body or organ weights or histopathology of any of the major organs such as liver, kidneys, pancreas, lung, heart, brain, bone marrow, and gonads. Long-term Kv1.3 blockade with PAP-1 further did not increase susceptibility to environmental pathogens as determined by the results a serology panel testing for Rat Corona Virus, Sialodacryoadenitis Virus, Sendai Virus, Pneumonia Virus of Mice, Kilham Rat Virus, H1 Virus, Rat Parvovirus, Mycoplasma pulmonis, Reovirus-3, Lymphocytic Choriomeningitis Virus, Theiler's Virus (GDVII), CAR- bacillus, and Hantaan virus.

Since we previously determined pharmacokinetics for PAP-1 in both rats and rhesus macaques and had observed that following oral application at 50 mg/kg PAP-1 plasma values rise to a peak of 6 – 10 μ M between 6 and 8 hours after application and then fall to 200 – 500 nM at 24 hours (Beeton et al., 2006), we also measured PAP-1 plasma trough concentrations at 24 hours throughout the toxicity study. On day-28 plasma trough concentrations determined by HPLC/MS were 310 ± 90 nM, at 3 months 350 ± 78 nM and at 6 months 290 ± 120 nM, demonstrating that PAP-1 does not seem to induce its metabolizing enzymes (presumably cytochrome P450 enzymes) and that it does not accumulate over time in keeping with its previously published two compartment model pharmacokinetics (Beeton et al., 2006; Azam et al., 2007).

Discussion

Previous studies demonstrate a relatively low bioavailability (10 – 20%) for PAP-1 in rats after oral administration although PAP-1 showed good absorption through the gastrointestinal tract in rhesus macaques (Schmitz et al., 2005; Pereira et al., 2007) and is predicted by QikProp® software to have an oral availability of 100% in humans. The observed low bioavailability of PAP-1 in rats was presumed to be mainly due to a large hepatic first-pass effect. When PAP-1 was orally administered to rats, PAP-1 and five phase-I metabolites (M1-M5) were structurally identified from bile. M1 was a carboxylic acid metabolite, while M2, M3, M4 and M5 were hydroxylated metabolites. While the metabolite M1 was reported in a previous study as an intermediate in the synthesis of α -methylene- γ -butyrolactone-psoralen (Ropp et al., 2004), the other four metabolites identified here are new chemical entities, i.e., 5-[4-(4-hydroxybutoxy)]psoralen (M2), 5-[4-(4-hydroxyphenoxy)]psoralen (M3), 5-[4-(3-hydroxyphenoxy)]psoralen (M4) and 8-hydroxyl-5-(4-phenoxybutoxy)psoralen (M5).

5-Methoxypsoralen (a.k.a. bergapten) and 8-methoxypsoralen (a.k.a. xanthotoxin), two compounds used for photo-therapy of psoriasis and naturally occurring in *Rutacea* plants such as citrus, are metabolized in rats by hydrolysis of the lactone ring and oxidation of the furan ring (Mays et al., 1986; John et al., 1992). Other studies further suggested that epoxidation of the furan ring of psoralens generated reactive intermediates responsible for the toxicity of psoralen compounds such as inactivation of CYP 2B1 and 2A6 (Koenigs and Trager, 1998a, 1998b; Caffieri, 2002). In contrast, PAP-1 maintained its intact psoralen structure during biotransformation based on the present study, and the major metabolite M1 was a polar carboxylic acid, which facilitated excretion without further metabolism, suggesting that PAP-1 should exhibit much less toxicity than other psoralen. In keeping with this suggestion, long-term administration of PAP-1 did not induce any changes in plasma liver enzymes and or liver histopathology. Since the psoralen core stayed intact during metabolism we thought it likely that several of the metabolites might still be active Kv1.3 blockers. In keeping with the SAR of phenoxyalkoxypsoralen-type Kv1.3 blockers (Schmitz et al., 2005) and the recently published binding-site model of PAP-1 (Zimin et al., 2010), the O-dealkylated M1 and M2 were found to be inactive. This observation was not surprising since the phenoxybutoxy side-chain of PAP-1 inserts into the lipophilic interphase between transmembrane segments S5 and S6 and the lack of the phenyl ring as well as the polarity of the carboxylic acid function of M1 would disrupt these hydrophobic interactions. The hydroxylated metabolites M3, M4 and M5 in contrast exhibited nanomolar potencies. The finding that the para-substituted M3 (IC₅₀ 7 nM) was more potent than the meta-substituted M4 (IC₅₀ 140 nM) was again confirmatory of the previously studied SAR of PAP-1 derivatives substituted on the side-chain phenyl ring (Schmitz et al., 2005).

