CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

22-081

PHARMACOLOGY REVIEW

MEMORANDUM
June 13, 2007

TO: File
FROM: Kenneth L. Hastings, Dr.P.H., D.A.B.T.

SUBJECT: NDA 22-081

I concur that the marketing application for ambrisentan (Letairis®) may be approved based on review of submitted nonclinical pharmacology/toxicology data. The label is acceptable.

_____
Kenneth L. Hastings, Dr.P.H., D.A.B.T.
Associate Director
Office of New Drugs

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This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

Kenneth Hastings
6/13/2007 05:41:06 PM
PHARMACOLOGIST

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY REVIEW AND EVALUATION

NDA NUMBER:	**22-081**
SERIAL NUMBER:	**000**
DATE RECEIVED BY CENTER:	**12/18/06**
DRUG NAME:	**Letairis™ (ambrisentan)**
INDICATION:	**Pulmonary hypertension**
SPONSOR:	**Gilead Science, Inc.**
DOCUMENTS REVIEWED:	**eCTD**
REVIEW DIVISION:	**Division of Cardi0-Renal Products (HFD-110)**
PHARM/TOX REVIEWER:	**William T. Link, Ph.D.**
PHARM/TOX SUPERVISOR:	**Albert DeFelice, Ph.D.**
DIVISION DIRECTOR:	**Norman Stockbridge, M.D., Ph.D.**
PROJECT MANAGER:	**Melissa Robb**

Date of review submission to Division File System (DFS): 5/1/07

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EXECUTIVE SUMMARY

Ambrisentan is an endothelin receptor antagonist (ET_A-selective) proposed for daily use the treatment of pulmonary arterial hypertension (PAH) to improve exercise capacity, delay clinical worsening ~~~~~~~~~~~~~~~ . A maximal recommended human dose (MRHD) of 10 mg is proposed. The mean AUC_{total} drug and $C_{max\ total\ drug}$ for PAH patients are 13.75 µg·hr/mL and 1.19 µg/mL, respectively. These values were used in calculation of all exposure ratios discussed herein, following adjustment for species variation in protein binding so that ratio of free (unbound) drug is used.

Pharmacology

Ambrisentan shows a high affinity for ET_A receptors (Ki = 0.63 nM) and a lower affinity for ET_B receptors (Ki = 48.7 nM) resulting in a 77-fold selectivity for ET_A receptors versus ET_B receptors, as determined in human ET receptors expressed in mammalian cells. The primary cytochrome p450 metabolites, 4-hydroxymethyl ambrisentan and O-demethyl ambrisentan were 64- and 35-fold less potent for ET_A binding, respectively, and 84- and 11-fold less potent for binding to ET_B, respectively. Therefore, these metabolites of ambrisentan are not expected to contribute greatly to the pharmacologic effects of ambrisentan in humans.

Ambrisentan either did not inhibit or only weakly inhibited (<50%) specific radioligand binding to over 100 other receptors and binding sites tested. These results suggest that ambrisentan is unlikely to produce receptor mediated biological effects that are not related to endothelin.

Orally dosed ambrisentan was shown to block the pressor effects of big-endothelin-1 (Big ET-1) in the rat in a dose- and time-dependent manner. Oral ambrisentan reduced arterial pressure in conscious, normotensive rats (300 mg/kg) and dogs (dose- dependent, 1, 10, 100 mg/kg), respectively. The depressor effects in rats were sustained for >24 hours; in dogs ambrisentan administration was associated with reduced arterial pressure at all doses for the six hour duration of the experiment.

Three secondary pharmacodynamics studies were performed. Two of these studies targeted ischemia of the rat kidney and heart, respectively. Ambrisentan was not consistently effective in attenuating functional damage to the kidney, and only moderately improved developed pressure upon reperfusion of the isolated heart. The third study evaluated the effect of ambrisentan on the hyperproliferation of the porcine coronary artery after balloon catheter injury. Doses of 10 and 30 mg/kg/day of ambrisentan administered for 4 weeks after injury attenuated arterial neointimal formation, thereby increasing total luminal cross-sectional area of the affected vessel compared to control.

