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Pharmacokinetics and Metabolism of Endothelin Receptor Antagonist: Contribution of Kidneys in the Overall *In Vivo* *N*-Demethylation

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In vivo clearance of BMS-182874 was primarily due to metabolism *via* stepwise *N*-demethylation. Despite *in vivo* clearance approached *ca* 50% of the total liver plasma flow, BMS-182874 was completely bioavailable after oral administration in rats. Saturable first-pass metabolism and the role of extrahepatic tissue were evaluated as possible reasons for complete oral bioavailability despite extensive metabolic clearance. Pharmacokinetic parameters were obtained after an intravenous and a range of oral doses of BMS-182874 in rats. Bile and urine were collected from bile-duct cannulated (BDC) rats and the *in vivo* metabolic pathways of BMS-182874 were evaluated. Pharmacokinetics of BMS-182874 were also compared in nephrectomized (renally impaired) *vs.* sham-operated control rats. Oral bioavailability of BMS-182874 averaged 100%, indicating that BMS-182874 was completely absorbed and the first-pass metabolism (liver or intestine) was negligible. The AUC and Cmax values increased dose-proportionally, indicating kinetics were linear within the oral dose range of 13 to 290 mmole/kg. After intravenous administration of BMS-182874 to BDC rats, about 2% of intact BMS-182874 was recovered in excreta, indicating that BMS-182874 was cleared primarily *via* metabolism *in vivo*. The major metabolite circulating in plasma was the mono-*N*-desmethyl metabolite and the major metabolite recovered in excreta was the di-*N*-desmethyl metabolite. *In vivo* clearance of BMS-182874 was significantly reduced in nephrectomized rats. These observations suggest saturable first-pass metabolism is unlikely to be a mechanism for complete oral bioavailability of BMS-182874. Reduced clearance observed in the nephrectomized rats suggests that extrahepatic tissues (*e.g.*, kidneys) may play an important role in the *in vivo* clearance of xenobiotics that are metabolized via *N*-demethylation.

Key words: Bioavailability, *N*-Demethylation, BMS-182874, Endothelin receptor antagonists

INTRODUCTION

Endothelin (ET) is a potent vasoconstrictor and is apparently produced in almost all tissues including the vascular endothelial cells (1). The level of plasma ET has been shown to be elevated in patients with numerous cardiovascular diseases (*e.g.*, myocardial infarction, diabetes and ischemia), and ET is perceived to play important pathophysiological roles (2). In an attempt to learn more about the origin of pathophysiology and perhaps to reverse the disease state, the search for ET receptor antagonists has been very active in recent years. BMS-182874, a 5-(dimethylamino)-*N*-(3,4-dimethyl-5-isoxazolyl)-1-naphthalenesulfonamide, is an orally active and non-peptidic endothelin receptor antagonist (Fig. 1). BMS-182874 is a highly ET_A selective antagonist (3). BMS-182874 had an *in vitro* inhibitory binding constant (Ki) of 55 nM to the ET_A receptor in cultured vascular smooth muscle A10 cell membrane and >200 μM to ET_B receptor on rat cerebellar membrane. Preliminary pharmacokinetic studies in rats suggested that the oral bioavailability of BMS-182874 was complete despite extensive metabolism *via* stepwise *N*-demethylation *in vivo*. Rapid clearance yet complete oral bioavailability suggested that the *in vivo* metabolism of BMS-182874 was either saturated in the liver and intestine during the absorption process or predominantly extrahepatic in origin. Therefore, the objectives of this report were 1) to evaluate the oral

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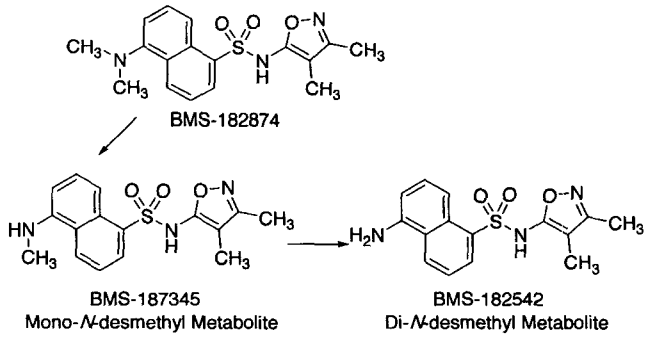

Fig. 1. *Structures of BMS-182874 and its N-desmethyl metabolites*

bioavailability of BMS-182874 after administration of a range of oral doses in rats and 2) to characterize the *in vivo* metabolism profiles of BMS-182874 and the routes of excretion after administration of BMS-182874 in the bile-duct cannulated rats, and 3) to investigate the role of extrahepatic tissues (*e.g.*, kidneys) in the overall *in vivo* clearance of BMS-182874.

