Identification of Two Novel Metabolites of SCH 486757, a Nociceptin/Orphanin FQ Peptide Receptor Agonist, in Humans

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ABSTRACT:

The study of human metabolism of endo-8[bis(2-chlorophenyl)methyl]-3-(2-pyrimidinyl)-8-azabicyclo[3.2.1]octan-3-ol (SCH 486757) after a 200-mg oral dose of the drug to healthy volunteers in the first-in-human study is presented. The structural elucidation of two unique metabolites, which were detected in the process of metabolite characterization in human plasma and urine by liquid chromatography-mass spectrometry (LC-MS), is described. These metabolites (M27 and M34) were initially detected in human plasma at high levels (>35% of the LC-MS response of the parent drug). Additional LC-MS experiments (hydrogen/deuterium exchange and accurate mass measurement) were used to determine structures of metabolites. It was found that both metabolites were formed through a loss of the C–C bridge from the tropone moiety with the conversion into a substituted pyridinium compound. This metabolic process has not been reported previously. Because of the apparent high abundance of metabolites based on the LC-MS response, actual circulating amounts of these metabolites relative to the parent drug were determined semiquantitatively to evaluate their coverage in preclinical species. With the use of reference standards, it was shown that the LC-MS response of M27 and M34 in human plasma was much higher than that of the parent compound. Actual amounts of M27 and M34 metabolites were less than 5% of the level of the parent drug; therefore, additional assessment was not required.

Introduction

Cough is one of the most common reasons for patients to contact their health care providers. Although coughing is an important way to keep a throat and airways clear, excessive coughing, even without serious underlying reasons, may cause discomfort. Severe coughs or coughs that interfere with sleep may need medications, which usually include classic opioids such as codeine. These remedies have been available for decades but have significant side effects, such as sedation, dizziness, nausea and vomiting, constipation, and respiratory depression, limiting their use (Benyamin et al., 2008). Another liability of opioids is development of physical dependence, leading to the possibility of abuse (Benyamin et al., 2008). Most common over-the-counter medications are relatively ineffective for the treatment of cough; therefore, there is a need for novel effective antitussive agents with improved safety profile and no abuse potential.

Endo-8[bis(2-chlorophenyl)methyl]-3-(2-pyrimidinyl)-8-azabicyclo[3.2.1]octan-3-ol (SCH 486757) (structure shown in Fig. 1) is a potent nociceptin/orphanin FQ peptide receptor (NOP) agonist being evaluated for the treatment of cough. Like nociceptin, SCH 486757 is effective against both irritant-induced and mechanically induced cough, and its antitussive activity preclinically is comparable to that of standard antitussive drugs. The pharmacology of nociceptin is distinct from that of opioids in that nociceptin does not have significant binding affinity for the classic opioid receptors; in turn, opioid antagonists do not block the biological activity of nociceptin (Varty et al., 2008; Ho et al., 2009). The antitussive activity of SCH 486757 is mediated by NOP receptors rather than by opioid receptors. SCH 486757 is not expected to have the side-effect liabilities of opioid antagonists.

The study of biotransformation plays an important role in evaluation of safety of a drug especially when drugs such as antitussive drugs are intended for the general population. Given the current regulatory environment, early read-out of human metabolism is desirable; therefore, profiling of human metabolites as part of the first-in-human studies has become a standard practice in many laboratories. Urine and plasma samples from the top three dose levels in rising single/multiple dose studies are usually collected, and then selected samples are profiled for metabolites. Toxicological testing of SCH 486757 resulted in an adequate safety profile to allow human dosing. It was safe and well tolerated by young healthy volunteers at single doses up to 400 mg. For this work, urine and plasma samples from subjects given 100-, 200-, and 400-mg doses of NOP agonist or placebo were collected.

