ACS Medicinal Chemistry Letters

Letter

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Synthesis, in Vitro Covalent Binding Evaluation, and Metabolism of ^{14}C-Labeled Inhibitors of 11β-HSD1

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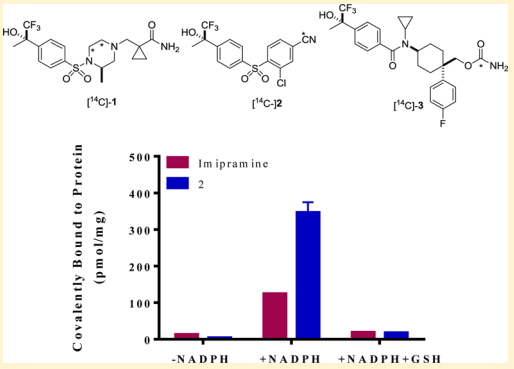
Supporting Information

ABSTRACT: In this letter, we reported the design and synthesis of three potent, selective, and orally bioavailable 11β-HSD1 inhibitors labeled with ^{14}C: AMG 456 (**1**), AM-6949 (**2**), and AM-7715 (**3**). We evaluated the covalent protein binding of the labeled inhibitors in human liver microsomes in vitro and assessed their potential bioactivation risk in humans. We then studied the in vitro mechanism of **2** in human hepatocytes and the formation of reactive intermediates. Our study results suggest that **1** and **3** have low potential for metabolic bioactivation in humans, while **2** has relatively high risk.



KEYWORDS: *11β-HSD1, 11β-HSD2, diabetes, metabolic syndrome, hydroxysteroid dehydrogenase, arylsulfonylpiperazine, diarylsulfone, 4,4-disubstituted cyclohexylbenzamides, radiolabeled inhibitor, covalent protein binding, bioactiviation, reactive metabolite*

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a key enzyme that acts as an NADPH-dependent reductase converting inactive glucocorticoids such as cortisone into their active form (e.g., cortisol) in specific tissues including liver, adipose, and brain.[1−4] However, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), an oxidase structurally related to 11β-HSD1, catalyzes the conversion of cortisol to the inactive cortisone using NAD^+ as a cofactor. Excessive levels of glucocorticoids greatly contributes to metabolic syndrome, including insulin resistance, visceral obesity, and type 2 diabetes.[5−9] Selective inhibition of 11β-HSD1 provides a promising therapeutic strategy for the treatment of obesity and the other elements of the metabolic syndrome. To date, several small molecule inhibitors of 11β-HSD1 have been tested in the clinic for the treatment of such conditions.[10−14]

Previously, we reported three series of potent, selective, and orally bioavailable 11β-HSD1 inhibitors exemplified by arylsulfonylpiperazine **1** (AMG 456),[15] diarylsulfone **2** (AM-6949),[16] and cyclohexylbenzamide **3** (AM-7715) (Figure 1).[17] All three compounds inhibited 11β-HSD1 significantly in the biochemical enzyme assay[18] with an IC_{50} of 0.5−0.9 nM but had no inhibition against HSD2 at a concentration of 10 μM. These molecules were also found to be extremely potent 11β-HSD1 inhibitors in the cell-based enzyme assays with human HEK 293 cells and fat cells.[18] Furthermore, like **1** and **2**,[15,16] compound **3** has a low clearance (CL = 0.12−0.41 L/h/kg) and excellent oral bioavailability (%F = 38−88) across three preclinical species (Table 1). Consistent with the ex vivo efficacy observed with **1** and **2**,[15,16] compound **3** achieved a nice dose-dependent inhibition of 11β-HSD1 compared to the vehicle in a cynomolgus monkey ex vivo pharmacodynamic model[19] when administered orally (Figure 2).

Nevertheless, small molecule drugs can undergo metabolic bioactivation.[20] Reactive drug metabolites may covalently bind to biological proteins to form a drug−protein adduct. The bioactivation is generally considered to be related to drug toxicity, although mechanism involved in the toxicity induced by the adduct is largely undefined.[21] In vitro covalent protein binding (CPB) study offers direct evidence of covalent binding of drug to proteins and is commonly used to measure bioactivation and assess potential toxicity risk in humans.[22,23] Radiolabeling a drug provides a convenient way to measure the metabolism of a drug and to quantify the extent of covalent binding to proteins. In this article, we describe the design and synthesis of the labeled 11β-HSD1 inhibitors **1**−**3**, their CPB evaluation, and the in vitro metabolism of **2** in human hepatocytes.

