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Metabolic activation of indole-containing prostaglandin D2 receptor 1 antagonists: Impacts of glutathione trapping and glucuronide conjugation on covalent binding

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Abstract—Some DP1 receptor antagonists from an indole-containing series were shown to cause in vitro covalent binding to protein in rat and human liver microsomes. Glutathione trapping experiments along with in vitro labeling assays confirmed that the presence of a strong electron withdrawing group was necessary to abrogate in vitro covalent binding, leading to the discovery of MK-0524. Hepatocyte incubations and in vivo studies showed that acyl-glucuronide formation did not translate into covalent binding.

The potential for idiosyncratic toxicity of a drug is an important issue for the pharmaceutical industry due to its lack of predictability, the potential side-effects for patients affected, and the financial impacts. Drugs showing idiosyncratic reactions were withdrawn from the market during the past decades due to adverse-events such as hepatotoxicity, allergic responses, and skin rashes observed in subsets of patients.[1] It is well accepted that bioactivation of molecules via phase I and II hepatic metabolism can lead to formation of reactive intermediates, which can bind to biomolecules such as protein or DNA, giving rise to side-effects and various toxicities.[2,3] Reactive intermediates containing moieties such as quinone methide, quinone imine, epoxide, imine methide, aldehyde or acyl-glucuronide were reported as potential precursors leading to drug-protein adduct formation.[4] Considering the important impact of metabolic bioactivation on the development of a drug candidate, the propensity for molecules to cause covalent binding is assessed early in the discovery stage at Merck Research Laboratories (MRL) and efforts are made to abrogate protein labeling.[5,6]

In summary, the potential of drug candidates to cause covalent binding is first evaluated in vitro by incubation of a radiolabeled analog in the presence of rat and human liver microsomes under oxidative conditions, and then in vivo in rats. In both cases, formation of covalent adducts with protein is determined by successive washing of protein pellets using either Brandel harvester technique or centrifugation-based methods.[7–9] A target value of 50 pmol-eq/(mg at 1 h) for the in vitro assay and of 50 pmol-eq/mg protein in vivo in rat (plasma and liver) was proposed by Evans et al., considering these values are approximately 10-fold over the background of the assays and represent 1/20th of binding obtained for known hepatotoxins.[5]

The paper herein describes an in vitro covalent binding issue that was observed during the course of developing a prostaglandin D_2 receptor 1 (DP1 receptor) antagonist for the treatment of seasonal allergic rhinitis and niacin induced flushing.[10–12] Work was first performed to identify what reactive species was responsible for protein labeling, using reduced glutathione (GSH) as trapping agent. Then, GSH trapping experiments were used

Keywords: Covalent binding; DP1 antagonist; Methylindole; MK-0524; Reactive intermediate; Metabolite.
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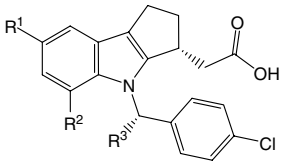
as a surrogate marker assay to help in the structure metabolism relationship (SMR) studies aimed at minimizing the in vitro protein labeling. Radiolabeled analogs of key compounds were prepared to confirm reduction of microsomal covalent binding. As a second step, hepatocyte incubations were used as an additional assay to evaluate the in vivo relevance of oxidative reactive intermediate formation and the potential impact of acyl-glucuronide and acyl-migration products on covalent binding. Ultimately, in vivo experiments were done to assess the propensity of lead DP1 antagonists to cause protein labeling. This work clearly highlights the direct impact of covalent binding on the course of a drug discovery program and the solutions found to abrogate labeling issues in an indole-containing series.

After initial efforts to identify a lead compound from the Merck sample collection and to improve potency and selectivity over other prostanoid receptors,[11,12] compounds **1**, **2**, and **3** were selected and further investigations were initiated to assess their potential to cause in vitro protein covalent binding.[13] Radiolabeled analogs of **1** and **2**, tritiated at the methylsulfone moiety,[14] and of **3** with ^{14}C at the carboxylic acid moiety,[15] were synthesized and in vitro liver microsomal covalent binding experiments were performed, as described elsewhere.[7] In summary, 10 μM of compound (1 μCi/mL) was added to a phosphate-buffered solution (125 mM; pH 7.4) containing 1 mg/mL of rat or human liver microsomes. Following a 15 min pre-incubation at 37 °C, NADPH (1 mM) or phosphate buffer was added (final incubation volume of 200 μL) and a 60-min incubation was carried out at 37 °C. Then, samples were quenched with acetone, the precipitated proteins were collected and washed using a Brandel cell harvester, and the presence of radiolabeled material bound to microsomal proteins was measured by scintillation counting after protein solubilization.