The parent drug PAP-1 and its five metabolites (M1-M5) were found in bile and feces samples, while only PAP-1 and metabolite M1 were detected in plasma derived from the mesenteric vein and abdominal artery and metabolite M1 alone was present in urine. No 5-hydroxypsoralen from dealkylation of the complete side-chain was detected in any samples. These results suggest that following absorption from the intestine PAP-1 was metabolized extensively in the liver, followed by two possible routes: 1) the five metabolites M1 - M5 were excreted across the canalicular membrane into bile; and 2) M1 was transported across the sinusoidal membrane of hepatocytes into the circulation. We hypothesize that the metabolites in the bile were excreted into the duodenum and most of M1 was reabsorbed later on. This is due to the facts that M1 was present in the mesenteric venous blood in high concentrations (similar to the bile) but little in feces, and that no M1 was detected following the incubation of PAP-1 with intestinal microsomes (data not shown). After effective absorption into the systemic circulation, M1 was predominantly eliminated by the kidneys. The other metabolites (M2 - M5) in contrast do not seem to have been reabsorbed in the intestine and were directly excreted through feces because they were not detectable in plasma from the mesenteric vein but appeared in large amounts in the feces. Based on these observations we propose that M1 is the major circulating metabolite of PAP-1 and its biological activity as well as toxicity should be a focus for further study although it was demonstrated to be ineffective on Kv1.3 and therefore likely devoid of any immunosuppressive activity. In addition, all five metabolites with hydroxyl groups may go through phase-II conjugation reactions with glucuronic acid or sulfuric acid. The significantly higher concentrations of M3, M4 and M5 in feces than in bile suggest that they were likely released from the hydrolysis of phase-II metabolites. The fact that the Kv1.3-active metabolites M3, M4 and M5 could not be detected in plasma further suggests that they do not contribute to the immunosuppressive activity of PAP-1.

The metabolic stability of PAP-1 and its five isolated metabolites (M1 - M5) was determined *in vitro* with liver microsomes. After incubation of PAP-1 in rat liver microsomes in the presence of NADPH, five metabolites were observed, in contrast to the

negative results in the control incubations without NADPH, indicating the formation of these metabolites was enzymatic and NADPH-dependent. The *in vitro* metabolites were the same five metabolites that we had found in bile following *in vivo* metabolism. Furthermore, M2 was converted to M1 in microsomal incubations, while both M3 and M4 were transformed to M2 and M1, suggesting that O-dealkylation is the major metabolic reaction of PAP-1 in the liver. Neither M2 nor M1 were detected in M5 incubations, confirming what we had observed during the NMR experiments, namely that the hydroxyl group of M5 was not located on the side-chain phenyl ring as in M3 and M4, but instead on the psoralen ring system. M1 and M2 could be formed from PAP-1, M3 and M4 through O-dealkylation and formation of the corresponding aldehydes that were immediately reduced to the alcohol M2 or oxidized to the acid M1. Thus hydroxylation and O-dealkylation would be the major metabolic pathways of PAP-1, and O-dealkylation would probably be the major pathway for inactivation of PAP-1 on Kv1.3, as shown in Fig. 7.

