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Metabolism and biological production of resolvins derived from docosapentaenoic acid (DPAn-6)

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ABSTRACT

17S-HDPAn-6 (17S-hydroxydocosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*-pentaenoic acid) and 10S,17S-HDPAn-6 (10S,17S-dihydroxydocosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*-pentaenoic acid) are potent anti-inflammatory resolvins derived from DPAn-6 (docosapentaenoic acid n-6) and are analogous in structure and action to DHA (docosahexaenoic acid)-derived resolvins. These resolvins have proven to be potential drug candidates, albeit with therapeutic profiles that need optimization. The main objectives of this study were to evaluate key features of DPAn-6 derived resolvins that are important for therapeutic efficacy, demonstrate that these DPAn-6 resolvins could be produced naturally, and could therefore have physiological significance. Here we demonstrate biological production, examine pharmacokinetic profiles and identify key routes of metabolic inactivation of DPAn-6 derived resolvins. We compare their metabolic stability to a known resolvin, 17S-HDHA (17S-hydroxydocosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid) and show that order of their stabilities is 10S,17S-HDPAn-6 > 17S-HDPAn-6 > 17S-HDHA. We show that both these compounds are not strong inhibitors of cytochrome-P450 enzymes. We evaluate activity of compounds in the delayed-type hypersensitivity model, results of which show that compounds need optimization for enhanced duration and magnitude of action. Analysis of the metabolic stability and identification of metabolites of these compounds could play an important role in the design of better analogs with longer durations of action and hence better efficacy.

1. Introduction

The inflammatory response plays a central role in the protection of the body against invading pathogens and tissue injury. The initiation of this response is an active process and recent research has now demonstrated that the resolution of inflammation, like its initiation, is also an active process, mediated by a number of endogenous lipid-derived molecules that "switch off" the inflammatory cascade and promote homeostasis [1–3]. These lipids are primarily derived from the enzymatic reaction of endogenous lipoxygenases and cyclo-oxygenases with C-20 and C-22 LC-PUFAs (long-chain polyunsaturated fatty acids) to produce oxygenated lipid products or oxylipins at the site of inflammation. Researchers have shown that a number of oxylipins, including prostaglandin D2 and lipoxins derived from arachidonic acid, as well as resolvins derived from the long-chain omega-3 fatty acids, DHA and EPA (eicosapentaenoic acid; 20:5n-3) promote the resolution of inflammation [2,3]. When administered to animals directly, lipoxins and resolvins possess potent anti-inflammatory activity [4–7].

Although both omega-6 and omega-3 fatty acids can be precursors to such anti-inflammatory molecules, scientists generally believe that omega-6 fatty acids, especially arachidonic acid, are pro-inflammatory, whereas the omega-3 fatty acids, especially DHA and EPA down regulate the inflammatory response [8]. More recently and rather surprisingly, Nauroth et al. demonstrated that in addition to DHA and EPA, another

Abbreviations: 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 17:0, heptadecanoic acid; 18:0, stearic acid; 20:0, arachidic acid; 22:0, behenic acid; 24:0, lignoceric acid; 14:1 n-5, myristoleic acid; 16:1 n-7, palmitoleic acid; 18:1n-7, cis-vaccenic acid; 18:1n-9, oleic acid; 20:1n-9, gondoic acid; 22:1 n-9, erucic acid; 24:1 n-9, nervonic acid; 18:3n-3, α-linolenic acid; 20:5n-3, eicosapentaenoic acid; 22:5n-3, docosapentaenoic acid n-3; 22:6n-3, docosahexaenoic acid; 18:2n-6, linoleic acid; 18:3n-6, α-linolenic acid; 20:2n-6, eicosadienoic acid; 20:3n-6, dihomogamma-linolenic acid; 20:4n-6, arachidonic acid; 22:4n-6, adrenic acid; 22:5n-6, docosapentaenoic acid n-6; CYP, cytochrome P450; FAME, fatty acid methyl ester; LC-PUFA, long-chain polyunsaturated fatty acids; LOD, limit of detection; NPD1, neuroprotectin D1; RVE1, resolvin E1; 15-PGDH, 15-prostaglandin dehydrogenase; PK, pharmacokinetic/s; SRM, selective reaction monitoring; 17S-HDPAn-6, 17S-hydroxydocosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*-pentaenoic acid; 10S,17S-HDPAn-6, 10S,17S-dihydroxydocosa-4*Z*,7*Z*,11*E*,13*Z*,15*E*-pentaenoic acid; 17S-HDHA, 17S-hydroxydocosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid; 10S,17S-HDHA, 10S*17S-dihydroxydocosa-4Z,7Z,11E,13Z,15E, 19Z-hexaenoic acid; LLQ, lower limit of quantitation.

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long-chain omega-6 fatty acid found in algal oil derived from *Schizochytrium* sp., namely DPAn-6 is more effective than either DHA or EPA at reducing pro-inflammatory cytokine production *in vitro*, and that *in vivo*, orally administered DPAn-6 appears to enhance the effect of DHA in reducing acute inflammation [9]. We postulated that the anti-inflammatory activity of DPAn-6 may in part be mediated by resolvins derived directly from this fatty acid. Indeed, Dangi et al. recently demonstrated that DPAn-6 is readily converted to two major resolvins, 17S-HDPAn-6 and 10S,17S-HDPAn-6 by soybean 15-lipoxygenase [10]. These resolvins were shown to possess potent anti-inflammatory action in two animal models of acute inflammation when administered locally or systemically. Whether similar resolvins are produced endogenously remained to be demonstrated since actual *ex vivo* or *in vivo* production of these DPAn-6 resolvins was not shown. In this study, we investigate whether human blood is capable of synthesizing DPAn-6 resolvins and also if such resolvins can be produced physiologically.

Physiological significance of these DPAn-6 resolvins or similar lipid mediators depends on their magnitude and duration of action. These parameters in turn are influenced by tissue membrane levels of the precursor fatty acids and the metabolic stability of the mediators formed. Humans can alter plasma as well as tissue levels of LC-PUFA (long-chain polyunsaturated fatty acids) through their diets [11]. Fatty acids become available for enzymatic conversion to inflammatory mediators after cleavage from membrane phospholipids by phospholipase 2, which is activated early in inflammation. The resulting resolvins are known to act locally to modify the inflammatory response. At this point, the resolvins can be inactivated by several enzymes by conversion to metabolites which have little or no activity. Many resolvins such as NPD1 (neuroprotectin D1) and RVE1 (resolvin E1) are being explored as therapeutic agents in conditions like colitis, periodontitis, and Alzheimer's disease [12–14], but such compounds are subject to metabolic inactivation [15,16]. Identification of metabolites of such new drug-leads is important as a first step in designing better analogs. For example, metabolic inactivation of RVE1 has been studied in great detail, using the enzyme 15-PGDH (15-prostaglandin dehydrogenase). The reaction of RVE1 with 15-PGDH mainly produces an inactive form of the compound referred to as 18-oxo-RVE1 [14]. This observation has helped in the design of a more stable analog, namely, 19-(p-fluorophenoxy)-RvE1 that resists rapid metabolic inactivation. This RVE1 analog retains biological activity reducing PMN infiltration and pro-inflammatory cytokine/chemokine productions *in vivo*. Similarly, analogs of lipoxin A4 with improved pharmacokinetic profiles have been developed [17]. DPAn-6 resolvins are potent anti-inflammatory compounds, but have therapeutic profiles which need optimization. Here, we present pharmacokinetic data for both 17S-HDPAn-6 and 10S,17S-HDPAn-6 that helps in addressing key features of the molecules that need enhancement. Also, we investigate the *in vitro* metabolic stability of 17S-HDPAn-6 and 10,17S-HDPAn-6, and identify metabolites that are produced when these compounds are incubated with rat or human liver microsomes. Identification of metabolites will facilitate in the designing of metabolically stable analogs with improved therapeutic profiles.