Safety pharmacology studies were conducted to examine the effect of ambrisentan on the central and peripheral nervous system, cardiovascular and respiratory, gastrointestinal

and renal systems, as well as uterine smooth muscle, blood coagulation, and spleen cell mitogenicity. The majority of these studies were conducted in compliance with the current GLP. In the *in vivo* studies, doses used were 10 – 300 mg/kg administered via intravenous (iv) or oral routes. Ambrisentan elicited only minor effects in most of the assays. In the rat gastrointestinal system high doses of ambrisentan delayed gastric emptying to the duodenum and increased gastric acid secretion. Ambrisentan administered as single iv doses reduced sodium and chloride excretion in both male and female rats in a dose-dependent manner. Single oral doses also significantly reduced excretion of sodium and chloride, and calcium excretion was significantly reduced in females at the highest dose administered.

These safety pharmacology tests indicate that high concentrations or doses of ambrisentan produced little to no effects in *in vitro, ex vivo* and in whole animal models. The safety pharmacology results suggest minimal risk for off-target biological effects with single dose ambrisentan in humans. However, large single doses of ambrisentan could lower arterial pressure and have the potential for causing hypotension and symptoms related to vasodilation.

Pharmacokinetics

The absorption, distribution, metabolism, and excretion (ADME) profile of ambrisentan has been assessed in the mouse, rat, rabbit, and dog, the main species used in the preclinical safety evaluation of the drug. The dose ranges and formulation for the ADME studies were similar or identical to those used in the toxicology studies. The pharmacokinetic/toxicokinetic characteristics of ambrisentan following repeat dosing were assessed in the toxicology studies. Single and repeat dose pharmacokinetic/toxicokinetic parameters were obtained using validated analytical methods for determination of plasma ambrisentan and metabolites.

Absorption

In the single oral dose studies, ambrisentan was rapidly absorbed in both rats and dogs. In rats, after administration of 30 mg/kg of ambrisentan by gavage, a mean maximum plasma concentration (C_{max}) of 32.4 µg/mL was achieved at 1 hour (t_{max}). In dogs, after a 30 mg/kg dose given in capsule, C_{max} was 43.6 µg/mL with t_{max} between 0.5 and 2 hours. The half-life ($t_{1/2}$) was determined to be 5.7 hours in rats and 7.9 hours in dogs. Ambrisentan had absolute oral bioavailability of 85% in rats and 72% in dogs.

In humans, after a single oral dose of 1, 5, 10, 15, 20, 50, and 100 mg of ambrisentan to healthy male volunteers (EE-001) the drug was rapidly absorbed into the systemic circulation, with peak plasma concentrations being attained after 1.5 hours post dose. Both C_{max} and AUC increased with dose in a linear manner. The elimination half-life for ambrisentan ranged from 5.75 to 12.71 hours.

The effects of repeated oral administration of ambrisentan were evaluated in toxicology studies ranging from 4 to 104 weeks in mice and rats at a target dose range of 1 to 2000

mg/kg; and 4 to 39 weeks in dogs at a target dose range of 30 to 1500 mg/kg. Toxicokinetic results demonstrated that escalation of ambrisentan dose generally resulted in a proportional increase in systemic exposure in all species. In several repeat dose studies in mice and dogs, there was evidence for greater systemic exposure in females than in males. In rats, however, there was evidence for greater systemic exposure in males than in females upon repeat dosing.

Results of drug accumulation for ambrisentan in the long term toxicity studies for each species were mixed. Ambrisentan exposures determined periodically over 52 weeks of dosing in both mouse and rat studies were decreased over time, although some doses showed little change. Ambrisentan exposures assessed over 39 weeks administration in the dog demonstrated reductions over time in the low dose group, but showed elevations in AUC in the mid and high dose groups as time progressed.

