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# In vitro biotransformations of the prostaglandin $D_2$ (DP) antagonist MK-0524 and synthesis of metabolites

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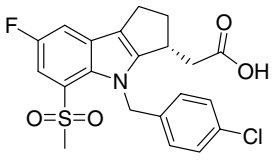
**Abstract**—Metabolites of the potent DP antagonist, **MK-0524**, were generated using in vitro systems including hepatic microsomes and hepatocytes. Four metabolites (two hydroxylated diastereomers, a ketone and an acyl glucuronide) were characterized by LC–MS/MS and $^1$H NMR. Larger quantities of these metabolites were prepared by either organic synthesis or biosynthetically to be used as standards in other studies. The propensity for covalent binding was assessed and was found to be acceptable (<50 pmol-equiv/mg protein).


Prostaglandin $D_2$, a major cyclooxygenase-derived prostanoid is implicated in congestion, inflammatory processes, and nicotinic acid-induced flushing.[1–3] **MK-0524**, [(3*R*)-4-(4-chlorobenzyl)-7-fluoro-5-methylsulfonyl)-1,2,3,4-tetrahydrocyclopenta[*b*]indol-3-yl)acetic acid)], is a potent and selective prostaglandin $D_2$ receptor (DP) antagonist that has been shown to inhibit antigen-induced increases in nasal resistance in animals[4] and nicotinic acid-induced flushing in mice and humans.[3]

As a part of drug discovery and development, the metabolic profiles of new drug candidates must be characterized. Drug metabolites are the products of enzymatic modifications such as oxidation or conjugate formation.



**MK-0524**

Intermediates in the metabolic reactions, or the products themselves, may be reactive and give rise to covalent protein binding. Evaluation of this is important since covalent protein modification may lead to unwanted toxicities or idiosyncratic reactions in humans.[5]

In order to generate metabolites for characterization, incubations of **MK-0524** were conducted under standard conditions using suspended hepatocytes or hepatic microsomal incubations (supplemented with NADPH or uridinediphospho-glucuronic acid (UDPGA)) from several species including human.[6,7] All incubates were analyzed by reverse-phase liquid chromatography-mass spectrometry on a 2790 HT liquid chromatograph coupled to a Waters 996 photodiode array detector and a Micromass Quattro LC triple quadrupole mass spectrometer operated in negative ion mode. Figure 1 shows the UV-chromatogram of a rat hepatocyte incubation.

HPLC–MS analysis indicated that two major metabolites, **M1** and **M4**, were a result of oxidation of **MK-0524** because the observed $[M-H]^-$ ions were 16 Da higher than **MK-0524**. Another oxidative metabolite (**M3**) was also observed with a mass 14 Da higher than **MK-0524**. MS fragmentation patterns for **M1**, **M3**, and **M4** were not diagnostic enough to pinpoint the sites of oxidation. In the hepatocyte incubations, conjugated

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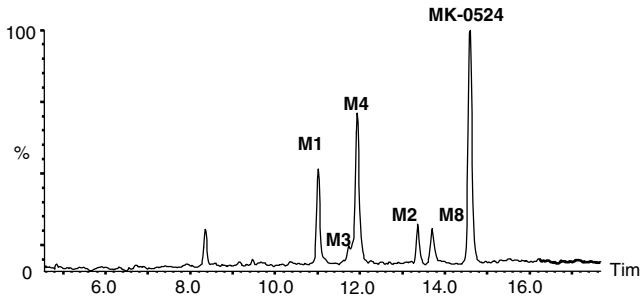



**Figure 1.** HPLC of a rat hepatocyte incubation of **MK-0524**. The incubation was conducted using 50 μM **MK-0524** and $10^6$ cells for 2 h at 37 °C. HPLC analysis was performed on a YMC ODS-A S5 column (4.6 × 150 mm) with UV detection at 305 nm. The mobile phase flow rate was 1 mL/min with a gradient for which solvent A was 20 mM ammonium acetate, 5% methanol in water and B was acetonitrile. The acetonitrile was increased from 5% to 65% over 17.5 min. The peak at 8.2 min was present in blank incubations and is not related to **MK-0524**.