Results and Discussion. *Chemistry.* We recently reported the design and synthesis of nonradiolabeled **1**−**3**;[15−17] however, those synthetic routes were not suitable for the synthesis of radiolabeled compounds. Since carbon and hydrogen atoms are present in all drug molecules, ^{14}C and

Received: August 12, 2014
Accepted: September 23, 2014
Published: September 23, 2014

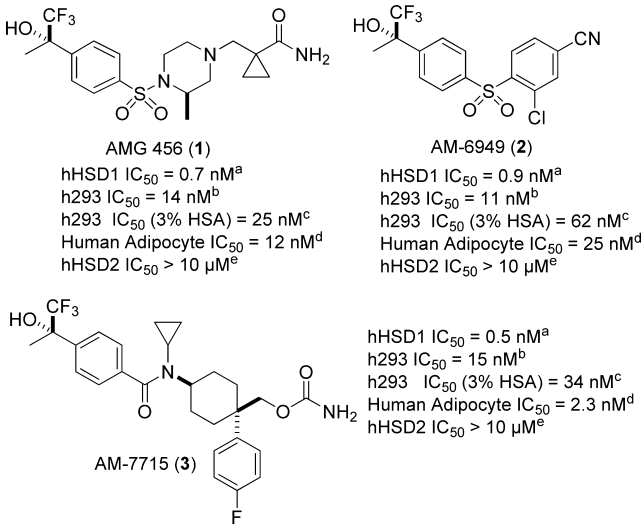


Figure 1. Chemical structures and potencies of **1**, **2**, and **3**. (a) IC_{50} in the human 11β-HSD1 biochemical scintillation proximity assay (SPA). (b) IC_{50} in the cell-based enzyme assays with human HEK 293 cells (serum free). (c) IC_{50} in the cell-based enzyme assays with human HEK 293 cells in the presence of 3% human serum albumin (HSA). (d) IC_{50} in the cell-based enzyme assays with human fat cells (serum free). (e) IC_{50} in the human 11β-HSD2 biochemical scintillation proximity assay. Values of IC_{50} are means of at least two determinations. Standard deviation was ±30%.

Table 1. Preclinical Pharmacokinetics of 3

	iv CL (L/kg/h)	Vdss (L/kg)	$T_{1/2}$ (h)	po F (%)	C_{max} (ng/mL)
rat[a,b]	0.041	2.3	43	72	1130
cyno[a,c]	0.029	1.0	27	38	211
dog[a,b]	0.012	1.2	78	88	1840

[a]iv dose (0.5 mg/kg) in 10% *N*,*N*-dimethylacetamide, 10% ethanol, 40% propylene glycol, and 40% water. [b]po dose (2.0 mg/kg) as a solution in 15% HPβCD in Ora-Plus. [c]po dose (2.0 mg/kg) as a suspension in 1% Tween 80/99% and 1% methylcellulose (4000 cps) in deionized water. Values are an average for three animals. Standard deviation was ±30%.

tritium (^{3}H) are widely used radioisotopes for labeling drug molecule. Because of the potential for label loss by metabolism,[24] we chose ^{14}C over tritium as a label isotope for the synthesis of radiolabeled compounds **1**–**3**. The strategies used to synthesize the radiolabeled **1**–**3** were (a) selecting appropriate label positions away from the metabolic soft spots; (b) design and synthesis of labeling precursors that allow ^{14}C incorporation; and (c) incorporating ^{14}C isotope at the end of the synthetic routes to minimize a number of radioactive intermediates in the synthesis. On the basis of the aforementioned strategies as well as the availabilities of ^{14}C labeling reagents, the label positions were designated and shown in the structures of [^{14}C]-**1**, [^{14}C]-**2**, and [^{14}C]-**3**, respectively. The synthesis of labeling precursors **9**, **16**, and **19** is described below in Schemes 1–3.