In the CYP isoenzyme inhibition studies, the selective rat CYP1A1/2 inhibitor alpha-naphthoflavone and rat CYP3A inhibitor ketoconazole at 30 μ M considered to be enzyme-selective (Baririan et al., 2006) significantly inhibited the formation of M1 and M2, suggesting that both CYP1A1/2 and CYP3A mediating the O-dealkylation of PAP-1. The involvements of CYP1A1/2 and CYP3A in O-dealkylation were also demonstrated by application of alpha-naphthoflavone and ketoconazole (Monostory et al., 2003; Baririan et al., 2006). In addition, the formation of mono-hydroxylated PAP-1 analogs was also inhibited by these two inhibitors while the CYP2D6 inhibitor quinidine, the CYP2E inhibitor diethyldithiocarbamate and the CYP2C9 inhibitor sulphaphenazole up to 50 or 100 μ M did not affect either O-dealkylation or hydroxylation of PAP-1. These results suggest that CYP1A1/2 and CYP3A appear to be the major CYP450 isoenzymes involved in the metabolism of PAP-1 via aromatic O-dealkylation and hydroxylation, which are also presented in Fig. 7. More studies are warranted for incubation of PAP-1 with rat (and human) pure CYP isoenzymes and *in vivo* inhibition of the metabolism of PAP-1.

In conclusion, our results show that hydroxylation and O-dealkylation principally via CYP1A1/2 and CYP3A are the major metabolic pathways for the experimental immunosuppressant PAP-1 in rats both in liver microsomes and *in vivo*. The five phase-I metabolites include 5-(oxybutyric-acid)psoralen (M1), 5-[4-(4-hydroxybutoxy)]psoralen (M2), 5-[4-(4-hydroxyphenoxy)]psoralen (M3), 5-[4-(3-hydroxyphenoxy)]psoralen (M4), and 8-hydroxyl-5-(4-phenoxybutoxy)psoralen (M5) with reduced Kv1.3 activity, and M1 being the major metabolite with entirely lost Kv1.3 activity. Our results further demonstrate that long-term administration of PAP-1 does not produce any significant changes in hematology, blood chemistry, histology of any major organ, or speeding up of metabolism indication enzyme (presumably cytochrome P450 enzymes) induction.

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ABBREVIATIONS

PAP-1 5-(4-phenoxybutoxy)psoralen

HPLC	high-performance liquid chromatography
ESI	electrospray ionization
MS	mass spectrometry
Kv	voltage-gated K ⁺ channel
SPE	Solid-phase extraction
CYP	cytochrome P450
ACD	allergic contact dermatitis

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Fig. 1. Solid-phase extraction (SPE) separation scheme for rat bile. Bile samples were loaded onto LC-18 cartridges. Three fractions (F-1, F-2 and F-3) were collected after elution with 10%, 50%, 80% methanol, respectively. F-1 of eluants were combined, acidified with 0.1 M HCl and then applied to a second LC-18 cartridge. After washing with 40% methanol, F-4 was eluted with 60% methanol.

Fig. 2.

Representative HPLC chromatogram of 5-(4-phenoxybutoxy)psoralen (PAP-1) and its metabolites in rats following oral administration at 50 mg/kg. **A:** PAP-1 and its five major metabolites (M1 - M5) in the rat bile continuously collected for 24 hrs after dosing. PAP-1 was eluted at 25.6 min, M1 at 10.7 min, M2 at 11.1 min, M3 at 18.5 min, M4 at 20.0 min and M5 at 20.7 min, respectively. **B:** M1, M2, M3, M4 and M5 in the feces collected from 0 to 24 h post-dosing. **C:** PAP-1 and M1 in the plasma from the mesenteric vein and abdominal arterial at 6 h after oral administration. **D:** M1 in the urine continuously for 24 hrs after dosing.

Fig. 3. MS¹ and MS² spectra of metabolites of 5-(4-phenoxybutoxy)psoralen (PAP-1) and proposed fragmentation pathways. The cleavage of the ether bond between the psoralen moiety and the butyl side chain was the main fragmentation pathway for each metabolite. **A:** M1; **B:** M2; **C:** M3; **D:** M4; **E:** M5.