MATERIALS AND METHODS

Materials

BMS-182874 and its *N*-desmethyl metabolites (Fig. 1) were synthesized at Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). NADPH (tetrasodium salt, 95%) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were reagent grade or better.

Pharmacokinetics and oral bioavailability in rats

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were surgically prepared with an indwelling jugular vein cannula one day prior to drug administration. Rats were fasted overnight prior to dosing and fed 8 h after dosing. They were allowed access to water *ad libitum* and were conscious and unrestrained during the study. Rats were given a single intravenous dose of BMS-182874 (100 μmol/kg; n=3). Separate groups of rats received several oral doses of BMS-182874 (13, 65 or 290 μmol/kg; n=3 for each dose level). In addition, a group of rats received either a single intravenous dose (100 μmole/kg; n=3) or a single oral dose (200 μmole/kg; n=3) of BMS-187345 (mono *N*-desmethyl metabolite of BMS-182874). Both BMS-182874 and BMS-187345 were administered as an aqueous solution in 5% (w/v) sodium bicarbonate. During the 48 h study, aliquots of 500 μL blood samples were collected at various time points and plasma prepared by centrifugation.

Pharmacokinetics in nephrectomized rats

The sub-total nephrectomy was performed by Hilltop Lab Animals (Scottdale, PA) and the surgical procedure were as follows. Male Sprague-Dawley rats (~300 g) were anesthetized with isoflurane. The right renal vessels were firmly ligated and the right kidney was removed. For the left kidney, approximately 3 mm was sliced off from each kidney pole. A pad of Gelform was applied to both sides of the stump and fixed in place with nylon netting and braided silk suture. The kidney stump was return to the abdominal cavity. Each rat was surgically prepared with an indwelling jugular vein cannula and the rats were fasted overnight prior to dosing. They were allowed access to water *ad libitum* and were conscious and unrestrained during the study. Two groups of rats (sham-operated control, n=4, and nephrectomized, n=3) were given a single intravenous dose of BMS-182874 (100 μmol/kg). For this study, blood samples were collected at various time points only up to 4 h because previous studies showed that the samples beyond 4 h were below the lowest quantifiable level (~0.5 μM).

Routes of excretion and metabolism in bile duct-cannulated (BDC) rats

Male Sprague-Dawley rats were surgically prepared with indwelling bile duct and duodenal cannulae two days prior to drug administration. Rats were fasted prior to the surgery. The cannulae were exteriorized and connected to allow for normal bile flow during the recovery period. One day after surgery, the cannulae were disconnected and control bile was individually collected and frozen at -20°C, after which the cannulae were reconnected 12 hr prior to dosing. During the 12-h study, bile was quantitatively collected in this same manner. Also during this time, the control bile was infused into the duodenum of the rat from which it was collected, at approximately 1 mL/h to avoid depletion of bile salts and provide intra-lumenal bile for drug solubilization. The rats were fasted overnight prior to dosing and fed 8 h after dosing. They were allowed access to water *ad libitum* and were conscious and unrestrained during the study. Both BMS-182874 and BMS-187345 were dissolved in 5% (w/v) sodium bicarbonate and each given to separate groups of rats intravenously to determine the routes of excretion and metabolism after systemic administration. After the dose was administered (140 μmol/kg, n=2), bile was quantitatively collected for 12 h and urine for 24 h. The bile and urine samples were quantitatively analyzed for intact drug and its metabolites.

In vitro metabolism in liver microsomes

Livers were obtained from male Sprague Dawley rats (n=6) after an overnight fast and immediately placed in ice-cold KCl solution (0.15 M). The tissue was homogenized in 0.15 M KCl and the homogenate centrifuged for 30 min at 10,000×*g*. The supernatant was then centrifuged for 1 h at 105,000×*g* and the microsomal pellet resuspended in 0.15 M KCl and stored at -80°C (4). Standard incubation solutions contained 50 mM potassium phosphate (pH 7.4), 5

mM NADPH, and 0.3 mg microsomal protein. The reaction was initiated by adding BMS-182874 to pre-warmed test tubes. The incubation mixtures were placed in a shaking water bath at 37°C. Aliquots were withdrawn at appropriate time intervals and the reaction quenched by adding an equal volume of acetonitrile.