Distribution

Distribution of radioactivity following a single oral administration of [^{14}C]-ambrisentan at a dose of 32 mg/kg was examined in male Wistar rats and pigmented Long Evans rats. The radioactivity was distributed widely into tissues with the highest concentrations observed at 1 hour post-dose in most tissues. Highest concentrations were observed in the GI tract, liver, plasma, lungs, blood, kidney and mesenteric lymph nodes in decreasing order. The levels of radioactivity in tissues declined with time, but low concentrations were still observed in the gastrointestinal tract at 48 and 72 hours post-dose along with trace amounts in liver and several other tissues. The terminal half-life values for decline in tissues radioactivity levels were estimated to be between 11 and 27 hours. No extraordinary affinity to melanin was observed in pigmented animals. The concentrations of radioactivity in the brain were very low at 1 hour and declined rapidly. Overall, the results of this study showed that [^{14}C]-ambrisentan related material had a wide distribution into tissues but elimination occurred relatively rapidly. There was no evidence of retention in any tissues, including melanin-containing tissues.

In vitro studies in mice, rats, rabbits, dogs and human plasma indicate that ambrisentan bound extensively to proteins, with mean binding values of 91.8%, 97.2%, 96.8%, 96.4% and 98.8%, respectively. Ambrisentan plasma protein binding in humans was greater than in animals. The results also suggested high capacity for binding since saturation occurred at very high concentrations (200 µg/mL) of ambrisentan, which is about 150-fold above expected human C_{max}. No sex-dependent differences in plasma protein binding were observed in rabbit, dog, and human.

Metabolism

In vitro and *in vivo* metabolism of [^{14}C]-ambrisentan was investigated in mouse, rat, rabbit, dog, and humans.

In rat hepatocytes, glucuronidation and hydroxylation occurred to approximately equal extent whereas glucuronidation was the preferred route of metabolism in dog and human

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hepatocytes, with only minor amounts of oxidative metabolites. Ambrisentan conversion rates were 15% to 25% over 24 hours in all three species. The primary metabolite identified was ambrisentan glucuronide (M2), with only trace amounts (<1%) of hydroxylated metabolite (M3) detected in dog and human hepatocytes. *In situ* perfused rat liver studies show a similar metabolite pattern to that observed in rat hepatocytes.

Microsomes expressing single human cytochrome p450 enzymes (CYPs) or uridine glucuronosyltransferase enzymes (UGTs) were used to identify the enzymes that metabolize ambrisentan. The results indicated that ambrisentan is glucuronidated via several UGT enzymes (1A9, 2B7, 1A3) and hydroxylated by all CYP enzymes tested with CYP3A4, CYP3A5 and CYP2C19 exhibiting the highest turnover rates in metabolizing ambrisentan. However, based on turnover rate and relatively large abundance in the liver, CYP3A4 appears to be the likely primary oxidative enzyme involved in metabolism of ambrisentan with CYP3A5 and CYP2C19 contributing to a lesser extent.

Administration of [^{14}C]-labeled ambrisentan at 30 mg/kg oral dose to mice, rats, rabbits, and dogs, and 10 mg to humans and subsequent radioprofiling of [^{14}C]-ambrisentan related materials indicated that metabolic pathways for ambrisentan were qualitatively similar across species. The metabolites identified for ambrisentan in these studies included 4,6 dimethyl-2- hydroxypryimidine (M1), ambrisentan glucuronide (M2), 4-hydroxymethyl ambrisentan (M3), O-demethyl ambrisentan (M4), 4,6-dihydroxymethyl ambrisentan (M5), 4,6-dihydroxymethyl ambrisentan glucuronide (M6), 4-hydroxymethyl ambrisentan glucuronide (M7), and O-demethyl-4-hydroxymethyl ambrisentan (M8). However, all metabolites were not identified in all species tested.