In this article, we present a study of the human metabolism of SCH 486757 with a particular emphasis on structural elucidation of two unique metabolites, which were detected in the process of metabolite characterization in human plasma and urine by liquid chromatography-mass spec-

ABBREVIATIONS: NOP, nociceptin/orphanin FQ peptide receptor; SCH 486757, endo-8[bis(2-chlorophenyl)methyl]-3-(2-pyrimidinyl)-8-azabicyclo[3.2.1]octan-3-ol; LC-MS, liquid chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; H/D, hydrogen/deuterium; ETOAc, ethyl acetate; MIST, metabolites in safety testing.
HPLC methods with an in-line radioactivity detector. Specific activity was 119 Ci/kg. Bile was collected for 8 h postdose and stored at 10°C until analysis. Bile samples were used for the development of the HPLC method and to assist with the structural identification of metabolites. A solution of an internal standard (ammoniated polyethylene glycol) was placed into a freezer overnight. After centrifugation, the supernatant was removed, and the remaining pellet was extracted again with 3 volumes of methanol. The extraction procedure was evaluated by spiking the 14C-labeled parent compound into blank human plasma and resulting in 96.7% recovery of the added radiocarbon. For metabolite profiling, supernatants from both extractions were combined, concentrated, and reconstituted in dimethyl sulfoxide followed by the addition of a 1:1 mixture of mobile phases A and B. Rat bile and human urine samples were pooled across animals/subjects and after centrifugation were directly injected onto the HPLC-MS system.

Sample Pooling and Processing. Plasma and urine samples from the 200-mg dose group were pooled across subjects dosed with SCH 486757 (n = 6) and placebo (n = 2). The drug-derived material from plasma was extracted with methanol. Methanol was added to each plasma sample with a 3:1 ratio, and the mixture was placed into a freezer overnight. After centrifugation, the supernatant was removed, and the remaining pellet was extracted again with 3 volumes of methanol. The extraction procedure was evaluated by spiking the 14C-labeled parent compound into blank human plasma and resulted in 96.7% recovery of the added radiocarbon. For metabolite profiling, supernatants from both extractions were combined, concentrated, and reconstituted in dimethyl sulfoxide followed by the addition of a 1:1 mixture of mobile phases A and B. Rat bile and human urine samples were pooled across animals/subjects and after centrifugation were directly injected onto the HPLC-MS system.

**Materials and Methods**

**Reagents and Chemicals.** [14C]SCH 486757 and SCH 486757 were synthesized at Schering-Plough Research Institute (Kenilworth, NJ). The radiochemical purity of [14C]SCH 486757 was 96.6 to 97.1% as determined by two HPLC methods with an in-line radioactivity detector. Specific activity was 119 μCi/mg or 52.4 mCi/mmol.

Acetonitrile and methanol, both high-purity grade, were purchased from Burdick and Jackson (Muskegon, MI). Ammonium acetate (certified ACS grade), ammonium-d4-acetate-d3, deuterated water (D2O), and acetic-d3 acid-d were obtained from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide and glacial acetic acid were from Thermo Fisher Scientific (Waltham, MA). A Milli-Q Water Purification System (Millipore Corporation, Billerica, MA) was used to produce deionized water. FLO-SCINT III scintillation cocktail was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).

**Clinical Samples.** The clinical phase of the study was conducted at Pharma Bio-Research Group B.V. (Zuidlaren, The Netherlands). Plasma (2 and 6 h postdose) and urine (0–24 h) samples from patients dosed with 200 mg of SCH 486757 or placebo were collected and stored at −20 ± 10°C until processing and analysis for metabolites. Plasma and urine samples were pooled across subjects and then profiled using LC-MS.

**Animals, Dosing, and Sample Collection.** An exploratory study was conducted before analysis of the samples from the first-in-human study to develop an LC-MS method, to assess extraction recovery, and to examine the stability of drug and metabolites in typical sample processing conditions. In this study, bile duct-cannulated male Sprague-Dawley rats (250 g) were purchased from Charles River Laboratories, Inc. (Wilmington, DE). Four rats received a single oral dose of [14C]SCH 486757. The dose was formulated in 0.4% methylcellulose at a target concentration of 15 mg/kg; a target-specific activity was 100 μCi/kg. Bile was collected for 8 h postdose and stored at −20 ± 10°C. Bile samples were used for the development of the HPLC method and to assist with the structural identification of metabolites.
to improve mass accuracy. The remaining effluent was directed to the flow scintillation analyzer or to waste. The QSTAR mass spectrometer was operated at a resolution of ~10,000 with a mass accuracy of <5 ppm.