Sample processing and analysis

Concentrations of BMS-182874 and its metabolites in plasma, bile and urine were determined by a specific HPLC-UV assay. To precipitate proteins and to eliminate UV-absorbing interfering substances, 600 µL of acetonitrile: water (2 : 1 v/v) was mixed with 200 µL of each sample. After centrifugation, 700 µL of the supernatant was transferred to a clean conical tube. The supernatant was then mixed with 300 µL of acetonitrile and centrifuged. The entire supernatant from the second precipitation step was transferred to a glass tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1.0 mL of water and an appropriate volume was analyzed by HPLC. A standard curve was prepared by analysis of plasma, bile and urine samples containing known amounts of analytes and was linear between 0.5 and 500 µM. The analytical HPLC column used was µ-Bondapak reverse phase C_{18} (3.9 mm × 30 cm; Waters Chromatography Division, Millipore Corp., Milford, MA). The mobile phase, consisting of solvent A (water: acetonitrile: trifluoroacetic acid, 95:5:0.115 v/v) and solvent B (water: acetonitrile: trifluoroacetic acid, 20:80:0.115 v/v), was programmed linearly from 90:10 to 30:70 (A:B) over 30 min. The flow rate was 1.2 mL/min and the absorbance was monitored at 220 nm. For metabolite elucidation, a 50 µL aliquot of the combined supernatant was directly injected on a Sciex API III LC/MS mass spectrometer (PE Sciex, Thornhill, Ontario, Canada).

Data analysis

Pharmacokinetic parameters were determined from plasma concentrations with standard methods (5). The area under the concentration *vs.* time curve (AUC) was calculated by the LaGrange integration method. Oral bioavailability in rats was estimated from plasma data by dividing the average dose-normalized $AUC_{0\text{-infinity symbol}}$ after oral administration by the average $AUC_{0\text{-}8}$ for the intravenous route. Values are reported as mean ± SD, unless otherwise indicated. Statistical comparison was performed with the two-tailed unpaired *t*-test; $p<0.05$ was considered statistically significant.

RESULTS

Pharmacokinetics/oral bioavailability

After intravenous administration, the plasma concentrations of BMS-182874 rapidly declined in a biexponential manner and the apparent elimination $t_{1/2}$ was relatively short (<1 h, Fig. 2). After oral administration, BMS-182874 was rapidly and completely absorbed from the gastrointestinal tract of rats (T_{max}~20 min). The apparent oral bioavailabilities averaged 100% at a range of oral doses (13 to 290 µmol/kg). The AUC values and C_{max} increased proportionally to the oral doses (Table I). After either route of administration, a single major metabolite was detected in the systemic circulation. Based on LC/MS analysis, this metabolite was identified as the mono-*N*-desmethyl metabolite (BMS-187345, Fig. 1). The apparent elimination $t_{1/2}$ of this metabolite in plasma after the administration of BMS-182874 was about 6 h, which was substantially longer than that of BMS-182874. Indeed, when BMS-187345 was dosed intravenously to a separate group of rats, its elimination $t_{1/2}$ was about 8 h. After intravenous administration, the plasma concentrations of BMS-187345 also declined in a biexponential manner and its systemic clearance (0.6 ± 0.1 ml/min/kg) was approximately 20-fold less than that of BMS-182874. BMS-187345 was well absorbed from the GI tract and its oral bioavailability averaged 90% in rats (Table II).

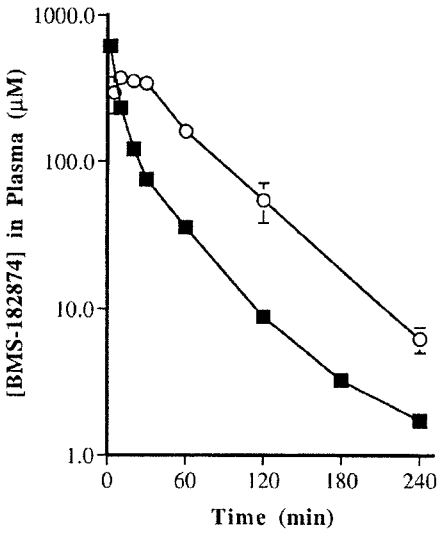

Fig. 2. Mean (± sem) plasma concentrations of BMS-182874 after an intravenous (■; n=3, 100 µmole/kg) and oral (○, n=3, 290 µmole/kg) administration of BMS-182874 in rats