Table 1. In vitro liver microsomal covalent binding (pmol-eq/(mg at 1 h))



Compound	R^1	R^2	R^3	Rat	Human
1	SO_2Me	CH(OH)Me	H	13 ± 5	9 ± 0.4
2	SO_2Me	COMe	H	23 ± 2	18 ± 1
3	F	COMe	H	290 ± 50	46 ± 3

As shown in Table 1, minimal in vitro covalent binding was observed for **1** and **2**, whereas substitution of the methylsulfone functionality at R^1 with a fluorine atom (**3**) led to a significant increase in protein labeling in both rat and human liver microsomes in an NADPH dependent manner. Additional in vitro covalent binding experiments were performed on **3** using phenotyped human liver microsomes ($n = 6$) and a correlation ($R^2 = 0.8633$) was obtained between testosterone 6β-hydroxylase activity and protein labeling, suggesting the implication of CYP3A in the reactive intermediate formation.

In vitro incubations of **3** were repeated using unlabeled material and analyzed by high performance liquid chromatography coupled to photodiode array and mass spectrometric detectors (HPLC-PDA-MS). Typical metabolic profiles are shown in Figure 1. Interestingly, the

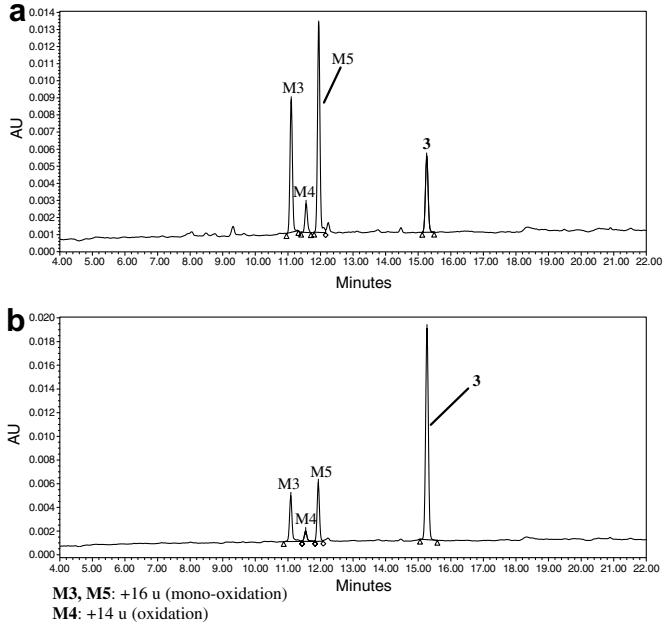


Figure 1. Metabolic profiles of **3** (10 μM) in rat (a) and human (b) liver microsomal incubations under oxidative conditions. HPLC-PDA conditions: YMC-ODS-A 4.6 × 150 mM 3μm, 1 mL/min; mobile phase composed of 20 mM NH_4OAc + 5% MeOH (c) and acetonitrile (d); Gradient: 10–60% D in 17 min, to 90% D at 18 min, then isocratic at 90% D for 4 min; λ 260 nm.

compound was metabolized to greater extents in rat than in human incubates, which correlates with the higher levels of protein labeling observed in rat. **M3** and **M5** were the main oxidative metabolites detected in both species by HPLC-PDA-MS/MS. In negative ion mode, both metabolites showed a +16 u mass shift versus **3**, as compared to a −2 u difference in positive ion mode, most likely due to water loss. A minor oxidative metabolite, **M4**, with a +14 u mass shift relative to **3** was also detected.

A mixture of metabolites produced by large scale incubation of **3** in rat liver microsomes under oxidative conditions and subsequent solid phase extraction was subjected to HPLC-UV-NMR analysis.[16] Two separate injections were performed and the pump stopped when the peak for either **M3** or **M5** was eluted into the 60 μL active volume NMR flow cell of a conventional room temperature flow probe. In each case, 1-D proton spectra and 2-D 32 scans gradient COSY spectra were acquired at 600 MHz.