2. Materials and methods

2.1. Materials

DHA-S^{TM} oil was obtained by controlled fermentation of *Schizochytrium* sp., followed by further downstream processing, and was manufactured by Martek Biosciences Corporation (Columbia, MD). This oil is used in food and dietary supplements and is Generally Recognized as Safe (GRAS) for food [11]. Composition of this oil has been previously described [11]. All other reagents were from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated.

2.2. Synthesis and purification of resolvins

17S-HDHA, 10S,17S-HDHA (10S,17S-dihydroxydocosa-4Z,7Z, 11E,13Z,15E,19Z-hexaenoic acid), 17S-HDPAn-6 and 10S,17S-HDPAn-6 were enzymatically synthesized using the appropriate LC-PUFA (NuChek Prep, Elysian, MN) and soybean lipoxygenase (Type 1B) and purified and characterized as described by Dangi et al. [10] Briefly, LC-PUFA was treated with soybean lipoxygenase in 0.05 M sodium borate buffer pH 9.0 for 30 min at 4 °C, followed by reduction of products by sodium borohydride and final purification of products by HPLC techniques.

2.3. Synthesis of oxylipin in human blood

The sodium salt of DPAn-6 (NuChek Prep, Elysian, MN) was dissolved in water and added to a final concentration of 100 μM in 10 ml of heparinized human blood (Innovative Research, Southfield, MI) and then incubated at 37 °C for 50–120 min. Three controls were set up. The first control was set up in order to evaluate baseline levels of 17-HDPAn-6 and/or 10,17-HDPAn-6 (if any) in blood, and was run with equivalent volumes of water added to blood. The second control used equivalent amounts of DPAn-6 added to heat-inactivated blood. For the heat-inactivated blood control, blood was heated to 100 °C for five minutes to inactivate enzymes, and used after cooling to room temperature. For the third control, 100 μM 17-HDPAn-6 and 10,17-diHDPAn-6 were freshly added to blood to represent 100% conversion. All reactions were stopped by pouring the entire blood solution into 10 volumes of acidified methanol (water:methanol:HCl, 10:80:1, v/v/v) followed by incubation at 4 °C for 30 min. Samples were then centrifuged at 6500 × *g* at 4 °C for 15 min, and for quantitative experiments, 19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-pentaenoic acid (Cayman Chemical Co., Ann Arbor, MI) at a final concentration of 1 p.p.m. (parts per million) was added as an internal standard to supernatants. Supernatants were diluted with 20 volumes of deionized water and purified on DSC18 SPE cartridges (Sigma–Aldrich, St. Louis, MO). The cartridges were washed with water and adsorbed material was eluted twice with 10 ml methanol. The eluate was dried and reconstituted with 1 ml methanol and analyzed by LC/MS/MS as described in Section 2.13.

2.4. Synthesis of oxylipin in human platelets

Fresh platelets (Innovative Research, Southfield, MI) were centrifuged (2000 × *g*, 25 min, 20 °C) and resuspended to 1.7×10^8/ml in 0.135 M NaCl, 12 mM TRIS, pH 7.4, 1.54 mM Na_2EDTA before incubation (0.5 ml) with the sodium salt of DPAn-6, (NuChek Prep., Elysian, MN) 100 μM final concentration, at 37 °C for 90 min. Incubations were terminated by adding 0.1 ml 2 M formic acid and extracted 3 times with 3 ml ethyl acetate. The ethyl acetate layer was washed with 1 ml water and then dried under N_2. The contents were resuspended in 0.3 ml of mobile phase (described below) and analyzed by LC/MS/MS as described in Section 2.13.

2.5. Half-life determination

The microsomal stability of 17S-HDHA, 17S-HDPAn-6 and 10S,17S-HDPAn-6 was investigated over a 60 min period using human (pooled and mixed) or rat (Sprague–Dawley, pooled male)

liver microsomes (BD Biosciences, San Jose, CA) containing Phase I enzymes. Each compound (10 μM final concentration) was incubated with microsomes (0.2 mg/ml final protein concentration) along with 12.5 μl of NADPH regenerating system A and 2.5 μl of NADPH regenerating system B (BD Biosciences, San Jose, CA) in 2.5 ml of 0.1 M phosphate buffer, pH 7.4 for one hour at 37 °C. Samples were removed at 10 min intervals, and analyzed by LC/MS/MS. All assays were run in duplicates and analyzed in triplicate.

2.6. Metabolite identification

Metabolites of 17S-HDPAn-6 and 10S,17S-HDPAn-6 were identified after incubating each compound (100 μM final concentration) with rat (Sprague–Dawley, pooled male) or human (pooled and mixed) liver microsomes (BD Biosciences, San Jose, CA) for 60 min. All other conditions were exactly the same as those described under half-life determination. The structures of the major rat and human metabolites were deduced based on data acquired by LC/MS/MS.

2.7. P450 inhibition

P450 inhibition studies were conducted at Cerep (Seattle, WA) based on a procedure described by Dierks et al. [18]. Studies were conducted by incubating test compounds (10 μM) with human liver microsomes (0.2 mg protein/ml, Xenotech, Lenexa KS) containing CYP enzymes and isozyme-specific substrates (10 μM), 1.3 mM NADP, 3.3 mM D-glucose-6-phosphate and 0.4 U/ml of glucose-6-phosphate dehydrogenase for 15 min at 37 °C, using LC/MS/MS detection of expected metabolites of the reference substrates. Percent of control activity was then calculated by comparing the peak areas obtained in the presence of the test compound to that obtained in the absence of the test compound. Subsequently, the percent inhibition was calculated by subtracting the percent control activity from 100 of each compound. All assays were run in duplicate.

2.8. Animal studies

All animal studies were conducted in accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals. Study protocols were approved by the animal facility's Institutional Animal Care and Use Committee.

2.9. Pharmacokinetic study in rats

The pharmacokinetic parameters of the two DPAn-6 oxylipin compounds were determined in Crl:Cd(SD) rats (Charles River Laboratories, Chicago, IL, $n = 3$ rats/administration route/compound along with two control animals/compound) by Cerep (Seattle, WA). 17-HDPAn-6 and 10,17-HDPAn-6 were diluted in DMSO/Solutol HS15/PBS, pH 7.4 (5/5/90, v/v/v) or in PBS (pH 7.4) alone, respectively, and administered intravenously at 1 mg/kg or by oral gavage at 5 mg/kg. Blood samples (300–400 μl) were collected by jugular vein catheter between 5 min and 24 h after resolvin administration into lithium heparin-coated tubes (BD Biosciences, San Jose, CA). Plasma was harvested following centrifugation (2500 × *g*, 15 min), and protein was precipitated by addition of acetonitrile (4:1, v/v), followed by centrifugation (6000 × *g*, 15 min, 4 °C). Supernatants were transferred and evaporated to dryness and then reconstituted in water/acetonitrile/methanol (2/1/1, v/v/v) containing 0.1% ammonium hydroxide in preparation for HPLC–MS/MS. Pharmacokinetic parameters were derived using a non-compartmental analysis of the plasma concentration data using WinNonlin (Pharsight Corporation, Mountain View, CA).