Based on the plasma concentrations and number of phase I metabolites formed, it was apparent that the extent of metabolism was the highest in the mouse. This was followed by rabbit, rat, human and dog. The only phase I metabolite (M3) observed in humans was also present in mouse, rat and rabbit. With the exception of the mouse, the parent drug was the prominent drug-related component in the plasma of all species, including humans. The remaining radioactivity in human plasma was accounted for by three metabolites, 4-hydroxymethyl ambrisentan (M3) ambrisentan glucuronide and a glucuronide of the 4-hydroxymethyl ambrisentan (M7). Based on plasma profiles, the overall pattern of metabolism in humans most closely approximated the metabolite pattern seen in the rat.

Excretion

The feces were the primary route of excretion of drug-related material in all species except the rabbit. In humans, mice, rat and dogs, the fecal recovery accounted for 66%-76% of the dose. Urinary excretion was a minor route of elimination in all animal species (7-23%) and humans (23%) except for the rabbit (44%). The collective evidence indicates that in all species, except the rabbit, the primary excretion pathway was via the feces.

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Potential for drug interaction

The induction effect of ambrisentan on hepatic phase I and II enzymes was examined in both rats and dogs. There was no evidence from these studies that ambrisentan induces cytochrome p450 enzymes, glutathione-S-transferase (GST), or UDP-glucuronosyltransferase (UDP-GT) concentration or activity at clinically relevant concentrations.

The potential of ambrisentan to inhibit various hepatic CYP450 and glucuronyltransferase enzymes was evaluated in microsomal assays. There was no marked inhibition observed for ambrisentan at concentrations below 300 μM. At that highest concentration, there was a slight inhibition of only CYP2A6 and CYP2C8 (13-25%) and UGT1A1, UGT1A6, UGT1A9, and UGT2B7 (10-30%). Given that the human C_{max} for ambrisentan at the highest dose (10 mg) was 3.2 μM, these results suggest a low potential for drug-drug interaction based on inhibition of CYP and UGT enzymes.

Toxicology

The toxicological profile of ambrisentan has been evaluated in single-dose, repeat-dose, carcinogenicity, and reproductive/developmental toxicity studies in mice, rats, rabbits, and dogs and genotoxicity studies *in vitro* (mammalian and bacterial cells) and *in vivo* (rats). Doses administered *in vivo* ranged from 1 to 2000 mg/kg. The repeat-dose studies ranged from 4 weeks to 39 weeks of treatment, and the carcinogenicity studies were 2 years in duration. With the exception of 2 single dose studies in which ambrisentan was administered intravenously, all other studies used an oral administration route (gavage, capsule, or diet admixture). Administration as a diet admixture was used for mice and rats in the longer duration studies (greater than 13 weeks). Toxicokinetic measurements were comparable to those obtained in gavage studies.

Single dose

In single-dose toxicity studies in mice and rats, the maximum non-lethal doses for orally administered ambrisentan were 1000 and 2150 mg/kg in the mouse (female and male, respectively) and 3160 mg/kg in the rat (male and female). The maximum non-lethal doses for intravenously administered ambrisentan were 511 and 619 mg/kg in the mouse (male and female, respectively) and 464 mg/kg in the rat (male and female). The clinical signs observed at toxic doses included lassitude, forced respiration, prone position, partial palpebral closure, and convulsions. Necropsy findings of the animals that died spontaneously revealed congestion in the lungs, liver and kidneys.

Repeat dose

Mice

In repeat-dose studies in mice, ambrisentan was administered orally by diet admixture for 6 or 13 weeks at doses of 60-2000 mg/kg/day. Overt clinical signs of toxicity, observed at

doses greater than 500 mg/kg/day, included respiratory effects, rough coat, gastrointestinal disturbances, and emaciation. The NOAEL reported in the 13 week mouse study was 60 mg/kg/day for males and females, corresponding to $AUC_{0-24hrs}$ of 7.2 and 13.5 (µg·hr)/mL in males and females, respectively. This NOAEL was based on nasal cavity findings and testicular atrophy. This dose represents 3.6- and 6.73-fold the calculated human exposure in PAH patients [based on free (unbound) drug at the MRHD of 10 mg] for male and female mice, respectively.