The synthesis of [^{14}C]-**1** and its labeling precursor **9** is outlined in Scheme 1. Sulfonylation of **4** with 4-acetylbenzenesulfonyl chloride provided the sulfonamide **5**. Treatment of **5** with trifluoromethyltrimethylsilane afforded the carbinol **6**, which was directly converted to the methanesulfonate **7**. Nucleophilic displacement of **7** with sodium azide, subsequent reduction of the azide, and alkylation of the resultant amine

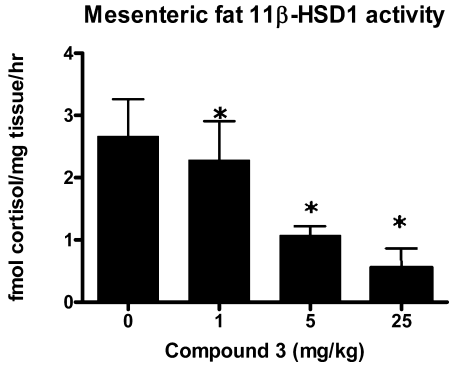
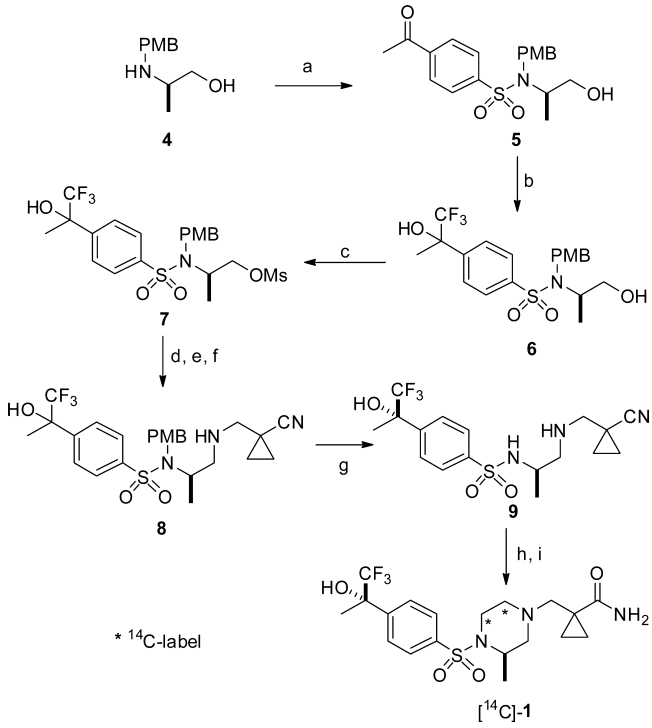


Figure 2. Compound **3** achieved a nice dose-dependent inhibition of 11β-HSD1 compared to the vehicle. *p < 0.07. Ex vivo 11β-HSD1 enzyme activity in intact mesenteric fat collected from cynomolgus monkeys dosed orally with compound **3**. Plasma and mesenteric fat samples were collected 4 h after **3** was administered orally in dosing vehicle (15% HPβCD in 50% Ora-plus). 11β-HSD1 enzyme activity was measured as the [^{3}H]-cortisol formed after 1 h incubation of fat samples in reaction buffer containing [^{3}H]-cortisone at 37 °C. Compound concentration in plasma was determined by LC–MS/MS. Average concentrations of **3** in plasma were measured to be 64 ng/mL (1 mg/kg), 524 ng/mL (5 mg/kg), and 4320 ng/kg (25 mg/kg), respectively.

Scheme 1. Synthesis of [^{14}C]-1[a]



[a]Reagents and conditions: (a) 4-acetylbenzene-1-sulfonyl chloride, triethylamine, 48%; (b) TMS–CF_3, TBAF, THF, 67%; (c) MsCl, triethylamine, CH_2Cl_2, 62%; (d) NaN_3, DMSO, 98%; (e) triphenylphosphine, THF, water, 79%; (f) 1-(iodomethyl)-cyclopropanecarbonitrile, *N*-ethyldiisopropyl amine, acetonitrile, 87%; (g) chiral separation, then ceric ammonium nitrate (CAN), acetonitrile, water, 15%; (h) [^{14}C]1,2-dibromoethane, K_2CO_3, acetonitrile; (i) KOH, *t*-BuOH, 90 °C, 23% from **9**.