In vivo routes of excretion and metabolism in BDC rats

The identification and recovery of drug-related materials in bile and urine in BDC rats are summarized in Table III. After intravenous administration of BMS-182874, trace quantities of intact BMS-182874 (less than 2% of dose) were recovered in excreta. The di-*N*-desmethyl metabolite (BMS-182542, Fig. 1), which is formed after further *N*-demethylation of BMS-187345, was the major metabolite recovered in excreta (*ca.* 35% of dose) and was excreted

Table I Pharmacokinetic parameters of BMS-182874 in rats (mean ± sd, n=3, except sham-operated, n=4)

Parameters	Unit	Normal				Sham-Operated	Nephrectomized
		iv	po	po	po	iv	iv
Dose	μmol/kg	100	13	65	290	100	100
Clearance	mL/min/kg	9.9±0.29				11.1±1.8	6.3±1.8[a]
V_{ss}	L/kg	0.34±0.036				0.32±0.046	0.39±0.086
$t_{1/2}$	hr	0.85±0.10				0.56±0.13	1.0±0.42
C_{max}	μM		20±5.5	75±12	390±82		
T_{max}	hr		0.28±0.19	0.17±0.0	0.28±0.19		
Dose-Normalized $AUC_{0-\infty}$	μM · hr/(μmole/kg)	1.7±0.049	1.7±0.33	2.1±0.38	1.6±0.087	1.5±0.23	2.9±0.54[a]
Bioavailability	%		100	120	95		

[a]$p<0.05$, nephrectomized vs. normal and sham-operated control.

Table II. Pharmacokinetic parameters of BMS-187345 in rats (mean ± sd, n=3)

Parameters	Unit	iv	po
Dose	μmol/kg	100	200
Clearance	mL/min/kg	0.6±0.1	
Vss	L/kg	0.3±0.1	
$t_{1/2}$	hr	8±1	
C_{max}	μM		535±41
T_{max}	hr		0.6±0.3
Bioavailability	%		90

about equally in bile and urine. Small amounts of BMS-187345 were recovered (less than 6% of dose) about equally from bile and urine. The other minor metabolites were glucuronide conjugates of BMS-187345 (less than 7% of dose) and were detected only in bile. After intravenous administration of BMS-187345, less than 10% of the dose was recovered as intact BMS-187345. BMS-182542 was the major metabolite recovered in excreta (*ca.* 50% of dose), and was excreted about equally in bile and urine. Glucuronide conjugates of BMS-187345 were minor metabolites (less than 10% of dose) and were recovered only in bile.

Microsomal incubations

The major product found in incubation mixtures of BMS-182874 and rat liver microsomes was BMS-187345. The rate of the first demethylation reaction, measured under linear conditions, was described by the standard Michaelis-Menten equation. The Vmax and Km values for this reactions were 1.8 nmol/min/mg microsomal protein and 21 μM, respectively. BMS-187345 was further metabolized to BMS-182542 (*via N*-demethylation) as well as a hydroxylated product after prolonged incubation.

Pharmacokinetics of BMS-182874 in nephrectomized rats

After intravenous administration, the plasma concentrations of BMS-182874 in sham-operated rats declined very similarly to the normal control rats (Fig. 3). However, BMS-182874 plasma levels in the nephrectomized rats were significantly higher than those found in either the sham-operated or normal control rats. Consequently, the total plasma clearance of BMS-182874 in the nephrectomized rats was significantly lower (*ca.*, 50% reduction) than that in sham-operated rats (Table I, $p<0.05$).

DISCUSSION

BMS-182874 is a highly ET_A selective endothelin receptor antagonist. It has been shown to be pharmacologically active after oral administration in rats (6). Consistent with the observed oral activity, BMS-182874 was completely bioavailable after oral administration. After intravenous and oral administration of BMS-182874, BMS-187345 quickly appeared in the systemic circulation and persisted at a much higher concentration than the parent compound.

Table III. Average recovery of drug-related materials after an intravenous dose to BDC rats (n=2).