In the case of the major metabolite **M5**, changes in the number and chemical shifts of the cyclopentyl protons were observed. In particular, the methylene protons at position 1 could no longer be found and a new multiplet consistent with a hydroxyl-methine was observed. All of the other protons of the parent **3** could be found in **M5**. Analysis of the gradient COSY spectrum demonstrated the presence of a CH_2CHCH_2CH spin system. Thus, the oxidation must be at the 1 position as shown in Figure 2. Unfortunately, the relative stereochemistry of the hydroxylation could not be determined from the coupling constants nor was there sufficient material to perform an NOE experiment. Spectra from the less abundant metabolite **M3** were very similar to those obtained from **M5**. This metabolite was also found to have the same CH_2CHCH_2CH spin system with slightly altered chemical shifts. Thus, this metabolite must be the other possible diastereomer formed by oxidation at C-1 of **3**.[17]

Chemical trapping agents such as GSH, cyanide, semicarbazide or methoxylamine have been used in the past to identify reactive metabolites.[5,8] The potential of GSH, a soft nucleophile, to trap the reactive metabolite of **3** was assessed by repeating the rat in vitro liver microsomal covalent binding assay in the presence or absence of physiologically relevant concentrations of GSH (5 mM), and analyzing in parallel incubates done with unlabeled material by HPLC-PDA-QTof.

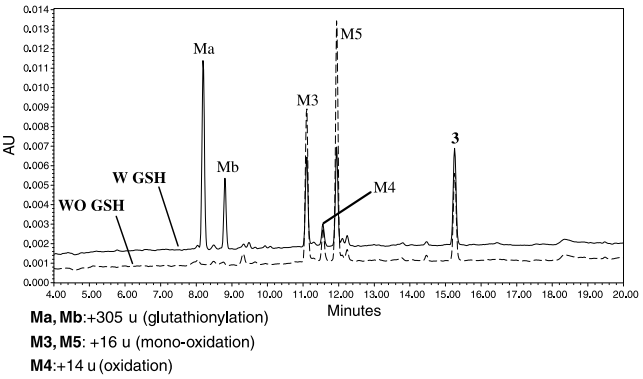


Figure 3. Metabolic profiles of **3** in rat liver microsomal incubations under oxidative conditions with/without GSH (HPLC-PDA; λ 260 nm).

A 4-fold decrease in protein labeling was observed in the presence of GSH (from 318 to 77 pmol-eq/(mg at 1 h)) and a concomitant formation of two new metabolites, both showing a +305 u mass shift relative to **3**, characteristic for glutathione adducts. In addition, a decrease of 31–51% in the **M3** and **M5** peak areas was observed in the presence of GSH, suggesting that **M3** and **M5** formation was diverted toward GSH adduct formation (**Ma** and **Mb**) under these conditions (Fig. 3).

The GSH adducts were analyzed by HPLC-UV-NMR following large scale incubation and solid phase extraction analogous to that used to produce **M3** and **M5**. Two separate injections were performed and the pump stopped when the peak for either **Ma** or **Mb** was eluted into the 60 μL active volume NMR flow cell of a cryogenically cooled flow probe. In each case, 1-D proton spectra and 2-D gradient COSY spectra were acquired at 600 MHz.

As was the case with **M3** and **M5**, it was clear from the gradient COSY data that **Ma** and **Mb** contained the same CH_2CHCH_2CH spin system identifying position 1 as the site for glutathione addition (Fig. 4). In addition to observing all of the remaining signals present in the parent, a complete set of resonances from glutathione could also be identified in each of the adducts, positively identifying **Ma** and **Mb** as the diastereotopic GSH adducts of **3**. Once again, the relative stereochemistry of the GSH addition could not be determined by NOE due to poor S/N in the 1-D NOE experiments.[18]

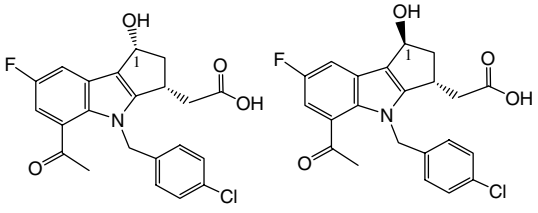


Figure 2. Chemical structures of **M3** and **M5**.