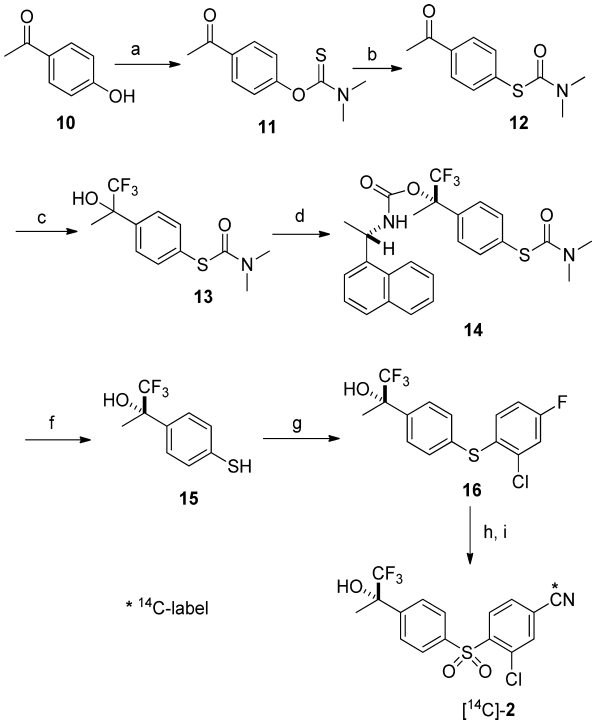
with 1-(iodomethyl)cyclopropane carbonitrile in acetonitrile provided a nitrile **8** as a mixture of diastereomers at the tertiary alcohol. The desired isomer was separated by chromatography

dx.doi.org/10.1021/ml500331y | ACS Med. Chem. Lett. 2014, 5, 1245–1250

on a chiral column, followed by removal of PMB group with ceric ammonium nitrate (CAN), to yield the diamine (labeling precursor **9**). Finally, [^{14}C]-**1** was obtained by alkylation of the precursor **9** with [^{14}C]1,2-dibromoethane, followed by basic hydrolysis of the cyano group.

The synthesis of [^{14}C]-**2** and its precursor **16** is detailed in Scheme 2. Reaction of phenol **10** with dimethylcarbamothioic

Scheme 2. Synthesis of [^{14}C]-2[a]

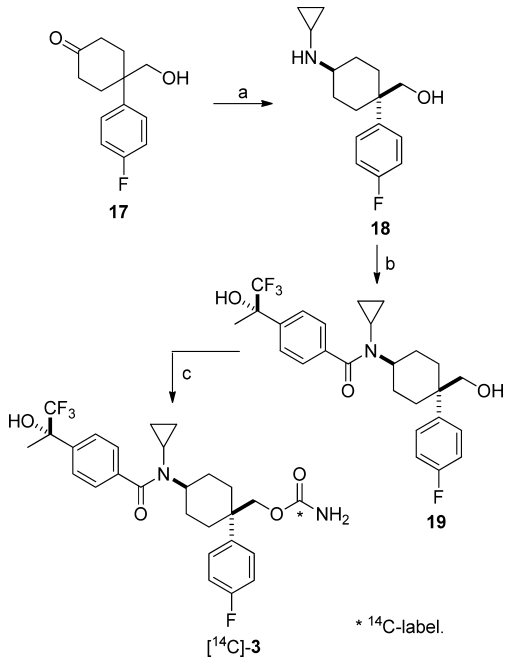


[a]Reagents and conditions: (a) dimethylcarbamothioic chloride, 1,4-diazabicyclo[2.2.2]octane (DABCO), DMF, 65 °C; 83%; (b) neat, 218 °C, 99%; (c) TMS−CF_3, TBAF, 94%; (d) (i) 4-nitrochloroformate, DMAP, CH_3CN; (ii) (*S*)-1-(naphthalen-1-yl)ethanamine, crystallization, 31%; (f) LiOH, 1,4-dioxane, H_2O, 100%; (g) 2-chloro-1,4-difluorobenzene, Cs_2CO_3, DMF, 110 °C, 74%; (h) *m*-CPBA, 82%; (i) [^{14}C]-KCN, 18-crown-6, DMF, 40%.