2.10. DHA-S™ feeding study

Diets were formulated by Research Diets (New Brunswick, NJ) and used AIN-76A as the base diet. Rats (Sprague–Dawley, males weighing 160–180 g from Charles River Laboratories, Chicago, IL) were divided into two groups ($n = 5$ each). Both groups were initially placed on a washout diet (containing no DHA or DPAn-6) for 7 days. The washout diet was depleted of any source of DHA or DPAn-6 and contained a corn-soybean oil blend as a source of fat, totaling 5% (w/w) of the total diet. The corn-soybean oil blend contained 49.5% of corn oil (Research Diets, New Brunswick, NJ), 49.5% of soybean oil (Research Diets, New Brunswick, NJ), 0.33% TAP 1010 (Vitablend, Wolvega, Netherlands), 0.37% Tocoblend (Vitablend, Wolvega, Netherlands) and 0.2% Herbalox (Kalsec, Kalamazoo, MI). Following the washout period, the test group was switched to a diet containing 3% (w/w) DHA-S™ with 2% w/w of the corn-soybean oil (equivalent to 1.2% of DHA/100 g of diet), while the control group was continued on the DHA-depleted diet. Antioxidants were matched to the control diet. At the end of 19 days, animals were sacrificed and various tissues isolated and frozen for further processing. Tissues were thawed and methanol or appropriate buffer was added. Samples were homogenized on ice for ~2–5 min using a Tissue Tearor (BioSpec Products, Inc., Bartlesville, OK). Samples were centrifuged and acidified supernatants diluted to a 10% methanol (in water) and then purified on 10 g/60 ml DSC-18 cartridges. Adsorbed material was eluted with methanol and finally dried under nitrogen. Dried material was reconstituted with 2 ml methanol and filtered using 0.22 μM GV Durapore, centrifugal filter units (Millipore, Bedford, MA) and further analyzed by tandem mass spectrometry.

2.11. FAME analysis of PUFAs in blood from DHA-S™ feeding experiment

Blood was obtained from rats by exsanguination after 19-days of feeding with either test or control diet as described in Section 2.10. Plasma was separated by centrifugation and frozen at −80 °C until analysis. Fatty acids were measured by FAME analysis in plasma phospholipids. Plasma phospholipids were extracted using the methods described by Folch et al. [19]. Fatty acids from plasma phospholipids were saponified with sodium hydroxide and methanol and then methylated with boron trifluoride (all reagents from Sigma–Aldrich, St. Louis, MO). Fatty acid methyl esters (FAMEs) thus formed, were identified by flame ionization detection. Retention times were compared to a mixed fatty acid methyl ester standard from NuChek Prep (Elysian, MN). Fatty acids were quantified by comparison to the 23:0 internal standard (NuChek Prep, Elysian, MN) as described by Arterburn et al. [11]. Statistical comparisons were done using single factor ANOVA using Excel 97-2003 software.

2.12. Evaluation of anti-inflammatory activity of DPAn-6 resolvins in a delayed-type hypersensitivity model in mice

DPAn-6 derived oxylipins, 17-HDPAn-6 and 10,17-HDPAn-6 were both tested at three doses (5, 50 and 500 μg/kg, i.v.) each in a delayed-type hypersensitivity model in mice (10 animals/group from The Jackson Laboratory, Bar Harbor, ME). Controls included PBS (i.v.), dexamethasone (MWI Veterinary Supply, Meridian, ID), 500 μg/kg (p.o.) and the NPD1 isomer, 10S,17S-dihydroxy DHA, at 300 μg/kg (i.v.). In brief, animals (Balb/C female mice, 25 g weight)

were quarantined for 7 days and sensitized with oxazolone (150 μl of a 5% solution) epicutaneously on their shaved abdomens. Seven days later, compounds were administered intravenously followed by resensitization with oxazolone (~15 min later) on their right ears. Resensitization was done with a 3% oxazolone solution, 10 μl applied both to the front and back of the right ears, whereas, the left ears were treated with an ethanol/acetone mixture. Caliper measurements were conducted 24 h later and were a preliminary gauge of anti-inflammatory activity of the compounds. A 7-mm disc of ear was punched out and weighed as a final indication of anti-inflammatory activity.

2.13. LC–MS/MS

Samples from PK (pharmacokinetic) experiments (Section 2.9) and from CYP inhibition studies (Section 2.7) were analyzed on a Thermo Finnigan TSQ Quantum series instrument (Thermo Scientific, Waltham, MA) using a Zorbax Extend-C18, 2 mm × 50 mm, 5 μ column (Agilent Technologies, Santa Clara, CA). For the PK studies, mobile phases comprised of 13.3 mM ammonium acetate, 6.7 mM formic acid in water and 6 mM ammonium acetate, 3 mM formic acid in water/acetonitrile (1:9, v/v), referred to as A and B respectively. The method used for analysis started at 20% B, 20% B at 1 min, 100% B at 2.5 min, 100% B at 3.4 min, 20% B at 3.5 min, and 20% B at 4.5 min at a flow rate of 0.5 ml/min. 17-HDPAn-6 was detected by setting up the SRM mode to monitor two transitions (345.2–327.1 and 345.2–283.2 *m*/*z*) and adding the signal intensities from the two scans. LLQ (Lower limit of quantitation) was 2.5 ng/ml by this method. 10,17-HDPAn-6 was detected using a multiple reaction monitoring mode (transition of 360.9 to 208.1 to 153.1 *m*/*z*) and had a LLQ of 1 ng/ml. All other LC/MS/MS analyses were carried out on an Agilent 1200 Series High Performance Liquid Chromatography Instrument interfaced with an Agilent LC/MSD Trap XCT Plus (Agilent Technologies, San Paulo, CA USA) operated in negative ion mode. Nitrogen was used as nebulizing and drying gas with nebulizer pressure at 20 p.s.i. and drying gas flow rate of 7 l/min. The interface temperature was maintained at 330 °C. Samples (25 μl) were injected onto a LUNA C18(2) column (250 mm × 4.6 mm, 5 μm, Phenomenex, Torrance CA, USA) using a mobile phase comprising solvent A (0.2% ammonium acetate, 30% methanol in water) and Solvent B (acetonitrile). The method used for separation of compounds was 48% B at 0 min, 48% B at 25 min, 90% B at 35 min, 90% B at 50 min, 48% B at 52 min and 48% B at 65 min using a flow rate of 0.4 ml/min. Identity of peaks was based on expected fragmentation, and quantitation was based on relative peak heights compared to that of internal and external standards. Chromatograms were acquired in selective ion mode using molecular ions ($[M\text{-}H]^-$) *m*/*z* 345 and 361 for 17-HDPAn-6 and 10,17-HDPAn-6, respectively. For the PK study, LC/MS/MS analysis was carried out using a TSQ Quantum triple quadrapole mass spectrometer (Thermo Scientific, Waltham, MA). Chromatographic separation was performed on a Zorbac Extend C18 column (2 mm × 50 mm, 5 μ particles) using buffer A consisting of 13.3 mM ammonium formate/6.7 mM formic acid/0.1% ammonium hydroxide in water with a gradient elution of 20–100% of Buffer B consisting of 6 mM ammonium formate/3 mM formic acid/0.1% ammonium hydroxide in water/acetonitrile (1:9 v/v) buffer. MS/MS was obtained using negative ion mode with 325 °C capillary temperature and 3.0 kV capillary voltage in multiple reaction monitoring mode. Compounds were quantified by comparison of peak areas of diagnostic ions to standard curves generated with authentic compound diluted in rat plasma. For the samples obtained from the DHA-S™, feeding study, scans were conducted in the SIM mode using two programs, one which scanned for mono-and dihydroxy-DHA compounds (mainly $[M\text{-}H]^-$ of 343 from 20 to 55 min and $[M\text{-}H]^-$ of 359 from 0 to 20 min respectively) and another which scanned for mono- and dihydroxy-DPAn-6 compounds (Mainly $[M\text{-}H]^-$ of 345 from 20 to 60 min and $[M\text{-}H]^-$ of 361 from 0 to 20 min respectively).