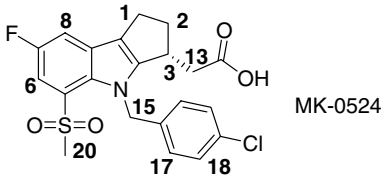
metabolites were also observed (**M2** and **M8**). HPLC–MS analysis indicated a 176 Da mass shift increment compared to **MK-0524**, indicating that glucuro-conjugates were formed. The mass spectrometric fragmentation pattern was not diagnostic enough to identify the site of conjugation. Dog and human hepatocyte incubations gave similar profiles. Hepatic microsomal incubations in various species (rat, rabbit, dog, sheep, squirrel monkey, rhesus monkey, and human) produced the oxidative metabolites in the presence of NADPH and the glucuronide in the presence of UDPGA, as assessed by HPLC–MS.

Incubations of **MK-0524** with microsomes expressing individual recombinant cytochromes P450 (rCYPs, GENTEST Corp., Woburn, USA) were performed under standard conditions.[7] CYP3A was by far the most active in producing **M1**, **M3**, and **M4**, while CYP1A and 2C9/19 also contributed to the formation of **M1** and **M4**, respectively. A CYP3A4 bioreactor[8] was therefore utilized to produce milligram quantities of **M1** and **M4** for $^1$H NMR characterization and biological testing. The bioreactor contained human CYP3A4, oxidoreductase (OR), and cytochrome $b_5$ ($b_5$) expressed with the baculovirus-SF-9 cell expression system. Typically, a 2 L culture of SF-9 cells was incubated at 27 °C and infected with three viruses expressing the CYP3A4, OR, and $b_5$ genes and 24 h post-infection, hemin was added. At 24 h post-addition of hemin, **MK-0524** was added and the culture was incubated at 27 °C for another 24 h or until the cell viability decreased below 50%. In a 2 L culture, which consisted of a 25 μM solution of **MK-0524** (22 mg), a turnover of 80% was achieved, which consisted of a mixture of 35% **M1**, 2% **M3**, and 43% **M4**, as indicated by reverse-phase HPLC–UV. Because the metabolites were partitioned between media and cells, the latter were harvested by centrifugation. The supernatant was set aside and the cell pellet was extracted with a 1:1 mixture of acetonitrile and water.

The metabolites were then isolated by using a combination of solid-phase extraction and preparative HPLC. Specifically, for preparative HPLC, a YMC ODS-AM column (50 × 20 mm) with 20 mL/min mobile phase flow rate was used. Electrospray mass spectrometric detection on a Micromass ZMD mass spectrometer was used to automate peak detection and collection. Prior to evaporation of the purified fractions, the pH was raised to ~9 to prevent metabolite degradation. Specifically, 200 μL of 8% aqueous ammonium hydroxide was added per 20 mL of HPLC eluant. Once the volume had been reduced to half by rotary evaporation, the remaining solvent was removed by lyophilization.

The $^1$H NMR spectra of the resulting solids were obtained on a Varian 600 MHz NMR. Initial attempts indicated that the metabolites were not stable in DMSO-$d_6$, however, 2 mM Tris-$d_{11}$ in $D_2O$ was used successfully (pH ~9.5). **MK-0524** was fully assigned and compared to the $^1$H NMR spectrum of **M1** and **M4**. All aromatic and benzylic protons were present in both metabolites (Table 1). The two protons at position 1 were replaced by a single proton shifted down field by more than 2 ppm, consistent with hydroxylation at carbon 1. The structures of **M1** and **M4** were proposed to be hydroxylated materials, as shown in Figure 2, based on these NMR data and the literature precedent of 3-methylindole oxidation.[9] The absolute stereochemistry of the hydroxylation products **M1** and **M4** could be determined by comparing vicinal proton coupling constants and molecular models of the metabolites. The vicinal coupling constants between the remaining proton at position 1 and the two geminal protons at position 2 were 6.4 and 3.4 Hz for **M1** and 6.9 and 1.3 Hz for **M4**. The calculated coupling constants from the molecular models allowed the identification of absolute stereochemistry of the new hydroxyl groups on **M1** and **M4** to