Fig. 4. HPLC chromatograms of 5-(4-phenoxybutoxy)psoralen (PAP-1) and its metabolites (M1 - M5) incubated with the rat liver microsomes. **A:** in the presence of NADPH; **B:** in the absence of NADPH; **C – G:** M1 - M5 in the presence of NADPH.

Fig. 5.

Effects of selective rat cytochrome P450 isoenzyme inhibitors on the metabolism of 5-(4-phenoxybutoxy)psoralen (PAP-1, 10 μ M) in rat liver microsomes ($n = 4$ in each point). Values are given as means $\pm$ standard deviation. **A:** alpha-naphthoflavone for CYP1A1/2 isoform, **B:** quinidine for CYP2D6, **C:** ketoconazole for CYP3A, **D:** diethyldithiocarbamate for CYP2E isoform, and **E:** sulphaphenazole for CYP2C9 isoform.

Fig. 6.

Kv1.3 blocking activity of the metabolites M1-M5 of 5-(4-phenoxybutoxy)psoralen (PAP-1). **A:** M1 (25 μ M) exerts no effect on Kv1.3 currents while subsequently applied PAP-1 potently blocks the current. **B:** Increasing concentrations of M3 reversibly block Kv1.3. **C:** Dose-concentration curves of PAP-1 (IC_{50} 2.0 ± 0.2 nM, $n_H = 1.8$), M3 (IC_{50} 6.5 ± 0.9 nM, $n_H = 1.6$), M4 (IC_{50} 139 ± 10 nM, $n_H = 2.0$) and M5 (IC_{50} 159 ± 15 nM, $n_H = 1.8$). Each data point is the mean $\pm$ SD from 2 – 3 measurements.

Fig. 7.
Proposed metabolic pathways of 5-(4-phenoxybutoxy)psoralen (PAP-1) in rats.

Table 1

¹H-NMR data for metabolites M1–M5 in rats (δ in ppm, J in Hz). All spectra were recorded on an Avance III NMR spectrometer at 400 MHz, in CD₃OD.

no.	M1	M2	M3	M4	M5	PAP-1
psoralen						
3	8.30 (<i>d</i> , 9.8)	8.30 (<i>d</i> , 9.8)	8.23 (<i>d</i> , 9.7)	8.30 (<i>d</i> , 9.7)	8.23 (<i>d</i> , 9.8)	8.22 (<i>d</i> , 9.8)
4	6.29 (<i>d</i> , 9.8)	6.28 (<i>d</i> , 9.8)	6.23 (<i>d</i> , 9.8)	6.22 (<i>d</i> , 9.7)	6.24 (<i>d</i> , 9.8)	6.22(<i>d</i> , 9.8)
8	7.18 (<i>s</i>)	7.18 (<i>s</i>)	7.16 (<i>s</i>)	7.16 (<i>s</i>)		7.15 (<i>s</i>)
4'	7.20 (<i>d</i> , 2.4)	7.19 (<i>d</i> , 2.4)	7.18 (<i>d</i> , 2.3)	7.19 (<i>d</i> , 2.2)	7.09 (<i>d</i> , 2.3)	6.90 (<i>d</i> , 2.3)
5'	7.80 (<i>d</i> , 2.4)	7.80 (<i>d</i> , 2.4)	7.76 (<i>d</i> , 2.3)	7.80 (<i>d</i> , 2.2)	7.78 (<i>d</i> , 2.3)	7.76 (<i>d</i> , 2.3)
alkene						
5-OCH ₂	4.59 (<i>t</i> , 6.16)	4.57 (<i>t</i> , 6.50)	4.62 (<i>t</i> , 5.99)	4.63 (<i>t</i> , 5.98)	4.41 (<i>t</i> , 5.60)	4.62 (<i>t</i> , 5.98)
5-OCH ₂ CH ₂ CH ₂		2.18 (<i>m</i>)	2.18 (<i>m</i>)	2.18 (<i>m</i>)	2.18 (<i>m</i>)	2.18 (<i>m</i>)
5-OCH ₂ CH ₂ CH ₂ CH ₂		3.65 (<i>t</i> , 6.44)	4.01 (<i>t</i> , 6.00)	4.14 (<i>t</i> , 5.98)	4.08 (<i>t</i> , 6.00)	4.09 (<i>t</i> , 6.00)
5-OCH ₂ CH ₂ CH ₂ COOH	2.18 (<i>m</i>)					
5-OCH ₂ CH ₂ CH ₂ COOH	2.6 (<i>t</i> , 7.16)					
aromatic ring						
2'',3'',5'',6''			6.70 (<i>m</i>)			
2'',4'',6''				6.76 (<i>m</i>)	6.89 (<i>m</i>)	
5''				6.91 (<i>m</i>)		
3'',5''					7.23 (<i>m</i>)	
2'',3'',4'',5'',6''						7.20(<i>m</i>)