3. Results

3.1. Ex vivo production of oxylipins

Ex vivo production of DPAn-6 oxylipins was examined by incubating DPAn-6 with fresh whole human blood, followed by identification of oxylipin products by HPLC–MS/MS. The main metabolite of DPAn-6 was 17-hydroxydocosa-4,7,10,13,15-pentaenoic acid (17-HDPAn-6) along with minor amounts of 14-hydroxydocosa-4,7,10,12,16-pentaenoic acid (14-HDPAn-6). These products were identified by molecular ions at *m*/*z* 345 ($[M\text{-}H]^-$), 327 ($[M\text{-}H]^-$-H_2O), 301 ($[M\text{-}H]^-$-CO_2), and 283 ($[M\text{-}H]^-$-CO_2-H_2O) along with diagnostic ions at 245, 229 (273-CO_2), and 201 (245-CO_2) for 17-HDPAn-6 and 205, 161 (205-CO_2), 233 and 189 (233-CO_2) for 14-HDPAn-6. DPAn-6 metabolites were not detected in untreated or control blood samples. To determine quantitative conversion rates of DPAn-6 to 17-HDPAn-6, the peak area for the 345 *m*/*z* molecular ion was compared with that produced by addition of a known amount of 17-HDPAn-6 directly to a control blood sample before processing. After 1 and 2 h of incubation with blood, 2.5 and 3%, respectively, of the DPAn-6 fatty acid was converted to 17-HDPAn-6. DPAn-6 was also incubated with fresh human platelets to determine if oxylipin metabolites were formed. As determined by LC/MS/MS analyses of the product metabolites, DPAn-6 was converted by platelets primarily to 14-HDPAn-6 (see spectral characteristics above). None of the controls showed production of resolvins, thus obviating the possibility that the above detected resolvins were synthesized through non-enzymatic auto-oxidation.

3.2. In vivo production of oxylipins

In vivo production of DPAn-6 and DHA resolvins was investigated by feeding rats a diet enriched in DHA and DPAn-6 for 19 days as described in Section 2. During the testing period, no adverse effects were seen in any of the test or control animals. Average intake of PUFA for the test group was 738 mg/kg/day. A FAME analysis of plasma revealed key differences in plasma phospholipid levels of PUFAs between the control and test group and results are outlined in Table 1. There was a drop in arachidonic acid levels (19%) whereas EPA, DPAn-6 and DHA levels were about 15, 3.5 and 2.7 fold of the controls respectively. We next examined whether the elevated levels of DHA and DPAn-6 contributed to detectable levels of oxylipins produced from these PUFAs. Limit of detection for most of the oxylipin compounds under consideration were in the range of 5–10 ppb in the final sample. Several tissue extracts were analyzed and results are outlined in Table 2. 10,17-HDPAn-6 and 10,17-HDHA concentrations were below LOD (limit of detection). Several tissues showed the presence of 17-HDPAn-6 as well as 17-HDHA. 17-HDPAn-6 could be unambiguously detected in the blood, trachea and heart whereas 17-HDHA was unambiguously detected in the lungs. Several other tissues also showed ions diagnostic of the above mentioned oxylipins (see Table 2), but could not be unambiguously assigned as such due to the presence of interfering ions. 7,17-HDPAn-6 was unequivocally detected in lungs. Although amounts of resolvins detected were below levels of precise quantitation, qualitatively, the levels of DPAn-6 resolvins were always higher than DHA resolvins, when both were found concomitantly in any specific tissue.

Table 1
Plasma phospholipid fatty acid levels[a].

	Control Mean (standard deviation)	Test Mean (standard deviation)
Fatty acid		
Total saturated[1]	43.08 (1.88)	49.11 (3.13)
Total mono-unsaturated[2]	11.84 (1.06)	8.89 (1.76)
n-3		
18:3n-3 (α-linolenic acid)*	0.08 (0.01)	0.11 (0.03)
20:5n-3 (eicosapentaenoic acid)***	0.11 (0.02)	1.62 (0.20)
22:5n-3 (docosapentaenoic acid n-3)**	0.54 (0.05)	0.68 (0.08)
22:6n-3 (docosahexaenoic acid)***	2.78 (0.40)	7.54 (0.63)
Total LC-PUFA n-3***	3.51 (0.48)	9.95 (0.94)
n-6		
18:2n-6 (linoleic acid)***	20.66 (1.39)	14.43 (1.28)
18:3n-6 (γ-linolenic acid)*	0.10 (0.04)	0.05 (0.00)
20:2n-6 (eicosadienoic acid)***	0.46 (0.09)	0.15 (0.01)
20:3n-6 (dihomogamma-linolenic acid)*	0.80 (0.15)	0.80 (0.04)
20:4n-6 (arachidonic acid)***	17.81 (1.03)	14.41 (1.06)
22:4n-6 (adrenic acid)*	0.45 (0.04)	0.00 (0.00)
22:5n-6 (docosapentaenoic acid n-6)***	0.54 (0.19)	1.90 (0.16)
Total LC-PUFA n-6***	40.81 (2.92)	31.73 (2.54)

[a] Blood was obtained from rats by exsanguination after 19-days of feeding with either test (diet containing 3%, w/w DHA-S™ as described in Section 2.10) or control diet. Statistical comparisons were done using single factor Anova using Excel-97-2003 software. Table shows percentage of the total fatty acids; means ± SD, $n = 5$ per group. [1]Saturated fatty acids include: 14:0 (myristic acid), 15:0 (pentadecanoic acid), 16:0 (palmitic acid), 17:0 (heptadecanoic acid), 18:0 (stearic acid), 20:0 (arachidic acid), 22:0 (behenic acid), 24:0 (lignoceric acid); [2]Monounsaturated fatty acids include: 14:1 n-5 (myristoleic acid), 16:1 n-7 (palmitoleic acid), 18:1n-7 (cis-vaccenic acid), 18:1n-9 (oleic acid), 20:1n-9 (gondoic acid), 22:1 n-9 (erucic acid), 24:1 n-9 (nervonic acid); *not statistically significant; **$P < 0.05$; ***$P < 0.01$

Table 2
Tissue distribution of selected oxylipins generated from DHA and DPAn-6[a].

Tissue/sample	17-HDHA	17-HDPAn-6
Small intestine	<LOD	Detected with interference
Large intestine	<LOD	<LOD
Blood	Detected with interference	Present
Liver	Detected with interference	<LOD
Lungs	Present	Detected with interference
Trachea	<LOD	Present
Kidneys	<LOD	Detected with interference
Heart	<LOD	Present
Brain	Detected with interference	<LOD
Feces	<LOD	<LOD

[a] Animals were fed a diet containing 3% DHA and DPAn-6 (Test) for 19 days. Resolvins were not detected in control (no DHA or DPAn-6 fed) samples. Limit of detection (LOD) was 25 ng/ml.