**Table 1.** $^1$H NMR chemical shift and multiplicity data for **MK-0524** and oxidative metabolites



MK-0524

| Protons | MK-0524 | M1 | M2 | M3 |
|---|---|---|---|---|
| **1a** | 2.91 (dd) | 5.49 (br s) | — | 5.28 (dd) |
| **1b** | 2.73 (m) | — | — | — |
| **2a** | 2.69 (m) | 2.57 (m) | 3.21 (dd) | 3.03 (m) |
| **2b** | 2.23 (m) | 2.42 (m) | 2.64 (d) | 1.97 (d) |
| **3** | 3.42 (m) | 3.54 (m) | 3.60 (m) | 3.25 (m) |
| **6** | 7.50 (d) | 7.65 (dd) | 7.59 (dd) | 7.66 (dd) |
| **8** | 7.48 (d) | 7.57 (dd) | 7.71 (dd) | 7.57 (dd) |
| **13a** | 2.51 (dd) | 2.48 (dd) | 2.58 (dd) | 2.58 (dd) |
| **13b** | 2.23 (m) | 2.03 (dd) | 2.17 (dd) | 2.24 (dd) |
| **15a** | 5.97 (d) | 5.91 (d) | 5.84 (m) | 5.88 (d) |
| **15b** | 5.68 (d) | 5.60 (d) | 5.79 (m) | 5.63 (d) |
| **17** | 6.70 (d) | 6.63 (d) | 6.74 (d) | 6.61 (d) |
| **18** | 7.20 (d) | 7.20 (d) | 7.2 (d) | 7.19 (d) |
| **20** | 2.98 (s) | 2.99 (s) | 2.76 (s) | 2.99 (s) |

All samples were dissolved in 2 mM Tris-$d_{11}$ in $D_2O$. Numbering of carbons is indicated below.

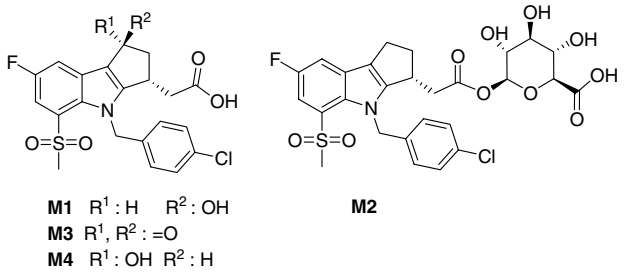



**Figure 2.** Structures of oxidative and conjugative metabolites of **MK-0524**.

be *syn* and *anti*, to the carboxyl-containing side chain, respectively.

**M1** and **M4** were found to interconvert under even slightly acidic conditions such as a mixture of 14 mM aqueous ammonium acetate and acetonitrile (65:35) at ambient temperature (Fig. 3). An equilibrium was reached after approximately 48 h, yielding an **M1** to **M4** ratio of 2:3. However, if **M1** and **M4** were dissolved in an acetonitrile solution containing ammonium hydroxide (0.01% v/v) minimal interconversion was observed after 24 h (<5%). Similar stability was observed in rat plasma. This is consistent with the fact that plasma becomes basic under ambient conditions, reaching pH values greater than 8 within a few hours of collection.[10]