Notes: s: singlet; d: doublet; t: triplet; m: multiplet.

Table 2

Hematology, blood chemistry, and histopathology from rats administered PAP-1 at 50 mg/kg by gavage for 6 months. Values are given as means $\pm$ standard deviation (* $p < 0.05$ by Student t-test).

	Females, vehicle (n=6)	Females, PAP-1 (n=6)	Males, vehicle (n=6)	Males, PAP-1 (n=6)
Hematology				
WBC (K/ul)	6.3 $\pm$ 0.5	5.6 $\pm$ 0.8	8.4 $\pm$ 1.3	8.2 $\pm$ 1.2
Neutrophils %	30.2 $\pm$ 7.8	35 $\pm$ 6.3	25.7 $\pm$ 5.6	22.7 $\pm$ 4.5
Lymphocytes %	59.5 $\pm$ 10.1	54.2 $\pm$ 7.2	64 $\pm$ 4.2	6.8 $\pm$ 4.1
Monocytes %	9.8 $\pm$ 4.8	10.1 $\pm$ 4.1	9.8 $\pm$ 2.9	9.2 $\pm$ 2.6
Eosinophils %	0.3 $\pm$ 0.5	0.7 $\pm$ 0.8	0.5	0.3 $\pm$ 0.5
Basophils %	0	0	0	0
Reticulocytes %	1.7 $\pm$ 0.2	1.5 $\pm$ 0.2	1.5 $\pm$ 0.5	1.5 $\pm$ 0.4
RBC (MM)	6.9 $\pm$ 0.3	6.9 $\pm$ 0.4	7.6 $\pm$ 0.7	7.5 $\pm$ 0.4
Hemoglobin (g/dl)	15 $\pm$ 0.8	14 $\pm$ 1.0	15.9 $\pm$ 1.7	14.7 $\pm$ 0.4
Hematocrit %	32.6 $\pm$ 2.5	30.1 $\pm$ 2.0	33.3 $\pm$ 3.3	30.9 $\pm$ 1.6
MCV (fl)	46.7 $\pm$ 1.6	44.3 $\pm$ 1.9	43.7 $\pm$ 1.3	41.2 $\pm$ 1.4
MCH (pg)	21.5 $\pm$ 1.0	20.6 $\pm$ 0.8	21.1 $\pm$ 0.9	19.6 $\pm$ 1.7
MCHC (g/dl)	46 $\pm$ 2.0	46.4 $\pm$ 1.6	48.4 $\pm$ 1.0	47.6 $\pm$ 1.7
Platelets (K/ul)	674 $\pm$ 54	620 $\pm$ 71	677 $\pm$ 173	736 $\pm$ 17
Blood Chemistry				
Aspartate Aminotransferase (U/I)	111.5 $\pm$ 29.2	97.7 $\pm$ 41.3	305 $\pm$ 163	207 $\pm$ 136
Alanine Aminotransferase (U/I)	61.6 $\pm$ 8.9	59.4 $\pm$ 14.4	116 $\pm$ 109	58 $\pm$ 25
Albumin (g/dl)	5.5 $\pm$ 0.5	6 $\pm$ 0.9	4.3 $\pm$ 0.3	4.3 $\pm$ 0.2
Alkaline phosphatase (U/I)	215 $\pm$ 60	235 $\pm$ 24	261 $\pm$ 22	243 $\pm$ 26