chloride in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) produced the carbamothioate **11**, which was transformed into the intermediate **12** by heating. Reaction of **12** with trifluoromethyltrimethylsilane in THF generated the carbinol **13**, which was successively treated with 4-nitrochloroformate and (*S*)-1-(naphthalen-1-yl)ethanamine to deliver a carbamate as a mixture of the two diastereoisomers at the tertiary alcohol. The desired isomer **14** was separated by fractional crystallization and hydrolyzed with lithium hydroxide to give the thiophenol **15**. The coupling of thiophenol **15** to 2-chloro-1,4-difluorobenzene in the presence of cesium carbonate yielded diphenylsulfide (labeling precursor **16**), which was subjected to *m*-CPBA oxidation to afford a sulfone intermediate. The preparation of [^{14}C]-**2** was achieved by replacement of the fluorine group in the sulfonyl benzene ring with [^{14}C]potassium cyanide.

The synthesis of [^{14}C]-**3** and its precursor **19** is described in Scheme 3. Reductive amination of **17**[17] with cyclopropylamine resulted in a 1:1.1 mixture of *cis*- and *trans*-cyclopropylamine intermediates, and the desired trans isomer **18** was separated by silica-gel chromatography. The *trans*-amine **18** was then

Scheme 3. Synthesis of [^{14}C]-3[a]



[a]Reagents and conditions: (a) cyclopropylamine, $NaBH(OAc)_3$, acetic acid, acetonitrile, 52%; (b) (*S*)-4-(1,1,1-trifluoro-2-hydroxypropan-2-yl) benzoic acid, EDC, HOAt, $NaHCO_3$, DMF, 44%; (c) [^{14}C] sodium cyanate, TFA, 40%.

coupled with (*S*)-4-(1,1,1-trifluoro-2-hydroxypropan-2-yl) benzoic acid[17] to afford the primary alcohol (labeling precursor **19**), which was treated with [^{14}C]sodium cyanate in the presence of trifluoracetic acid to provide [^{14}C]-**3**.

*In Vitro Covalent Protein Binding (CPB) Evaluation of [^{14}C]-**1**, [^{14}C]-**2**, and [^{14}C]-**3** in Human Liver Microsomes.* To measure drug bioactivation, an in vitro CPB study of **1**−**3** in human liver microsomes was carried out by incubating 10 μM of the labeled test article in the (−) or (+) NADPH-regenerating system and in the presence of both 1 mM NADPH and 1 mM glutathione for 1 h.[25] In the study, imipramine or naphthalene was used as a positive control and tested along with compounds [^{14}C]-**1**, [^{14}C]-**2**, and [^{14}C]-**3**. CPB of the labeled inhibitors or positive controls in the (−) NADPH was measured to be low in all cases (Figure 3), showing that their CPB contained trace level of NADPH-independent processes. In the presence of NADPH, the CPB of **1**, **2**, and **3** to human microsomal protein was determined to be 70, 370, and 15 pmol drug equiv/mg protein, respectively. Imipramine had moderate CPB of 124.5 pmol drug equiv/mg protein, while naphthalene showed relatively high CPB of 420 pmol drug equiv/mg protein, which are in the line with the data reported in the literature.[26] Both compounds **1** and **3** have a considerably low level of CPB compared to imipramine and naphthalene, respectively (70 pmol drug equiv/mg protein of **1** vs 125 pmol drug equiv/mg protein of imipramine in Figure 3a; 15 pmol drug equiv/mg protein of **3** vs 420 pmol drug equiv/mg protein of naphthalene in Figure 3c). Moreover, the CPB of **1** and **3** in human microsomes is also lower than or comparable to a proposed conservative threshold value of 50 pmol drug equiv/mg protein, an upper limit considered to be low risk by Evans et al.[22] These findings suggest that both **1** and **3** have low potential bioactivation risk in humans. In contrast, compound **2** exhibited a high level of CPB relative to imipramine (350 and