Drug-Related Material Recovered	BMS-182874 (140 μmol/kg)			BMS-187345 (135 μmol/kg)		
	% in bile 0-12 hr	% in urine 0-24 hr	Total (%)	% in bile 0-12 hr	% in urine 0-24 hr	Total (%)
BMS-182874	0.90	0.70	1.6			
BMS-187345	2.1	3.5	5.6	4.5	3.3	7.8
BMS-182542	17	17	34	27	24	51
BMS-187345-glucuronide	5.2	<0.1	5.2	6.8	<0.1	6.8
BMS-187345-OH-glucuronide	1.4	<0.1	1.4	2.6	<0.1	2.6
Total	27	21	48	41	27	68

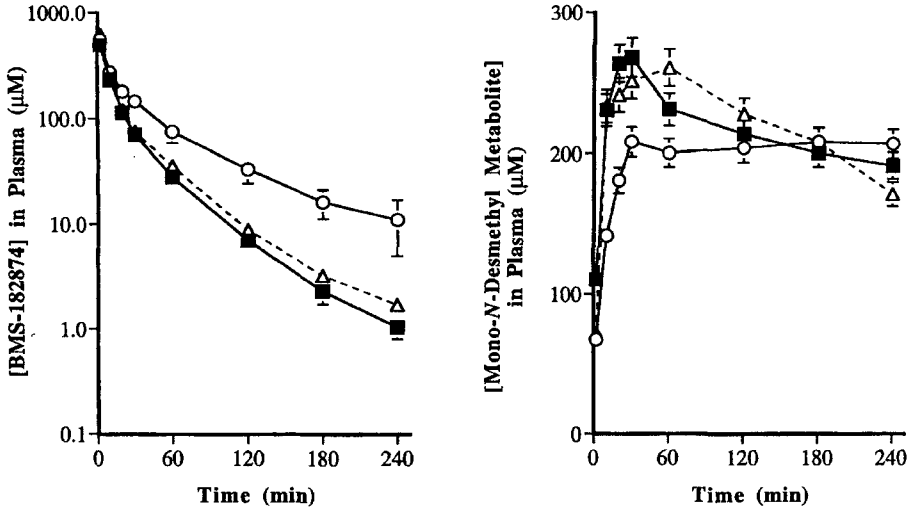

Fig. 3. (A) Mean (± sem, n=3) plasma concentrations of BMS-182874 after intravenous administration of BMS-182874 (100 µmol/kg) in normal (△) sham-operated (■), and nephrectomized (○) rats. (B) Mean (±sem, n=3) plasma concentrations of mono-*N*-desmethyl metabolite, BMS-187345, after intravenous administration of BMS-182874 in normal (△), sham-operated (■), and nephrectomized (○) rats.

Consistent with these observations, BMS-187345 had a much longer elimination $t_{1/2}$ in rats. BMS-187345 had similar *in vitro* and *in vivo* potencies to those of BMS-182874. Thus, it is likely that the high plasma levels of the pharmacologically active metabolite, BMS-187345, contributes to the long duration of action (>24 h) after administration of BMS-182874 in rats.

In pharmacokinetic studies in rats, BMS-182874 was rapidly converted to BMS-187345, which circulates in plasma at relatively high levels. To further characterize the metabolic pathways and routes of excretion, BDC rats were dosed with either BMS-182874 or BMS-187345 intravenously. The total biliary (0-12 h) and urinary (0-24 h) recovery of drug-related materials after administration of either BMS-182874 or BMS-187345 is incomplete (*ca.* 50 and 70%, respectively, Table III) partly because of the long elimination $t_{1/2}$ (*ca.* 8 h) of BMS-187345. Although the recovery was incomplete, the results clearly suggest that both BMS-182874 and BMS-187345 had negligible renal and biliary excretion because no appreciable amount of either compound was detected in excreta. The studies done in BDC rats also suggested that BMS-182874 and BMS-187345 were cleared exclusively *via* metabolism (*i.e.*, *N*-demethylation) *in vivo* . Consistent with the BDC rat data, the results obtained in NADPH-fortified rat liver microsomal incubations suggested that BMS-182874 undergoes oxidative metabolism *via* *N*-demethylation to generate BMS-187345. The liver microsomal results together with the data obtained in BDC rats clearly suggest that the liver might be the major metabolizing organ responsible for the overall *in vivo* clearance of BMS-182874 in rats.