Blood Urea Nitrogen (mg/dl)	17.8 $\pm$ 3.0	18.6 $\pm$ 1.6	16.7 $\pm$ 1.5	16.4 $\pm$ 1.7
Total (mg/dl)	0.2 $\pm$ 0	0.2 $\pm$ 0.4	0.18 $\pm$ 0.1	0.15 $\pm$ 0.1
Ca ²⁺ (mg/dl)	11.3 $\pm$ 0.2	11.1 $\pm$ 0.4	11.2 $\pm$ 0.5	10.5 $\pm$ 0.2*
Cholesterol (mg/dl)	103 $\pm$ 10	123 $\pm$ 16*	69.7 $\pm$ 6.8	74 $\pm$ 6.3
Creatinine (mg/dl)	1 $\pm$ 0.1	0.8 $\pm$ 0.2	0.8 $\pm$ 0.1	0.8 $\pm$ 0.1
Glucose (mg/dl)	143 $\pm$ 19	155 $\pm$ 34	175 $\pm$ 19.3	147 $\pm$ 19.4*
Phosphorus (mg/dl)	10.4 $\pm$ 0.8	9.6 $\pm$ 0.5	9.2 $\pm$ 0.5	9.7 $\pm$ 0.5
Total Protein (g/dl)	7.3 $\pm$ 0.2	7.7 $\pm$ 0.3*	6.5 $\pm$ 0.1	6.6 $\pm$ 0.1
Triglyceride (mg/dl)	140 $\pm$ 31	122 $\pm$ 50	150 $\pm$ 73	129 $\pm$ 20
Body and Organ Weights				
Body weight (g)	266.5 $\pm$ 5.5	264.8 $\pm$ 13.1	501 $\pm$ 16	493 $\pm$ 40
Right kidney (g)	0.99 $\pm$ 0.06	1.01 $\pm$ 0.08	1.94 $\pm$ 0.09	1.90 $\pm$ 0.17
Left kidney (g)	0.96 $\pm$ 0.05	1.04 $\pm$ 0.08	1.95 $\pm$ 0.10	1.87 $\pm$ 0.20
Spleen (g)	0.44 $\pm$ 0.02	0.44 $\pm$ 0.01	0.64 $\pm$ 0.02	0.67 $\pm$ 0.04
Liver (g)	9.96 $\pm$ 1.32	11.48 $\pm$ 1.15	19.44 $\pm$ 2.04	20.65 $\pm$ 2.23
Thymus (g)	0.25 $\pm$ 0.04	0.24 $\pm$ 0.06	0.49 $\pm$ 0.12	0.43 $\pm$ 0.04
Comparative Histopathology				

	Females, vehicle (n=6)	Females, PAP-1 (n=6)	Males, vehicle (n=6)	Males, PAP-1 (n=6)
Gastrointestinal tract	NS	NS	NS	NS
Pancreas	NS	NS	NS	NS
Proximal and distal lymph nodes	NS	NS	NS	NS
Heart	NS	NS	NS	NS
Thymus	NS	NS	NS	NS
Lung	NS	NS	NS	NS
Spleen	NS	NS	NS	NS
Liver	NS	NS	NS	NS
Kidney	NS	NS	NS	NS
Adrenal glands	NS	NS	NS	NS
Urinary bladder	NS	NS	NS	NS
Gonads	NS	NS	NS	NS
Brain	NS	NS	NS	NS
Bone marrow	NS	NS	NS	NS

NS; no significant findings.