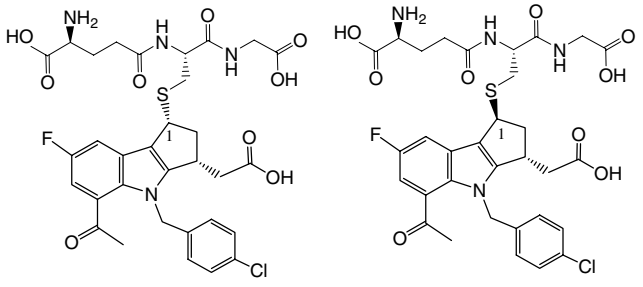


Figure 4. Chemical structures of GSH adducts of **3**, **Ma** and **Mb**.

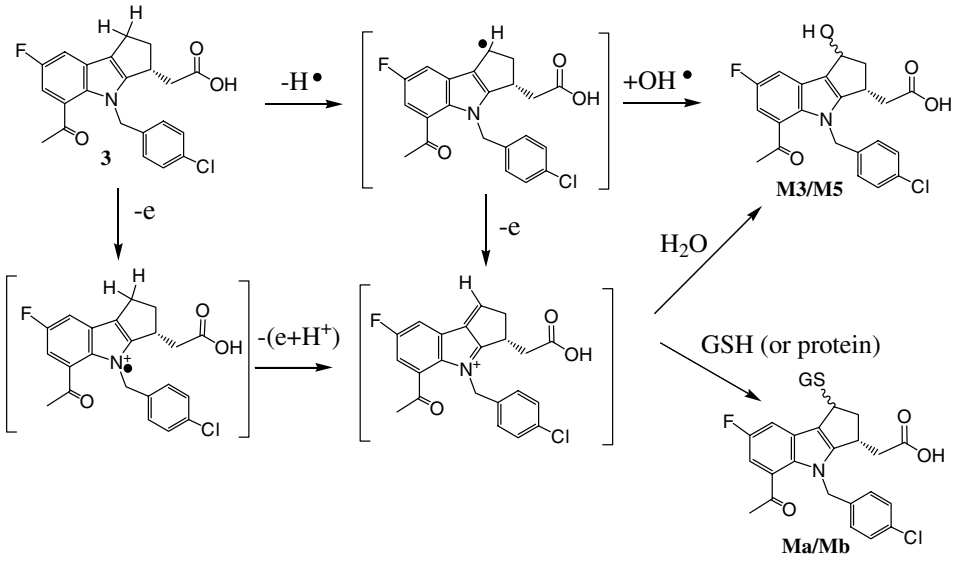


Figure 5. Proposed mechanism leading to **M3/M5** and **Ma/Mb** formation.

Interestingly, **3** contains a 3-alkyl-indole sub-structure. 3-Methyl-indole (3MI) is a pneumotoxic metabolite of tryptophan fermentation known to cause lung injury and covalent binding in vivo in goats as well as in vitro covalent binding in lung and liver microsomes from various species.[19–21] As observed for **3**, addition of millimolar quantities of GSH led to decreases in the extents of in vitro lung and liver covalent binding for 3MI in parallel with formation of GSH adducts, including a 3-[(glutathion-*S*-yl)-methyl]indole.[21–23] A mechanism implicating CYP-catalyzed dehydrogenation, most likely via hydrogen atom abstraction and subsequent one-electron oxidation, leading to formation of the electrophilic imine methide species, 3-methyleneindolenine, was postulated.[23,24] Considering the structural similarities of **3** with 3MI and of **Ma/Mb** with the 3-[(glutathion-*S*-yl)-methyl]indole of 3MI, it is highly probable that a similar mechanism would be involved in the bioactivation of **3** (Fig. 5). Analogous observations and conclusions were drawn for Zafirlukast, which also contains a 3-alkyl-indole moiety.[25]

GSH trapping experiments with unlabeled DP1 antagonists were then used as a surrogate assay to evaluate their propensity to generate reactive intermediates in rat and human liver microsomes. Following 60 min incubation of compounds **1**–**7** in the presence of rat or human liver microsomes, +/−NADPH, and +/−GSH, HPLC-PDA analysis of quenched samples was performed and presence of GSH adducts was determined by comparing chromatographic profiles of incubates +/−GSH.