3.3. Metabolism of DPAn-6 resolvins by liver microsomes and effect on cytochromes P450

Table 3 shows the calculated half life for the three compounds tested as described in Section 2.5. The results indicate that under the conditions of the study, 10,17-HDPAn-6 has a longer half life (110–270 min) than 17-HDPAn-6 (<30 min) with both rat and human microsomes which in turn appears to be more stable than 17-HDHA (~13 min). Metabolites produced on incubating DPAn-6 resolvins with liver microsomes were identified based on MS fragmentation patterns as described in Section 2. The main metabolites resulted from dehydrogenation reactions, ω-hydroxylations, and further lipoxygenase-like hydroxylations with minor epoxidation and hydrolysis products. In the case of 10,17-HDPAn-6, metabolites with $[M-H]^-$ of 377 (trihydroxy products as a result of lipoxygenase-like activity) and $[M-H]^-$ of 395 (tetrahydroxy products as a result of epoxidation and hydrolysis) were also seen. However, sites of hydroxylation and epoxidation could not be identified due to low levels of product formed. Metabolites are outlined in Figs. 1 and 2. Effects of both DPAn-6 resolvins on Cytochrome P450 activity were evaluated as described in Section 2. Table 4 shows the percent change in CYP activity relative to control in which no oxylipin was added. There was a modest reduction in CYP3A activity with both compounds. However, neither the 17-HDPAn-6 nor the 10,17-HDPAn-6 at 10 μM resulted in >50% inhibition of any of the test CYP enzymes at this 10 μM concentration, indicating that neither would be considered a strong CYP inhibitor [18].

Table 3
In vitro microsomal stability of 17S-HDPAn-6, 10S,17S-HDPAn-6 and 17S-HDHA[a].

Compound	$T_{1/2}$ with rat liver microsomes (min)	$T_{1/2}$ with human liver microsomes (min)
17-HDHA	13.6	13.0
17-HDPAn-6	17.5	25.8
10,17-HDPAn-6	110.0	266.0

[a] Compounds were incubated with microsomes at 37 °C for 60 min and were analyzed at 10 min intervals by LC/MS/MS techniques. All assays were run in duplicate and analyzed in triplicate. Values represent averages.

3.4. Pharmacokinetic behavior of DPAn-6 resolvins

The pharmacokinetics of the compounds were explored in rats by administering 17-HDPAn-6 or 10,17-HDPAn-6 either intravenously (1 mg/kg) or orally (5 mg/kg), and plasma concentrations of the compounds determined over the next 24 h by HPLC–MS/MS. Fig. 3 shows the concentration of the parent compounds in plasma ($n = 3$ rats per group) at each time point. Pharmacokinetic parameters, determined using a one-compartment model, are shown in Table 5. With oral dosing, maximum plasma concentrations were attained at 30 and 45 min for 17-HDPAn-6 and 10,17-HDPAn-6, respectively. Per this study, the oral bioavailability based on comparison of total area under the curve (AUC_∞) for the i.v. and oral administration of 17-HDPAn-6 and 10,17-HDPAn-6 were 30% and 2%, respectively. Interestingly, the terminal elimination half lives for both compounds was considerably shorter following oral administration (50 and 27 min for the mono- and dihydroxy compounds) compared to intravenous administration (approximately 10 and 13 h for 17-HDPAn-6 and 10,17-HDPAn-6, respectively), despite achieving similar maximum plasma concentrations for 17-HDPAn-6 (601 vs. 686 ng/ml, respectively). The pharmacokinetic parameters did not change appreciably when considered in 2- and 3-compartment models (data not shown).

3.5. Anti-inflammatory activity in the DTH model (mice)

Graphical results are shown in Fig. 4. 17-HDPAn-6 showed a reduction in ear core weights by 31% and 25% at doses of 5 and 500 μg/respectively. The 50 μg/kg dose of 17-HDPAn-6 showed a 10% reduction (not statistically significant). However one should note that the standard errors associated with the 50 μg/kg group were higher than in the other groups. The positive controls, Dexamethasone and NPD1 showed reductions of 29% and 7% (not statistically significant) respectively. None of the doses of 10,17-HDPAn-6 showed any statistically significant reductions in ear core weights as compared to the vehicle controls. Hence,