**Chromatographic Conditions.** A Luna Phenyl-Hexyl column (250 × 4.6 mm, 5 μm particle size; Phenomenex, Torrance, CA) with a Polaris C18-A guard column (Varian Inc., Palo Alto, CA) was used for separation of drug-derived material with mobile phases as follows: (A) 98% 20 mM ammonium acetate (pH adjusted to 4.2 by glacial acetic acid) and 2% acetonitrile and (B) 98% acetonitrile and 2% 20 mM ammonium acetate (pH adjusted to 4.2 by glacial acetic acid). For hydrogen/deuterium (H/D) exchange experiments, fully deuterated analogs of ammonium acetate, glacial acetic acid, and water were used for mobile phase preparation. The flow rate for all experiments was 1 ml/min; column temperature was 40°C. Separation was achieved using a linear gradient as follows: 0 min, 90% A; 2 min, 90% A; 5 min, 85% A; 51 min, 50% A; 57 min, 2% A; 61.5 min, 2% A; 62 min, 90% A; and 65 min, 90% A.

**Synthesis of M27.** To a mixture of 2-bromopyrimidine (1, 1.57 g, 9.85 mmol), toluene (9 ml), ethanol (9 ml), and H2O (3.2 ml), 2-(2-methylpyridin-4-yl)boronic acid (0.69 g, 0.98 mmol), and Na2CO3 (3.13 g, 29.5 mmol) and stirred at 100°C overnight (Scheme 1). The mixture was treated with EtOAc (60 ml) and filtered. The precipitate was dissolved in CH2Cl2, concentrated, and purified by HPLC (5–100% CH3CN-water) to give 2-(2-methylpyridin-4-yl)pyrimidine (0.31 g, 0.99 mmol). 

**Synthesis of M34.** Following the procedure for the synthesis of 3 using 1 (1.28 g, 8.04 mmol), 2-methylpyridin-4-ylboronic acid (5, 1.65 g, 12.06 mmol), trans-Pd(PPh3)2Cl2 (0.56 g, 0.80 mmol), and Na2CO3 (2.56 g, 24.12 mmol) in toluene (8 ml), ethanol (8 ml), and H2O (3.2 ml), 2-(2-methylpyridin-4-yl)pyrimidine (6) was prepared (Scheme 2). 1H NMR (CDCl3): δ 8.73 (d, J = 4.9 Hz, 2H), 8.54 (d, J = 4.6 Hz, 1H), 8.04 (s, 1H), 7.98 (d, J = 4.6 Hz, 1H), 7.18 (t, J = 4.8 Hz, 1H), 2.54 (s, 3H).

A solution of 6 (0.114 g, 0.66 mmol) and 4 (0.31 g, 0.99 mmol) in nitromethane (3.5 ml) in a sealed tube was stirred at 100°C overnight. The mixture was treated with EtOAc (60 ml) and filtered. The precipitate was dissolved in CH2Cl2, concentrated, and purified by HPLC (5–100% CH3CN-H2O) to afford M34 (1.3 mg). 1H NMR (CDCl3): δ 8.94 (d, J = 4.9 Hz, 2H), 8.76 (dd, J = 6.8 Hz, 1.6 Hz, 1H), 8.51 (d, J = 6.8 Hz, 1H), 8.26 (s, br, 1H), 7.64 (s, 1H), 7.44–7.59 (m, 5H), 7.36 (dd, J = 7.5 Hz, 1.3 Hz, 2H), 6.84 (dd, J = 7.5 Hz, 1.4 Hz, 2H), 2.85 (s, 3H).