As shown in Table 2, GSH adducts were observed only for compounds **3**, **4**, and **5**, and higher levels of GSH adducts were detected in rat compared to human microsomal incubations for all three compounds, in agreement with in vitro covalent binding results previously reported for **3**. However, such a high amount of GSH adduct formation should in theory translate into high in vitro covalent binding values (e.g., 4800 and 1755 pmol-eq/(mg at 1 h) for **3** in rat and human, respectively), if all reactive intermediates trapped by GSH would react with liver microsomal proteins in GSH deficient incubates. Similar observations were made for 3MI in lung and liver microsomal incubations. It was postulated that GSH may act directly on bioactivation enzymes to stimulate production of a reactive intermediate or it may serve to protect activating enzymes (CYPs) from destruction by trapping any electrophilic metabolites produced.[22] Minimal CYP3A4 time-dependent inhibition was observed for **3** in human liver microsomes (results not shown). Another possibility could be that reactive intermediates of **3**, **4**, and **5** may have a higher propensity to bind covalently to GSH than to liver microsomal proteins in vitro, because of the presence of a soft nucleophilic moiety (thiol) and the smaller chemical structure of GSH.

Table 2. Extents of GSH adduct formation in rat and human liver microsomal incubations under oxidative conditions for **1**–**7**

Compound	R^1	R^2	R^3	Rat	Human
1	SO_2Me	CH(OH)Me	H	<0.5%	<0.5%
2	SO_2Me	COMe	H	<0.5%	<0.5%
3	F	COMe	H	41%	15%
4	F	Br	H	22%	5%
5	F	CH(OMe)Me	H	44%	30%
6	F	SO_2Me	H	<0.5%	<0.5%
7	F	SO_2Me	Me	<0.5%	<0.5%

Radiolabeled analogs of **4**–**7**, tritiated at the methylsulfone moiety (**6** and **7**) or with ^{14}C at the carboxylic acid group (**4** and **5**), were prepared and tested in the in vitro liver microsomal covalent binding assay.[13,14] As expected, based on GSH trapping experiments, higher extents of labeling were obtained for **4** and **5**, whereas **6** and **7** showed low levels of covalent binding (<50 pmol-eq/(mg at 1 h); Table 3). When these results

Table 3. In vitro liver microsomal covalent binding (pmol-eq/(mg at 1 h))

Compound	R^1	R^2	R_3	Rat	Human
4	F	Br	H	460 ± 8	33 ± 1
5	F	CH(OMe)Me	H	301 ± 7	156 ± 11
6	F	SO_2Me	H	33 ± 1	16 ± 1
7	F	SO_2Me	Me	27 ± 3	27.8 ± 0.4

were put into perspective with the electron withdrawing potential (σ)[26] of substituents at C-5 or C-7 on the indole core, compounds such as **1**, **2**, **6**, and **7** having a strong electron withdrawing group (methylsulfone; $\sigma = 0.73$) gave lower protein labeling than their analogs differing by the presence of moieties with lower σ (fluorine or bromine; $\sigma = 0.15$ and 0.26, respectively). The current SMR would relate the electron density of the indole ring to the propensity of DP1 antagonists to cause liver microsomal protein labeling. An electron deficient system would disfavor the formation of cationic reactive intermediates (see Fig. 5), leading to lower levels of protein labeling or GSH trapping.

Certain acyl-glucuronides are known to be implicated in the formation of covalent adducts with proteins via either a trans-acylation mechanism, or a Shiff base.[27,28] Assays have been implemented to monitor the potential reactivity issues for discovery of compounds suspected to form acyl-glucuronide metabolites, and it was shown that non-steroidal anti-inflammatory drugs (NSAIDS) containing an acetic acid group were more prone to acyl-glucuronide migration and therefore, more reactive.[29,30] Considering the presence of acetic acid moiety in molecules from this DP1 antagonist series, further studies were initiated to evaluate the reactivity of their acyl-glucuronide metabolites in hepatocytes, a glucuronidation-competent in vitro model.