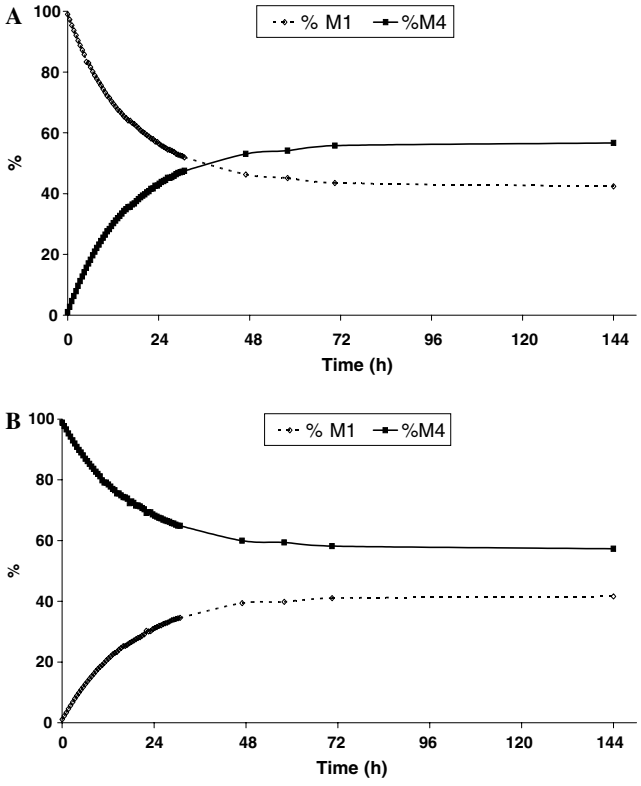



**Figure 3.** Stability study of **M1** (A) and **M4** (B) in 14 mM aqueous ammonium acetate and acetonitrile (65:35) at ambient temperature. Analysis was conducted by HPLC–UV.

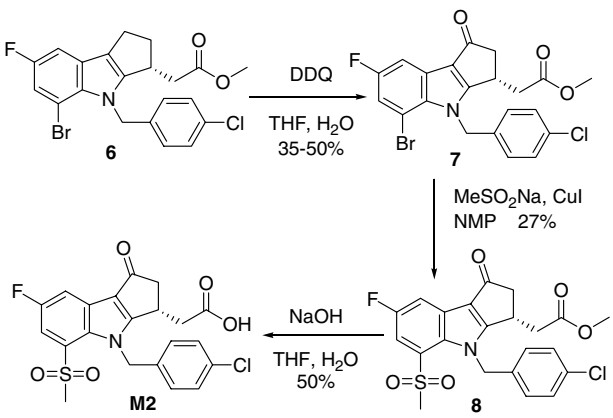
The small amount of **M3** generated in the CYP 3A4 bioreactor precluded isolation for NMR characterization. However, based on MS data, it was speculated that **M3** was the ketone resulting from oxidation of **M1** and/or **M4**.

A standard of **M3** was prepared by the synthesis as shown in Scheme 1. Briefly, a DDQ oxidation of the ester **6**[11] afforded the ketone **7**. Copper coupling of the bromine with methanesulfinic acid sodium salt yielded compound **8**. Saponification of **8** afforded the synthetic metabolite, **M3**. The structure of **M3** was confirmed by an HPLC co-injection of the authentic standard with a rat hepatocyte incubation.

Attempts to produce the alcohol metabolites, **M1** and **M4**, by reduction of **M3** were unsuccessful, presumably due to the electronic nature of the vinylogous indole amide nitrogen. Also, attempts to oxidize **MK-0524** using a variety of synthetic protocols failed to yield the hydroxylated metabolites, thus confirming the necessity of the CYP3A4 bioreactor synthesis, described above.