Rats

In rats, repeat-dose toxicity studies of oral ambrisentan ranged from 4 to 26 weeks of treatment with doses of 1-2000 mg/kg/day. In these studies, the target organs were the gastrointestinal tract (distension and dilation), nasal cavity (osseous hyperplasia) and testes (testicular tubular atrophy). Mortality was observed at doses greater than 300 mg/kg/day. In longer duration studies ($\geq$13 weeks) and at the highest doses tested (500 or 2000 mg/kg/day), elevations in liver enzymes (alkaline phosphatase [AP or ALP], serum alanine aminotransferase [ALT or ALAT], or serum aspartate aminotransferase [AST or ASAT]) were observed. These elevated values were less than 2-fold concurrent control values and were considered within normal ranges for the laboratory. When histopathology revealed changes in the liver, the changes were hypertrophy not necrosis of the liver and did not correlate with liver enzyme elevations.

The NOAEL dose in the 26-week study was 5 mg/kg/day for males and females based on osseous hyperplasia of the nasal turbinates. This dose corresponds to $AUC_{0-24hrs}$ of 9.6 and 8.0 (µg·hr)/mL for males and females, respectively and C_{max} values of 0.5 and 0.4 µg/mL, respectively. Calculated exposure ratios are 1.9- and 1.6-fold for males and females, respectively.

Dogs

In dogs, repeat-dose studies of oral ambrisentan (capsule or gavage) ranged in duration from 4 to 39 weeks of treatment with doses of 30-1500 mg/kg/day. The target organs were the gastrointestinal tract, kidneys, and heart at doses $\geq$1000 mg/kg/day. Mortality was observed at 1500 mg/kg/day in a 4-week study.

The NOAEL in the 39-week dog study was 300 mg/kg for males and females based on the weight decreases and decreased food consumptions at higher dose. The dose corresponds to AUC_{total} of 476.8 and 674.7 (µg·hr)/mL, and Cmax of 257.1 and 260.3 µg/mL for males and females, respectively. It should be noted that testicular atrophy was reported in the 30 mg/kg male group, but not at the higher doses. Calculated exposure ratios are 104- and 147-fold for males and females, respectively.

Genetic toxicology

The genotoxic potential of ambrisentan was assessed in the following assays: Ames test using *S. typhimurium* strains; an *in vitro* chromosome aberration assay; a test for bone

marrow micronucleus formation in the rat after oral administration; and an evaluation of unscheduled DNA synthesis (UDS) in the rat after oral administration.

Ambrisentan induced chromosome aberrations in the *in vitro* chromosome aberration assay using human lymphocytes. The concentrations of ambrisentan evaluated ranged from 208 to 2025 μg/mL. Structural chromosome aberrations were seen in male donors in a specific pulse-chase scheme: 4-hour culture with ambrisentan in the presence or absence of S-9 activation, followed by 16 hour with culture medium alone. The ambrisentan concentrations that yielded significant increases in structural aberrations were 1300 and 1600 μg/mL in the presence of S-9 mixture and 1600 μg/mL or more in the absence of S-9 mixture. Ambrisentan induced structural chromosome aberrations, but not numerical aberrations, in concentration-dependent fashion, in this *in vitro* assay. Moderate levels of cytotoxicity were present at these concentrations.

All three remaining genetic toxicology studies were adequately performed and all were determined to be negative.

Carcinogenicity

Carcinogenic potential was evaluated in the rat and mouse. In the rat study, ambrisentan was administered daily for 104 weeks as a diet admixture in doses of 0, 10, 30, and 60 mg/kg/day to rats. Body weight gain and food consumption were dose-dependently reduced for both sexes at the mid and high dose levels ($p < 0.01$), and hunched posture, labored respiration, rales and emaciation were evident in these groups before the end of the first year of dosing. The high and mid-dose male and female groups had their doses lowered to 40 and 20 mg/kg/day, respectively, in week 51. The high dose males and females were taken off drug completely in weeks 69 and 93, respectively. Effects on survival of these groups became evident within the first 6 months. With continued mortality, ambrisentan administration to the high dose rat group was stopped in an attempt to retain as many of these animals as possible for scheduled termination. The only evidence of ambrisentan-related carcinogenicity was a positive trend ($p < 0.025$) for the combined incidence of benign basal cell tumor and basal cell carcinoma of skin/subcutis in male rats when the high dose group was eliminated from the analysis (1 animal with each tumor at the mid-dose, none in any other group, $p < 0.025$) and the occurrence of mammary fibroadenomas in male rats of that same high dose group (4 animals with the tumor in that group, none in any other male group, $p < 0.05$, pairwise comparison with controls).