Despite the evidence described above, the primary metabolizing organ responsible for the *in vivo* *N*-demethylation does not appear to be the liver. Because the systemic plasma clearance of BMS-182874 in rats approached 50% of the total liver plasma flow, *ca* 25 ml/min/kg (7), and if the liver is the exclusive metabolizing organ, roughly 50% of the oral dose should have been extracted by the liver during first-pass (*e.g.*, total plasma clearance = hepatic plasma flow * hepatic extraction ratio). However, the oral bioavailability of BMS-182874 was 100%, indicating that BMS-182874 had negligible liver or intestinal first-pass metabolism. There are at least two possible explanations for the lack of first-pass metabolism. One possible explanation might be that the concentration of BMS-182874 reaching the portal circulation after a relatively high oral dose (290 µmol/kg) is much higher than the *in vivo* Km value, such that the demethylation pathway is saturated, allowing more intact BMS-182874 to reach the systemic circulation. Alternatively, a high concentration of BMS-187345 builds up after an oral administration of BMS-182874. BMS-187345 is subsequently further converted to BMS-182542 *via* *N*-demethylation with much less efficiency. Thus, the product inhibition after a high dose of BMS-182874 might be a viable explanation. To test the saturation and/or product inhibition as potential underlying mechanisms, the oral bioavailability of two lower doses was examined. However, lower oral doses (13 and 65 µmol/kg) also failed to show first-pass metabolism and AUC and C_{max} were proportional to the dose, suggesting that neither saturation nor product inhibition can explain the apparent discrepancy. To further demonstrate the dose linearity, separate groups of rats (n=2 each dose level) received an intraportal dose of 20 or 300 µmol/kg. The dose normalized AUC 0-4h values were identical between two dose levels (24 and 371 µM/h after 20 and

300 μmol/kg, respectively). The AUC values after an intraportal dose was very similar to that found after an oral dose, indicating that BMS-182874 was rapidly and completely absorbed in the GI tract. It is recognized, however, that the Km value for *N*-demethylation pathway of BMS-182874 *in vivo* might be sufficiently lower than the concentrations of BMS-182874 reaching the portal circulation, so that saturation could still occur even at the lowest oral dose tested (*e.g.*, 13 μmol/kg). Due to limits of assay sensitivity, lower dose level could not be examined.

The other plausible explanation is that extrahepatic tissues (*e.g.*, kidneys and lung) may play more important role in clearing BMS-182874 than the liver. During the pharmacological evaluation of BMS-182874 in bilaterally nephrectomized dogs, it was noted that the plasma level of BMS-187345 after intravenous administration of BMS-182874 was substantially lower than expected. To examine the role of the kidneys in the *in vivo* metabolism of BMS-182874, the pharmacokinetics of BMS-182874 after an intravenous administration was examined in nephrectomized rats. The results showed that the systemic clearance of BMS-182874 was substantially reduced compared to that in sham-operated control rats, (6.3 *vs.* 11.1 ml/min/kg, respectively, $p<0.05$). The plasma concentration of intact BMS-182874 was significantly lower in the sham-operated control rats than the nephrectomized rats throughout the sampling intervals. Consistently, the rate of appearance of BMS-187345 in plasma was significantly slower in the nephrectomized rats (Fig. 3). These limited data suggested that the kidneys might be, at least in part, an important contributing organ toward overall *in vivo* clearance of BMS-182874. Evaluation of the lung as a potential site of *N*-demethylation of dimethylamino containing drug is presently under investigation.

Mono- and di-methylamino group containing drugs are typically metabolized *via* stepwise *N*-demethylation reaction and liver plays an important role in this reaction. Consequently, these drugs are efficiently extracted by the liver and the oral bioavailability is often less than complete. For example, mifepristone (RU486), a dimethylamino group containing steroidal antiglucocorticoid, undergoes stepwise *N*-demethylation reaction in rats (8) and humans (9). The oral bioavailability of mifepristone is about 40% due to the first-pass metabolism both in rats and humans (10). BMS-182874 represents an unique example in which its *in vivo* clearance is mainly due to metabolism *via* *N*-demethylation, yet it completely escapes liver first-pass elimination. Further investigation of *N*-demethylation reaction with BMS-182874 may provide some structural requirements (if any) in designing xenobiotics that may escape liver first-pass metabolism.

In conclusion, an endothelin receptor antagonist, BMS-182874, was completely bioavailable after oral administration, and it is exclusively cleared *via* metabolism *in vivo*. Extrahepatic tissues (*e.g.*, kidneys) might be the responsible organ(s) for the *in vivo* metabolism (*i.e., N*-demethylation) of BMS-182874.

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