**Scheme 1.** Schematic presentation of the synthesis of M27.

**Scheme 2.** Schematic presentation of the synthesis of M34.

**Results**

**Metabolic Profiles.** Plasma and urine samples from the drug- and placebo-dosed subjects were pooled and extracted as described under Materials and Methods. The resulting extracts were analyzed by LC-MS.

Analysis of plasma and urine showed that SCH 486757 is extensively metabolized in humans. Overall, more than 40 metabolites were detected in human plasma and urine. The parent compound with m/z of 440 was the major drug-related component in plasma at both the 2 and 6 h time points. Although many metabolites were detected, only two metabolites, M27 and M34, appeared to be major, each representing approximately 38% of the peak area of the parent drug’s LC-MS response at 6 h postdose (Fig. 2). Other less prominent metabolites included mono- and dihydroxylated SCH 486757 and metabolites formed by the following mass shift relative to the parent compound: −18, −12, +13, +18, +24, and +46 Da. Structural elucidation of these metabolites by MS/MS alone was difficult because their MS/MS spectra poorly reflected metabolic modification sites as described below.

**MS/MS Spectra and Precursor Ion Scans.** SCH 486757 produced a protonated molecular ion, [M + H]+ at m/z 440/442, consistent with the molecular formula C25H26Cl2N3O. The MS/MS spectrum of SCH 486757 contains one characteristic ion at m/z 235, which corresponds to a stable carbocation formed by the bis(2-chlorophenyl)methyl group (Fig. 1). The parent compound has a labile bond between the carbon atom of the bis(2-chlorophenyl)methyl group and the nitrogen of the tropine moiety. This C–N bond is highly susceptible to fragmentation, which results in formation of two major fragments: a stable carbocation representing the only major peak in the MS/MS spectrum and a nondetectable neutral fragment corresponding to a substituted tropane-pyrimidine moiety.

The fragmentation pattern of the parent compound and the metabolites followed only two types of MS/MS spectra (Fig. 3). A majority of human metabolites have a spectrum of type I, which indicates that biotransformation occurred at the tropane-pyrimidine moiety. A few minor metabolites displayed an MS/MS spectrum of type II. Biotransformation in these metabolites results from oxidation of the bis(2-chlorophenyl)methyl group to M + 16 metabolites or, in addition to that, by metabolism on the tropane-pyrimidine moiety.

Because of a distinct fragmentation pattern, an initial search for metabolites of SCH 486757 in human samples was conducted by precursor ion scans for m/z 235 and 251. As shown in Fig. 2, a number of circulating metabolites of SCH 486757 are characterized by an MS/MS spectrum of type I and could be easily detected in a precursor ion scan experiment for m/z 235. However, in this article we focus primarily on the elucidation of structures of M27 and M34 and...
estimation of their circulating levels. The formation of these metabolites represents a previously unidentified pathway and from LC-MS data they appear to be major metabolites.

Structural Elucidation of M27 and M34. M27 and M34 metabolites produced protonated molecular ions, [M + H]+ at m/z 392/394 and 406/408, respectively. Relative to the parent compound, the
decrease in molecular mass for these metabolites was 48 and 34 Da, respectively. Similar to the parent compound SCH 486757, MS/MS spectra of M27 and M34 contain a characteristic ion at \( m/z \) 235 (Fig. 2), which indicates that the bis(2-chlorophenyl)methyl group is unchanged. For both metabolites, modifications occurred at the tropane-pyrimidine moiety, but no additional structural information could be obtained from MS/MS spectra. Additional experiments were performed to further elucidate the structures of M27 and M34.

Limitied amounts of clinical samples did not allow application of the full spectrum of techniques available for structural elucidation. In an earlier exploratory metabolism study in the rat, both M27 and M34 metabolites were detected in the bile. The level of both metabolites was not sufficient to allow their isolation in quantities necessary for NMR analysis, but was sufficient to use techniques such as H/D exchange experiments to further understand structural changes in these molecules.