In a second carcinogenicity study in mice 0, 50, 100, and 250 mg/kg/day was administered. Increased incidences of hunched posture, emaciation and rales were observed in high and mid dose males and high dose females. The high dose male and female groups had their dose lowered to 150 mg/kg/day in week 39 and were taken off drug completely in week 96 (males) or week 76 (females). Effects on survival became evident within the first six months in males and females. Only 11 high dose males, compared with at least 25 in each of the other main study male control and treated groups, survived to scheduled sacrifice at 24 months. None of the main study high dose

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females survived to 24 months as all 9 surviving members of this group were sacrificed at 84 weeks. Only 14 mid dose females and 18 low dose females survived to 24 months compared with at least 24 females in each of the concurrent control groups. Statistical analysis revealed no evidence of drug-related tumorigenesis, whether or not the high dose groups were included.

Reproductive toxicology

Ambrisentan affected female fertility in rats as evidenced by increases in pre-implantation losses in females at the higher doses tested. There were no test-item related effects on embryos when oral ambrisentan was administered directly to pregnant female rats (up to gestation day 6), or indirectly via treatment of males. The effect on male fertility in rats is less consistent. In one study, males demonstrated lower fertility indices and developed diffuse testicular tubular atrophy that was not consistently associated with infertility. In a second study, there was no treatment effect on male fertility, although testicular findings were present.

Ambrisentan is teratogenic in rats and rabbits when administered at any dose (7-150 mg/kg/day) between gestation days 6 and 15 and it is toxic to pregnant rabbits as evidenced by the maternal toxicities observed at doses greater than 21 mg/kg/day. Pregnant rats tolerate ambrisentan up to doses of 150 mg/kg/day. The fetal abnormalities consistently observed involved the lower jaw and/or palate. Additional findings present in rats included abnormalities of the major vessels and thymus. There was no dose in these studies at which fetal abnormalities were not observed.

When administered by daily oral gavage to pregnant rats from gestation day 15 through postpartum day 21, ambrisentan did not have any adverse effects on the specific pre- or post-natal developmental milestones of the offspring. A decrease in pup survival was present at 0-4 days post-partum at the higher dose that may represent toxicity affecting maternal behavior. An effect on pup nursing behavior cannot be ruled out, however. Male offspring at this high dose exhibited small testicles and decreased fertility rates.

Overall conclusions and recommendations

The pre-clinical development plan for ambrisentan demonstrates that the compound is a specific and selective antagonist of the endothelin ET_A receptor. Ambrisentan was rapidly absorbed with high bioavailability. Distribution, metabolism and excretion were generally comparable among species examined, and consistent with clinical observations.

Safety pharmacology studies did not reveal a large potential for adverse side effects. The observed reductions in blood pressure are consistent with the compound's pharmacological effects.

The demonstrated effects on reproduction are of primary concern to this reviewer. Principally, the teratogenic effects present the greatest area of concern and have no safety

margin identified. These effects are recognized for the entire class of these compounds and are adequately addressed in the labeling as well as the marketing plans.

The observed effects on testicular histopathology are more sporadic than those observed regarding teratogenicity. The data are consistent with those of other members of this class of compounds. It is difficult to define a No-Effect dose in this finding. Similarly, the effect appears to reduce fertility in some cases and is, therefore, biologically relevant despite the inconsistency of the observed histopathological observations. A No-Effect dose is easier to apply to the fertility findings, relative to the histopathology. However, the male rat is highly fertile, relative to humans, so any reduction in rat fertility is of concern to this reviewer. These findings, and the potential class effect, will need to be addressed in the proposed labeling.