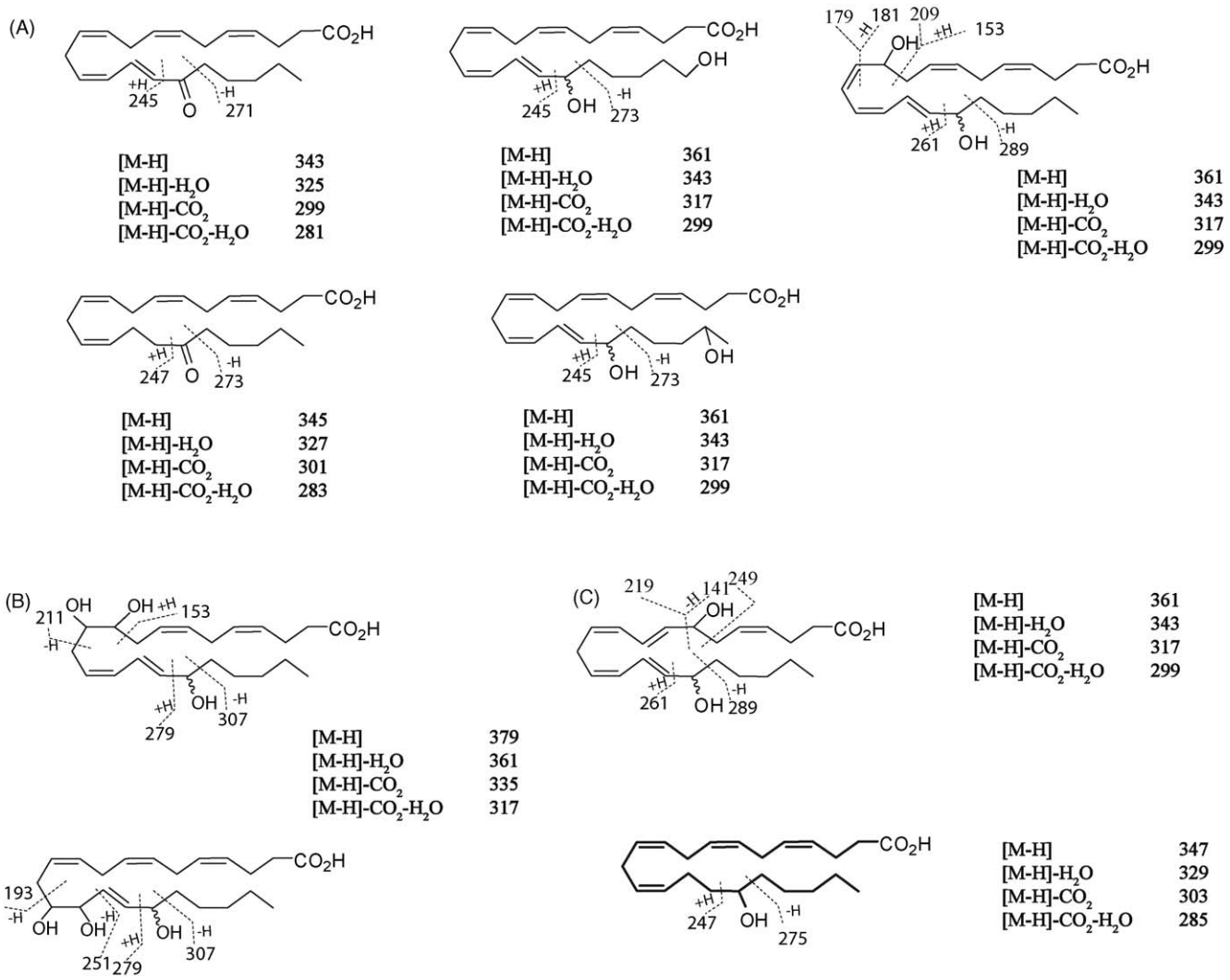

Fig. 1. Putative metabolites formed on incubation of 17-HDPAn-6 with rat and human liver microsomes. 17S-HDPAn-6 was incubated with rat liver or human microsomes for 60 min as described under Section 2. The structures of the major rat and human metabolites were deduced based on data acquired by LC/MS/MS. Metabolites seen with both human and rat liver microsomes (A); metabolites seen only in human liver microsome incubations (B); metabolites seen only with rat liver microsomes (C). Some of the diagnostic fragments used for determination of structures are shown for each structure. All fragments were detected in the negative ion mode.

17-HDPAn-6, at 5 μg/kg has anti-inflammatory activity in this DTH mouse model, comparable to activity shown by the positive control dexamethasone. Also, at this dose the compound exhibits better efficacy than the NPD1 isomer tested.

4. Discussion

A large amount of work has been done by Serhan and coworkers on EPA and DHA derived resolvins. It has been demonstrated that these resolvins can be produced endogenously by the action of lipoxygenases and cyclo-oxygenases and that the resulting compounds are key physiological modulators of inflammation [7,20,21]. We postulated that since DPAn-6 is similar in structure to DHA, resolvins derived from DPAn-6 could possibly be synthesized in an analogous manner and could also have important biological roles. Although it has been shown that 17S-HDPAn-6 and 10S,17S-HDPAn-6 are potent anti-inflammatory compounds, their biological production remained to be shown [10]. Here we demonstrate that DPAn-6-derived resolvins can be produced endogenously as well as by human blood. When DPAn-6 is added to fresh human blood *ex vivo,* 17-HDPAn-6 and 14-HDPAn-6, products known to be produced by 15- and 12-lipoxygenase activity, respectively, were formed [10]. These lipoxygenases are present in eosinophils, monocytes, and reticulocytes (15-lipoxygenase) and platelets, megakaryocytes and eosinophils (12-lipoxygenase) and were therefore expected products [22–24]. Hong et al. [6] noted that the major products of DHA in whole human blood were 17-HDHA and 10,17-HDHA, along with small amounts of 14- and 11-hydroxy DHA as well as some trihydroxy derivatives [6,14]. We did not see evidence of dihydroxy metabolites in our experiment with whole blood, but they are generally less abundant than the mono-hydroxy metabolites of DPAn-6, since they are formed as a secondary metabolite of the fatty acid. Plasma levels of DPAn-6 increase, albeit modestly, with increased intake of this fatty acid [11], suggesting that dietary intervention with DPAn-6 could result in increased synthesis of DPAn-6-derived oxylipins that in turn may modify the inflammatory response.

The most abundant source of DPAn-6 are algal oils derived from the Thraustochytrids, especially *Schizochytrium* sp. oil, which contains approximately 15% of total fatty acids as DPAn-6 along with 40–45% DHA [25]. Other dietary sources of DPAn-6 include some seafoods, with lesser amounts also found in poultry and egg yolks [26,27]. Although we did not detect any DPAn-6-derived

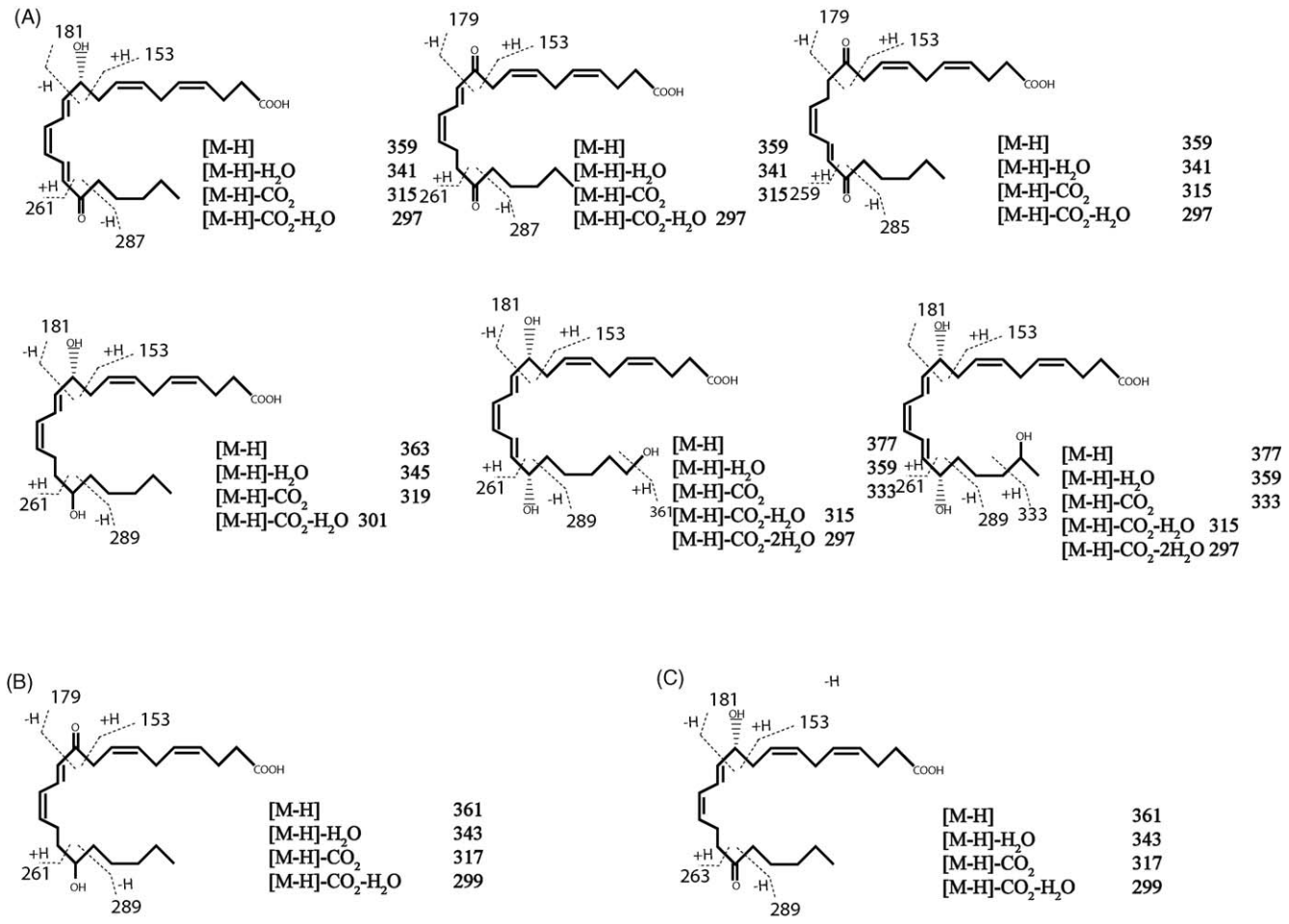

Fig. 2. Putative metabolites formed on incubation of 10S, 17S-HDPAn-6 with rat and human liver microsomes. 10S,17S-HDPAn-6 was incubated with rat liver or human microsomes for 60 min as described under Section 2. The structures of the major rat and human metabolites were deduced based on data acquired by LC/MS/MS. Metabolites seen with both human and rat liver microsomes (A); metabolites seen only in human liver microsome incubations (B); metabolites seen only with rat liver microsomes (C). Some of the diagnostic fragments used for determination of structures are shown for each structure. All fragments were detected in the negative ion mode.