dx.doi.org/10.1021/ml500331y | ACS Med. Chem. Lett. 2014, 5, 1245−1250

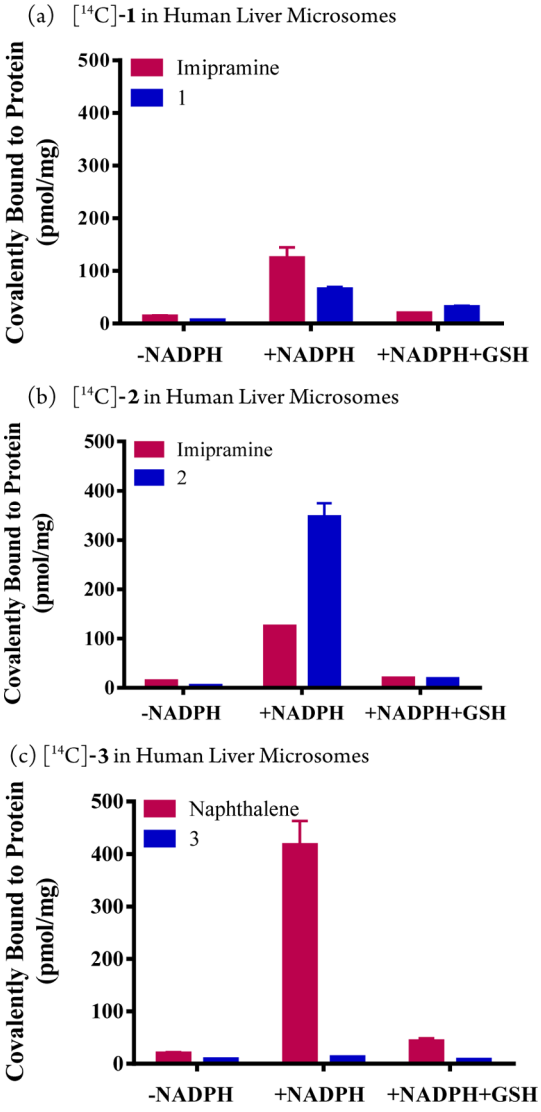


Figure 3. In vitro covalent binding evaluation of radioactivity to human liver microsomes upon incubation with [^{14}C]-labeled inhibitors at 10 μM concentration for 1 h. (a) [^{14}C]-**1** was incubated with human liver microsomes in the (−) or (+) of NADPH, and in the presence of both 1 mM NADPH and 1 mM glutathione, [^{14}C] imipramine was used as a positive control. (b) [^{14}C]-**2** was incubated with human liver microsomes in the (−) or (+) of NADPH, and in the presence of both 1 mM NADPH and 1 mM glutathione, [^{14}C] imipramine was used as a positive control. (c) [^{14}C]-**3** was incubated with human liver microsomes in the (−) or (+) of an NADPH, and in the presence of both 1 mM NADPH and 1 mM glutathione, [^{14}C] naphthalene was used as a positive control. CPB values are means of at least two determinations.

125 pmol drug equiv/mg protein for **2** and imipramine, respectively), indicating a high potential bioactivation risk of **2** in humans (Figure 3b). Furthermore, formation of chemically reactive intermediates was confirmed by a glutathione (GSH) trapping study. The high CPB of **2** in human microsomes was significantly reduced to 20 pmol drug equiv/mg protein by adding a natural trapping agent GSH (Figure 3b).

In Vitro Metabolism of [^{14}C]-2 in Human Hepatocyte Incubation. To identify the possible reactive intermediate, we decided to investigate the metabolic mechanism of **2** in human hepatocytes. After incubation of [^{14}C]-**2** with human hepatocytes, radiolabeled components were analyzed for the parent and its metabolites using tandem LC−MS/MS radiometric detection. M2 was identified as the major metabolite (5.3% of administered dose) along with M3 (1.3% of administered dose) and an *O*-glucuronide M1 (3.2% of administered dose) (Figure 4). In this study, a 9.8% total