**H/D Exchange Experiments.** H/D exchange can be conducted by replacing hydrogen-containing salts and solvents in the mobile phase with fully deuterium-substituted ones (Nassar, 2003; Liu and Hop, 2005). For H/D exchange experiments the chromatographic column was equilibrated with deuterated mobile phases before the injection of study samples. H/D exchange is usually fast, and samples can be injected directly onto the HPLC system without additional pretreatment.

Mass spectra of the parent compound (\( m/z \) 440), M27 (\( m/z \) 392), and M34 (\( m/z \) 406) in rat bile before and after deuterium exchange are shown in Fig. 4. The 2-Da mass increase for \([M + H]^+\) of SCH 486757 is consistent with the exchange of a hydrogen atom at the OH group attached to the tropane moiety and the ionizing proton in \([M + H]^+\). Unexpectedly, no change of \( m/z \) for either M27 and M34 was observed in the H/D exchange experiment, which implies that M27 and M34 do not have any exchangeable protons and do not require an ionizing proton, suggesting that these molecules most probably contain a quaternary amino group in their structures and the hydroxyl group on the tropane moiety no longer exists. Therefore, H/D exchange experiments provided crucial structural information about M27 and M34 metabolites.
Accurate Mass Measurements. Accurate mass measurements were conducted on the QSTAR mass spectrometer. For M27 and M34, the measured accurate masses were 392.0715 and 406.0872 Da, respectively. The most probable molecular formulas were C\textsubscript{22}H\textsubscript{16}N\textsubscript{3}Cl\textsubscript{2} and C\textsubscript{23}H\textsubscript{18}N\textsubscript{3}Cl\textsubscript{2}, respectively. M34 appears to contain an additional CH\textsubscript{2} group compared with M27.

On the basis of data from MS/MS, H/D exchange, and accurate mass experiments, the structure shown in Scheme 3 was proposed for the M27 metabolite. This structure contains an intact bis(2-chlorophenyl)methyl group and quaternary amine, a pyridinium group, in the molecule. The compound shown was synthesized as described under Materials and Methods. The mass and MS/MS spectra and the retention time of the synthesized standard matched those observed for the M27 metabolite of SCH 486757. The extracted ion chromatograms for M27, M34, and the parent drug from a mixture of synthetic standards are shown in Fig. 2C for comparison.

Structural Elucidation of M34. Similar to M27, M34 has intact bis(2-chlorophenyl)methyl groups and does not possess any exchangeable protons. This observation suggests that the structures of M27 and M34 are related. From the accurate mass experiments, the difference between elemental composition of M27 and the most probable candidate for M34 suggested the presence of an extra CH\textsubscript{2} group in M34. The
Because metabolite profiling was relative to the parent drug, spectra matched those initially observed for M34. The compound was synthesized; its retention time, LC/MS, and MS/MS for M27. The structure shown in Scheme 4 was proposed for M34. This metabolite was synthesized to elucidate elemental composition. Mass spectrometric techniques, such as neutral loss and precursor ion scans, are extensively used for searching “unknowns” (Ma et al., 2006; Segall et al., 2006; Prakash et al., 2007) using triple quadrupole mass spectrometers.

SCH 486757 was extensively metabolized in humans. The metabolite profiles showed the presence of many minor metabolites. However, two metabolites, M27 and M34, yielded LC-MS signals that were ~38% of the LC-MS response of the parent drug in plasma at 6 h. Therefore, attempts were made to elucidate their structures. LC-MS/MS experiments were not very informative for structural identification of these metabolites. MS/MS spectra of M27 and M34 contained only one characteristic ion at m/z 235, which indicates that the bis(2-chlorophenyl)methyl group is unchanged. For both metabolites, modifications occurred at the tropane-pyrimidine moiety, but no additional structural information could be obtained from MS/MS spectra. The H/D exchange experiments showed that these metabolites did not require an ionizing proton and the hydroxyl group on the tropane moiety was not present. These were critical findings on the putative structures of the metabolites. H/D exchange was previously used for metabolite profiling to help in the structural elucidation of small molecule compounds (Nassar, 2003; Liu and Hop, 2005; Prakash et al., 2007; Chen et al., 2009). During H/D exchange, hydrogen atoms attached to heteroatoms (O, N, and S) undergo exchange with deuterium, which results in the increase of molecular ion mass observed in a mass spectrometer.