First, compounds **1**–**6** were incubated at 10 μM (1 μCi/mL) in presence of fresh rat and human hepatocytes (0.25×10^6 cells in 0.25 mL of Krebs–Henseleit buffer) at 37 °C under 95/5 O_2:CO_2 for 2 or 120 min. Then, covalent adduct formation was determined using the Brandel cell harvester technique. Interestingly, lower levels of protein labeling were detected for compounds with the methylsulfone moiety (**1**, **2**, and **6**) than those with more electron-rich indole rings (**3**, **4**, and **5**; Table 4).

Moreover, the extent of covalent labeling was generally lower in hepatocytes than that reported in liver microsomes under oxidative conditions. In vitro incubates were analyzed by HPLC-PDA-MS and major metabolites were glucuronidation and mono-oxidation products and minor levels of glucuroconjugated mono-oxidative metabolites were detected, as reported previously for **6** (MK-0524).[12,31,32] No glutathione adducts were detected. When incubates were left at room temperature, the glucuronide conjugates led to formation of three other conjugates, as determined by HPLC-PDA-MS, suggesting potential acyl-migration. The structure of the glucuronide conjugate of **6** was later confirmed as being the acyl-glucuronide.[31] The hepatocyte covalent binding assay was repeated for **6** in presence of dog hepatocytes, where high levels of glucuronidation were obtained (77% of total area), but this did not translate into increased levels of covalent protein labeling (24 pmol-eq/(mg per h); $n = 2$). Since only minor levels of oxidative metabolites of **6** were formed and it is known, based on microsomal experiments, that minimal covalent binding is associated with their formation, these results clearly highlight the low propensity of these acyl-glucuronides to react with proteins. The presence of glucuronidation as an alternative metabolic pathway to oxidative metabolism as well as millimolar levels of natural trapping agents inside the cells could in part explain the lower in vitro protein labeling observed in hepatocytes as compared to liver microsomal incubations under oxidative conditions for compounds such as **3**, **4**, and **5**.

Table 4. In vitro covalent binding in hepatocytes (pmol-eq/(mg per h))

Compound	R^1	R^2	Rat	Human
1	SO_2Me	CH(OH)Me	9 ± 4	<5 ± 0.4
2	SO_2Me	COMe	8 ± 0.4	5 ± 0.5
3	F	COMe	45 ± 19	16 ± 4
4	F	Br	74 ± 49	33 ± 7
5	F	CH(OMe)Me	109 ± 5	43 ± 2
6	F	SO_2Me	38 ± 0.4	15 ± 1

The in vivo covalent binding of **3** and **6** was assessed in rats upon per os dosing of 10 mg/kg (100 μCi/kg). Following plasma and liver tissue collection 24 h post-dose, liver homogenates and plasma proteins were precipitated with acetone, washed repeatedly with methanol/diethyl ether mixture (3:1) until minimal residual radioactivity was extracted.[9] The protein pellet was solubilized and protein binding determined by scintillation counting. Minimal levels of covalent binding were observed in vivo for both compounds (<10 pmol-eq/mg).[31] An excretion study was also performed in bile duct cannulated rats (5 mg/kg (100 μCi/kg) of **3** or **6** iv) and multiple glucuroconjugates, most likely acyl-migration products, were the main products excreted in bile, along with low levels of parent, oxidative, and glucuroconjugated oxidative metabolites. This further demonstrates that acyl-glucuronide formation for **3** and **6** did not translate into labeling issues and that glucuronidation, as an alternative metabolic pathway, may diminish formation of reactive oxidative metabolites in vivo.

In summary, some DP1 antagonists containing a 3-alkyl-indole substructure were shown to cause protein labeling in presence of rat and human liver microsomes under oxidative conditions. Presence of GSH as a trapping agent led to reduction of in vitro covalent binding and to concomitant formation of two glutathione adducts at the C-1 of the cyclopentenyl ring, suggesting the potential implication of an iminium methide reactive species. GSH trapping experiments and in vitro labeling assays confirmed that the presence of a strong electron withdrawing group was necessary to abrogate in vitro covalent binding. Further in vitro (fresh hepatocytes)

and in vivo (rat) studies clearly showed that acyl-glucuronide formation did not translate into covalent binding. Protective mechanisms, such as reactive metabolite scavengers, or the presence of non-oxidative alternative metabolic pathways, such as phase II biotransformation, may prevent or minimize protein covalent adduct formation both in hepatocytes and in vivo for DP1 antagonists from this indole series.