Maximal production of the glucuronide conjugate, **M2**, was achieved using dog hepatic microsomes fortified with UDPGA.[12] Other species gave lower turnover. A 25 mL incubation containing 2 mg/mL of dog hepatic microsomal protein and 1.1 mg **MK-0524** (100 μM) afforded a 91% yield of **M2** after 2 h, as indicated by reverse-phase HPLC–UV. The product was isolated using a combination of solid-phase extraction and preparative HPLC conditions similar to those described above, except that the pH was not adjusted. $^1$H NMR of the isolated material at 600 MHz (DMSO-$d_6$) showed characteristic resonance for the anomeric protons at ~5.3 ppm. The coupling constant was 8.1 Hz, consistent with the *trans*-diaxial coupling expected for the formation of a β-linked *O*-acyl glucuronide as shown in Figure 2.[12] Synthetic acyl glucuronide, **M2**, was produced according to previously described methodology.[13]

Based on the known phenomena of acyl migration observed with β-linked *O*-acyl glucuronides,[14] **M8** is proposed to be the 2-*O*-linked acyl glucuronide. LC–MS




**Scheme 1.** Synthesis of the ketone metabolite **M2**.

**Table 2.** Covalent protein modification in vitro and in vivo

| Test system | Rat | Human |
|---|---|---|
| Microsomes (with NADPH)[a] | 33 | 16 |
| Hepatocytes[b] | 38 | 14 |
| In vivo[c]: | | |
| Liver (24 h) | 5 | |
| Plasma (24 h) | ND | |

ND, not detected (limit of detection 5 pmol-equiv/mg protein).
[a,b]Values reported as pmol-equiv binding/mg protein at 1 h.[18]
[c]Values reported as pmol-equiv binding/mg protein.[18]

spectra of this metabolite gave mass spectral data indicating a molecular weight of M+176 and microsomal incubations to form the glucuronide initially yielded only **M2**. **M8** was produced upon standing, with concomitant loss of **M2**, consistent with it being an **M2** degradant. A stability study was performed at 37 °C under slightly basic conditions (1 mM **M2** in 125 mM phosphate buffer (pH 7.4) and DMSO-$d_6$ (70:30). **M2** converted to **M8** and **MK-0524** with a degradation half-life of 95 min. A similar half-life has been reported for the acyl glucuronide of indomethacin in phosphate buffer at pH 7.4.[14] It is known, however, that β-linked *O*-acyl glucuronides are stable under acidic conditions[15] and this was found to be the case with **M2** (data not shown).

The formation of CYP3A4-mediated oxidative metabolites on the 3-methylene indole core[9,16] and formation of the acyl glucuronide and acyl migration products[14,17] raised concerns that reactive species may be formed as a result of metabolism. A tritiated analog of **MK-0524** was synthesized[4] and tested for covalent protein modification according to standard procedures in liver microsomes fortified with NADPH and in hepatocytes using a semi-automated membrane harvester to trap precipitated proteins.[5,18] Despite the fact that significant amounts of 3-methylene indole hydroxylation metabolites were formed in microsomal incubations and acyl glucuronide and rearranged glucuronides were formed in hepatocytes (cf. Fig. 1), the levels of binding were below the target threshold of <50 pmol-equiv/mg protein at 1 h (Table 2). This could be due to substitution of the indole core with electron-withdrawing fluorine and methyl sulfone moieties.[4] No tritiated water was detected by HPLC in the incubates indicating that the amount of covalent binding was not underestimated due to loss of the tritium label.

In rats, after a 10 mg/kg po dose of tritiated **MK-0524**, covalent binding was measured in liver homogenate and plasma at 24 h post-dose. The procedure involved exhaustive extraction of the protein pellet formed by acetonitrile precipitation of the plasma or liver homogenates followed by exhaustive extraction with methanol-ether (3:1).[19] Covalent binding was shown to be low or not detectable (Table 2). According to these results, there is no reason to believe that reactive metabolite intermediates should mediate adverse events via covalent modification of proteins.

The biosynthetic and synthetic metabolites prepared as described herein were used as standards for additional **MK-0524** metabolism studies.[20–22]

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