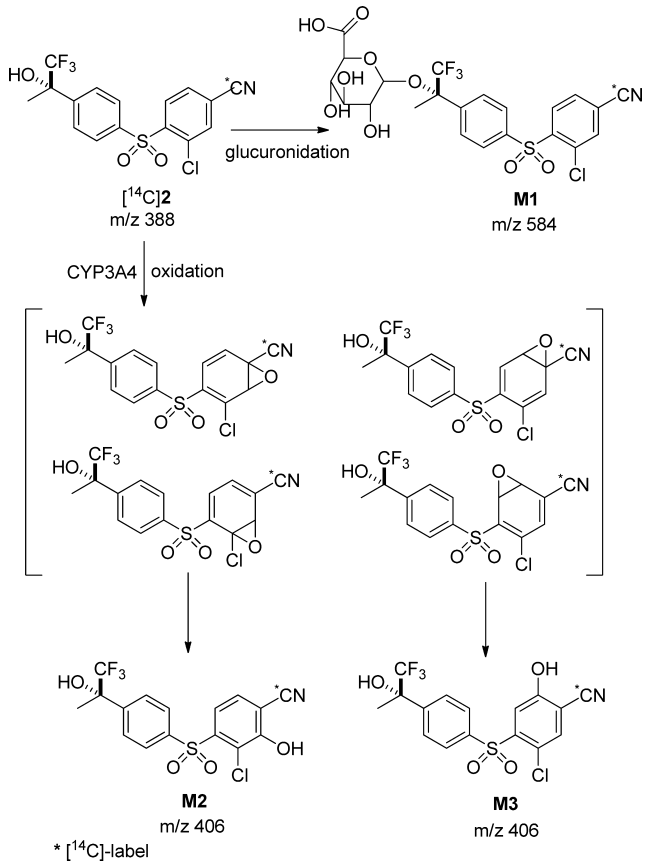


Figure 4. Major metabolites of [^{14}C]-**2** following incubation with human hepatocytes. Structures shown in bracket represent proposed reactive intermediates, which were not isolated.

turnover was observed upon incubation of **2** with human hepatocytes. Both metabolites M2 and M3 were isolated after incubation of **2** with human microsomes for 2 h. The chemical structures of M2 and M3 were resolved by comparing their radiochromatographic retention times, mass spectra, NMR proton chemical shift, and splitting patterns with those of **2** (see the Supporting Information). The reaction phenotyping study indicated that CYP3A4-mediated oxidation of **2** generated the highly electrophilic epoxides shown in brackets, which proceed by a 1,2-hydride shift followed by isomerization, leading to the hydroxyl metabolites M2 and M3. These data clearly indicated that **2** might cause metabolic activation in humans by the formation of reactive epoxides, which covalently modify proteins.

Conclusions. In summary, we successfully developed the synthetic routes to three potent [^{14}C]-labeled 11β-HSD1 inhibitors and used them for in vitro human microsomal protein covalent binding assessment. The covalent binding data suggest that both **1** and **3** have low potential for metabolic bioactivation in humans, while **2** has a relatively high risk for the associated liabilities of drug bioactivation. Furthermore, the metabolism of **2** in human hepatocytes in vitro was

dx.doi.org/10.1021/ml500331y | ACS Med. Chem. Lett. 2014, 5, 1245−1250

investigated, and the possible sites and metabolites were also researched.

■ ASSOCIATED CONTENT

Supporting Information

(i) In vitro biological assays. (ii) Cynomolgus monkey tissue ex vivo protocol. (iii) Covalent binding assays. (iv) Metabolite ID. (v) Synthesis and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Tim Carlson for helpful discussions.

■ ABBREVIATIONS

AcCN, acetonitrile; CAN, ceric ammonium nitrate; CL, clearance; *m*-CPBA, *m*-chloroperoxybenzoic acid; CYP3A4, cytochrome P450 3A4; DABCO, 1,4-diazabicyclo[2.2.2]octane; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide;; EDC, *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide; EtOAc, ethyl acetate; HPβCD, hydroxypropyl-beta-cyclodextrin; MsCl, methanesulfonyl chloride; MTBE, methyl *t*-butyl ether; TBAF, tetrabutylammonium fluoride; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMS-CF3, trimethyl-(trifloromethyl)silane

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(18) Experimental details of the in vitro biological assays can be found in the Supporting Information.

(19) Experimental details of cynomolgus monkey tissue ex vivo studies can be found in the Supporting Information.

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