Based on the accurate mass, H/D exchange data, and MS/MS spectra, the structures of these metabolites were proposed and confirmed after synthesis of reference standards. M27 and M34 were found to be formed through a loss of the C–C bridge from the tropane moiety following aromatization to pyridine with a tertiary ammonium ion, consistent with the absence of the hydroxyl group and lack of necessity for an ionizing proton in the LC-MS spectra.

The tropane group is a part of many biologically active compounds such as opioids (e.g., codeine or morphine), alkaloids, and drugs (maraviroc). Although these compounds are usually extensively metabolized by various types of enzymes as part of the natural biotransformation process for elimination from the body, all published reports indicate that the bicyclic aliphatic moiety stays metabolically intact (He et al., 1995; Dräger, 2002; Chen et al., 2007; Fattorusso and Taglialetela-Scafati, 2008; de Simone et al., 2008). Cleavage of a carbon–carbon bond in the process of metabolism is relatively rare and is often performed by an enzyme with a very specific function, such as aromatase, which is responsible for converting androgens into estrogens (Graham-Lorence et al., 1995).

To our knowledge, a loss of the C–C bridge from the tropane moiety with conversion into a substituted pyridinium compound during a metabolic process has not been reported previously. Limited in vitro work conducted with SCH 486757 showed that the formation of M27 occurs during incubation with human liver microsomes and is P450-mediated. M34 was not detected in in vitro incubations. The mechanism of this conversion and specific enzymes involved are yet unknown and will be examined in the future.

Before correction for the LC-MS response difference between the parent drug and M27 or M34 metabolite, both metabolites appeared to be major circulating metabolites. The LC-MS signal for both metabolites in human plasma was found to be approximately 11 times greater than the response of the parent drug. It was not unexpected to have high LC-MS sensitivity for M27 and M34 metabolites relative to those for the parent drug because both metabolites possess a permanent charge in the molecule.

**Discussion**

Characterization of human metabolism of drugs as part of first-in-human studies is becoming a routine practice in the pharmaceutical development process. Urine and plasma samples from healthy volunteers in rising single and rising multiple dose human safety and tolerance studies are collected, and metabolite profiling is performed to provide an early assessment of major human and potentially human-specific metabolites and to address potential metabolites in safety testing (MIST)-related questions (Smith and Obach, 2006; U.S. Department of Health and Human Services, 2008).

Metabolite profiling of nonradiolabeled samples presents a challenging task. In this study, a triple quadrupole mass spectrometer was used to generate nominal mass and MS/MS spectra and to perform the precursor ion and neutral loss scan searches. Targeted MS/MS scans were used to determine the presence of predicted metabolites and to characterize novel metabolites. Accurate masses of the metabolites were determined using a QSTAR high-resolution mass spectrometer to elucidate elemental composition. Mass spectrometric techniques, such as neutral loss and precursor ion scans, are extensively used for searching “unknowns” (Ma et al., 2006; Segall et al., 2006; Prakash et al., 2007) using triple quadrupole mass spectrometers.

Before correction for the LC-MS response difference between the parent drug and M27 or M34 metabolite, both metabolites appeared to be major circulating metabolites. The LC-MS signal for both metabolites in human plasma was found to be approximately 11 times greater than the response of the parent drug. Taking into account the response ratio of M27 and M34 to parent drug, the actual amounts of M27 and M34 were less than 5% relative to the parent drug. Therefore, neither M27 nor M34 would require quantitative determination of their amounts using validated bioanalytical methods or assessment...
of their quantitative coverage in preclinical safety evaluation. This study clearly indicates that the LC-MS response alone cannot be used reliably to estimate metabolite levels without response correction with reference standard, radioactivity, or another technique that provides quantitative determination. At a minimum, the LC-MS response relative to the parent drug should be determined before one makes any decision on whether a metabolite requires a MIST-related evaluation.

References

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