oxylipins in untreated blood samples, these experiments raise the possibility that DPAn-6 oxylipins are biologically relevant molecules that can be synthesized endogenously from DPAn-6 present in human blood or tissues and act locally to modify an inflammatory response. This is further supported by the fact that 17-HDPAn-6 was produced in blood and tissues such as the trachea and lungs when rats were fed a diet enriched in DHA and DPAn-6. Another DPAn-6 resolvin, 7,17-HDPAn-6, analogous in structure to Resolvin-D5 was also detected in the lungs of rats fed with diets enriched with DHA and DPAn-6 [28]. We do not know whether this analogous compound has any anti-inflammatory activity at this point, but copious amounts of this compound can easily be made based on methods described by Dangi et al. [10] and further tested when needed. It is important to note that although levels of DHA

Table 4
Effect of 17-hydroxy DPAn-6 and 10,17-dihydroxy DPAn-6 on cytochrome P450 activity[a].

CYP isozyme	Substrate	Metabolite detected	Compound tested	% Inhibition*
CYP1A	Phenacetin	Acetaminophen	17-HDPAn-6	−7
			10,17-HDPAn-6	−5
CYP2C9	Diclofenac	4′-Hydroxydiclofenac	17-HDPAn-6	28
			10,17-HDPAn-6	−1
CYP2C19	Omeprazole	5-Hydroxyomeprazole	17-HDPAn-6	−27
			10,17-HDPAn-6	15
CYP2D6	Dextromethorphan	Dextrorphan	17-HDPAn-6	−7
			10,17-HDPAn-6	4
CYP3A	Testosterone	6β-Hydroxytestosterone	17-HDPAn-6	30
			10,17-HDPAn-6	44

[a] 17-HDPAn-6 and 10,17-HDPAn-6 were incubated with human liver microsomes (10 μM) and reference substrates for each CYP isozyme (shown in parentheses) for 15 min at 37 °C ($n = 2$). Metabolites of the reference substrates were detected by LC/MS/MS. Average percent inhibition was calculated based on ability of the test compound to inhibit the conversion of the substrate to the expected metabolite. *Positive values indicate inhibition.

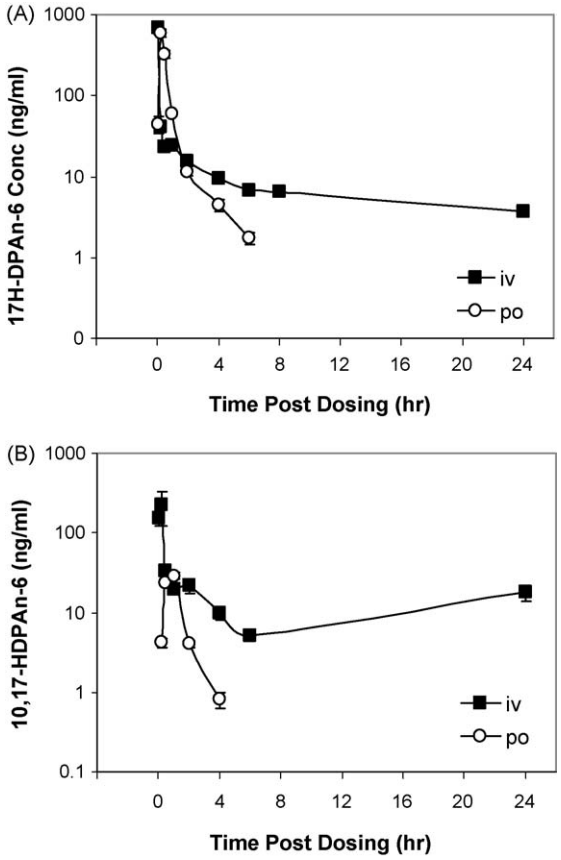

Fig. 3. Plasma concentrations of 17S-HDPAn-6 and 10S,17S-HDPAn-6 after intravenous or oral administration of oxylipin compounds. 17-HDPAn-6 (A) or 10,17-HDPAn-6 (B) were administered at 1 mg/kg intravenously (filled squares) or 5 mg/kg by oral gavage (open circles). Blood samples were obtained at times up to 24 h post administration and processed for quantitation of parent compounds by HPLC–MS/MS. Figure shows means ± SE (n = 3/group) of plasma concentrations of the parent oxylipin compounds. Plasma concentrations of the orally administered compounds fell below the limit of quantitation after 4 and 6 h for 10,17-HDPAn-6 and 17-HDPAn-6, respectively.

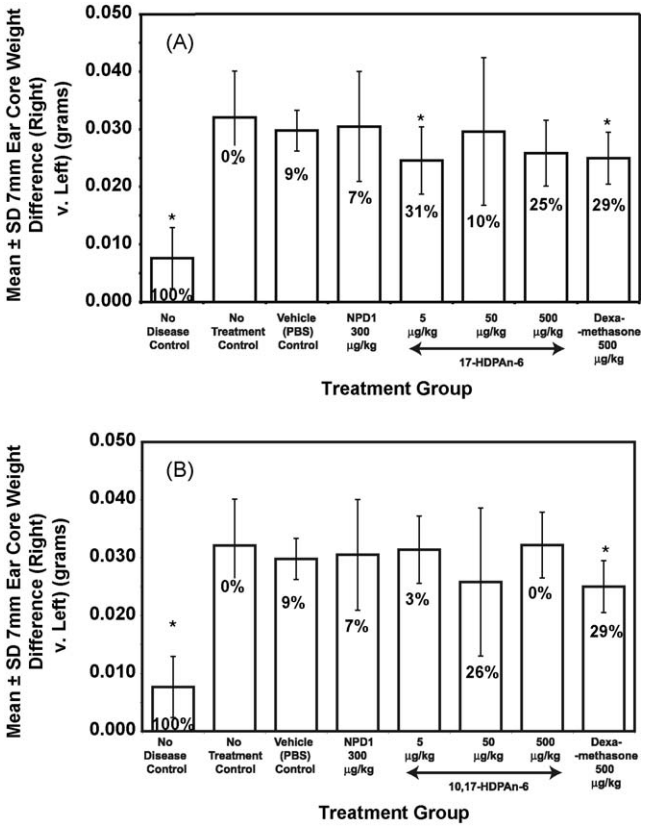

Fig. 4. Anti-inflammatory Evaluation of DPAn-6 resolvins in a delayed-type hypersensitivity model in mice. 17S-HDPAn-6 and 10S,17S-HDPAn-6 were both tested at three doses (5, 50 and 500 μg/kg, i.v.) each in a delayed-type hypersensitivity model in mice (10 animals/group). Controls included PBS (i.v.), dexamethasone, 500 μg/kg (p.o.) and the NPD1 isomer, 10S,17S-dihydroxy DHA, at 300 μg/kg (i.v.). Animals were sensitized with oxazolone and seven days later, compounds were administered intravenously followed by resensitization with oxazolone (~15 min later) on their right ears. Caliper measurements were conducted 24 h later and were a preliminary gauge of anti-inflammatory activity of the compounds (data not shown). A 7-mm disc of ear was punched out and weighed as a final indication of anti-inflammatory activity. Shown in the figure are ear core weights for animals administered 17S-HDPAn-6 (A) and 10S,17S-HDPAn-6 compared to appropriate controls. $^{*}P < 0.05$. Percent reductions with reference to the control are indicated for each group.