Acknowledgments

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16. HPLC conditions for HPLC-NMR analysis: YMC-ODS-A 4.6 × 150 mM 3 μm, 1 mL/min; mobile phase composed of 20 mM ammonium acetate-d_7 in D_2O + 5% CD_3CN (A) and 89:11 CD_3CN:D_2O (B); Gradient: 6–55% B in 14.5 min, to 100% B at 15 min, then isocratic at 100% B for 1 min; λ 254 nm.
17. (**M3**): ^{1}H NMR (500 MHz, D_2O): δ 7.47 (dd, J = 9.01, 2.54 Hz, 1H); 7.16 (d, J = 8.17 Hz, 2H); 7.10 (dd, J = 9.97, 2.39 Hz, 1H); 6.55 (d, J = 8.20 Hz, 2H); 5.43–5.40 (m, 1H); 5.24–5.14 (m, 2H); 3.76–3.71 (m, 1H); 3.41 (t, J = 6.93 Hz, 1H); 2.89 (t, J = 7.03 Hz, 1H); 2.61–2.53 (m, 1H); 2.52–2.46 (m, 1H). (**M5**): ^{1}H NMR (500 MHz, D_2O): δ 7.46 (dd, J = 9.02, 2.43 Hz, 1H); 7.15 (d, J = 8.21 Hz, 2H); 7.10 (dd, J = 9.82, 2.43 Hz, 1H); 6.54 (d, J = 8.21 Hz, 2H); 5.24–5.13 (m, 3H); 3.49–3.44 (m, 1H); 3.08–3.03 (m, 1H); 2.66 (dd, J = 14.84, 3.86 Hz, 1H); 2.32 (dd, J = 14.92, 10.62 Hz, 1H); 2.15–2.09 (m, 1H).
18. (**Ma**): ^{1}H NMR (600 MHz, CD_3CN): δ 7.49 (dd, J = 8.82, 2.58 Hz, 1H); 7.17 (d, J = 8.29 Hz, 2H); 7.14 (dd, J = 9.73, 2.58 Hz, 1H); 6.54 (d, J = 8.20 Hz, 2H); 5.21–5.14 (m, 2H); 4.57 (td, J = 6.27, 1.64 Hz, 1H); 4.42 (dd, J = 8.24, 5.53 Hz, 1H); 3.89–3.81 (m, 2H); 3.75–3.68 (m, 2H); 2.89 (dd, J = 13.70, 5.55 Hz, 1H); 2.84 (dd, J = 13.67, 8.28 Hz, 1H); 2.78 (t, J = 6.03 Hz, 2H); 2.72 (dd, J = 16.37, 3.82 Hz, 1H); 2.45–2.36 (m, 3H); 2.06–1.99 (m, 5H). (**Mb**): ^{1}H NMR (600 MHz, CD_3CN): δ 7.42 (dd, J = 8.82, 2.55 Hz, 1H); 7.16 (d, J = 8.21 Hz, 2H); 7.14 (dd, J = 9.73, 2.51 Hz, 1H); 6.54 (d, J = 8.18 Hz, 2H); 5.18 (s, 2H); 4.57 (dd, J = 8.51, 5.40 Hz, 1H); 4.47 (dd, J = 7.51, 1.70 Hz, 1H); 3.91–3.83 (m, 2H); 3.72 (t, J = 6.55 Hz, 1H); 3.61 (t, J = 9.09 Hz, 1H); 3.25 (dt, J = 14.22, 7.78 Hz, 1H); 3.11 (dd, J = 13.80, 5.35 Hz, 1H); 2.86 (dd, J = 13.73, 8.61 Hz, 1H); 2.78 (dd, J = 15.91, 4.23 Hz, 1H); 2.67 (dd, J = 16.46, 10.68 Hz, 1H); 2.43 (t, J = 7.65 Hz, 2H); 2.32 (d, J = 14.20 Hz, 1H); 2.09–2.03 (m, 2H); 2.01 (s, 3H).
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