intake far exceeded DPAn-6 intake, the amount of DPAn-6 resolvins detected were higher than those produced from DHA, indicating again that DPAn-6 may be a preferred substrate for lipoxygenase-like enzymes as was suggested by Dangi et al. Another reason for this difference in these levels could be due to superior stability of DPAn-6 resolvins as compared to DHA resolvins in this particular physiological milieu.

Microsomal incubations of DPAn-6 resolvins suggests that metabolic stabilities of the compounds is in the order, 10,17-HDPAn-6 > 17-HDPAn-6 > 17-HDHA. These stabilities do not necessarily translate to order of potencies, since several other features of the molecules such as solubility and stability in physiological fluids, permeability through membranes, possible active transport mechanisms, and affinities between these molecules and their receptors, in addition to metabolism play

Table 5
Pharmacokinetic parameters 17-HDPAn-6 and 10,17-HDPAn-6 in rats[a].

Compound	Route of admin.	Dose (mg/kg)	$T_{1/2}$ (min)	Clearance (ml/min/kg)	Vol. of Distrib. (ml/kg)	Vol. of Distrib.-ss (ml/kg)	AUC-all (min ng/ml)	AUC∞ (min ng/ml)
17-HDPAn-6	IV	1	607	43	38,352	24,996	22,026	25,259
10,17-HDPAn-6	IV	1	799	35	34,470	43,470	19,469	41,864

Compound	Route of admin.	Dose (mg/kg)	$T_{1/2}$ (min)	Tmax (min)	Cmax (ng/ml)	Bioavail. (%)	AUC-all (min ng/ml)	AUC∞ (min ng/ml)
17-HDPAn-6	PO	5	50	30	601	30	33,181	33,421
10,17-HDPAn-6	PO	5	27	45	29	2	1,562	1,653

[a] Rats (n = 3/group) were dosed with 17-HDPAn-6 or 10,17-HDPAn-6 and plasma concentrations of the compounds were quantified by HPLC–MS/MS over a 24-h period. Pharmacokinetic parameters were determined using WinNonlin software using a one-compartment model. $T_{1/2}$—terminal elimination half life; clearance—total body clearance; vol. of distrib—volume of distribution calculated based on terminal phase of plasma concentration from iv dosing; vol. of distrib-ss—volume of distribution at steady state; AUC-all—area under the curve from the time of dosing to the time of last observation; AUC∞—area under the curve from the time of dosing extrapolated to infinity; Cmax—maximum plasma drug concentration obtained after oral dosing; Tmax—time to maximum observed plasma concentration (Cmax); Bioavail.—oral bioavailability.

an important role in contributing to final activity. Nevertheless, metabolic stability is an important factor that is taken into consideration while designing superior analogs. Such enhancements are possible if one identifies molecular modification sites commonly accessed during metabolism. Metabolism of 17-HDPAn-6 and 10,17-HDPAn-6 on microsomal incubations resulted in compounds that were expected based on what has been observed before with DHA itself as well as resolvins and lipoxins. Oxidoreductase like activity characteristically seen due to activity of prostaglandin dehydrogenase type enzymes was manifested by production of metabolites containing characteristic oxo or oxodiene functionalities from both the DPAn-6 resolvins. Additionally, reduction of the double bonds at C-15 for 17-HDPAn-6 and 10,17-HDPAn-6 or C-11 for 10,17-HDPAn-6 is commonly seen reduction reactions as a result of such enzyme activity. Although we do not know whether the corresponding metabolites have reduced/no activity in the case of DPAn-6 resolvins, significantly reduced activity has been observed for similar metabolites in the case of RVE1 as well as LXA4. Other metabolites that are commonly seen for compounds of this nature are a result of hydroxylation at the terminal portions of the molecule, specifically at C-21 and C-22 in the case of DPAn-6 resolvins. Again, similar compounds have been detected, while evaluating the metabolome of RVE1, produced using human polymorphonuclear leukocytes and whole blood, as well as in murine inflammatory exudates, spleen, kidney, and liver. A large number of these RVE1 metabolites had reduced activities *in vivo* [29]. The metabolites produced in this study can be produced and purified and tested for activity, thus setting the stage for careful delineation of features of these molecules that are important for activity. This can be achieved by testing an array of metabolites either *in vitro* or *in vivo* for potentially reduced activity, and then designing molecules that would be protected from inactivation. For example, the hydroxyl groups are both subject to oxidation. If these oxidized molecules are inactive, one could design substituents at these positions that would block oxidation. A similar strategy could be employed for the terminal portions of the molecules. A prodrug or bioisosteric approach could be employed at suitable positions.

DPAn-6 resolvins have shown potent anti-inflammatory activity in several models of acute inflammations such as the mouse air pouch, rat hind paw edema, and the mouse delayed-type hypersensitivity model [10]. Compounds are not strong inhibitors of Cytochromes P450, an important characteristic that indicates lowered probability of drug–drug interactions. As described by Dangi et al., the compounds also fulfill most of the Lipinski's rules for an ideal drug molecule [10,30,31]. These DPAn-6 derived oxylipins have better PK parameters than LXA4, and in fact have comparable bioavailabilities and half lives to the much more stable lipoxin analogs [17]. These molecules are several fold more potent than LC-PUFAs such as DHA/DPAn-6. For example, as reported by Dangi et al., these compounds are effective at concentrations in the range of 12.5–1250 μg/kg in the rat hind paw edema model. A dose of around 1 g/kg is required to elicit a similar effect if PUFAs such as DHA or DPAn-6 were to be administered in this model [9,32]. In humans, common doses of EPA and DHA that have shown benefit in conditions associated with chronic inflammation, such as rheumatoid arthritis generally exceed a gram [33]. Nevertheless, several features of the molecules still need optimization. For example, both compounds exhibit some unusual PK behavior. In particular, the oral half lives of the compounds are 15–20 times shorter than with intravenous administration. Although the mechanism explaining these differences in half lives cannot be deduced from these study data, one possible explanation is an acute auto-induction or auto-activation of compound clearance that occurs more rapidly following oral administration due to a liver first pass phenomenon. Although very efficacious in the rat hind-paw edema model, both compounds exhibited a non-monotonic dose response. In the DTH model, although 17-HDPAn-6 showed anti-inflammatory activity at 5 μg/kg, activity for 10,17-HDPAn-6 was not significant 24 h post-administration. Therefore, it is desirable to synthesize derivatives or analogs with improved bioavailabilities and stability profiles for possible pharmaceutical applications. The data gleaned from the metabolism studies described in this paper is an excellent starting point for this endeavor.

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