There appears to be little evidence for hepatic injury potential with ambrisentan, based on animal studies. Histopathological findings were limited to hepatocellular hypertrophy, consistent with minimal enzyme induction, and there was no evidence of necrosis

The positive findings of human chromosomal aberrations are observed in the context of moderate cell toxicity and at high concentrations relative to plasma concentrations of ambrisentan in patients. Based on a weight-of-evidence approach considering all genetic toxicology studies, there does not appear to be a large potential for genetic toxicity associated with clinical use of ambrisentan. Nevertheless, the findings should be mentioned in the labeling, if only in the interest of full disclosure.

The findings in the rat carcinogenicity study should also be addressed in labeling. The findings of benign fibroadenoma in males at the high dose are only seen in the context of lethal toxicity, and the basal cell lesions at the mid dose are only significant when the high dose is excluded from analysis. The Executive CAC opinion on the rat study was that both findings were at doses above a maximal tolerated dose and do not provide adequate evidence for positive tumorigenicity.

The findings of the nasal epithelium in rodents are of lesser concern to this reviewer. These appear to be a rodent-specific phenomena and would easily be detected in human patients should it occur. It appears that the majority of the toxic signs in rodents are related to the respiratory distress associated with their obligate nasal breathing and the obstructive nature of this toxicity.

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Labeling recommendations:

1) The following should be used as a total replacement for the **NONCLINICAL TOXICOLOGY** section (**13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility**, Lines 323-339)

Oral carcinogenicity studies, for up to two year duration were conducted at starting doses of 10, 30 and 60 mg/kg/day in rats ▬▬▬▬▬▬▬▬ the maximum recommended human dose [MRHD], m^2 basis) and at 50, 150 and 250 mg/kg/day in mice (▬▬▬▬▬▬▬▬ MRHD). In the rat study, the high and mid-dose male and female groups had their doses lowered to 40 and 20 mg/kg/day, respectively, in week 51 due to effects on survival. The high dose males and females were taken off drug completely in weeks 69 and 93, respectively. The only evidence of ambrisentan-related carcinogenicity was a positive trend in male rats, for the combined incidence of benign basal cell tumor and basal cell carcinoma of skin/subcutis in the mid-dose group (high-dose group excluded from analysis), and the occurrence of mammary fibroadenomas in males in the high–dose group. In the mouse study, high dose male and female groups had their dose lowered to 150 mg/kg/day in week 39 and were taken off drug completely in week 96 (males) or week 76 (females). Ambrisentan was not associated with excess tumors in any dosed group.

Positive findings of clastogenicity were detected, at drug concentrations producing moderate to high toxicity, in the chromosome aberration assay in cultured human lymphocytes. There was no evidence for genetic toxicity of ambrisentan when tested *in vitro* in bacteria (Ames test) or *in vivo* in rats (micronucleus assay, unscheduled DNA synthesis assay).

The development of testicular tubular atrophy and impaired fertility has been linked to the chronic administration of endothelin receptor antagonists in rodents. Testicular tubular degeneration was observed in rats treated with ambrisentan for two years at doses ≥10 mg/kg/day (7.9-fold MRHD). Increased incidences of testicular findings were also observed in mice treated for two years at doses ≥50 mg/kg/day (27.7-fold MRHD). Effects on sperm count, sperm morphology, mating performance and fertility were observed in fertility studies in which male rats were treated with ambrisentan at oral doses of 300 mg/kg/day (236-fold MRHD). At doses of ≥10 mg/kg/day, observations of testicular histopathology in the absence of fertility and sperm effects were also present. There are insufficient data on the effects of ambrisentan or other endothelin receptor antagonists on testicular function in man.

2) **Section 8.3 Nursing Mothers**, lines 235-236 – a statement should be added:

A preclinical study in rats has shown decreased survival of newborn pups and effects on testicle size and fertility of pups following maternal treatment with ambrisentan from late gestation through weaning.

PHARMACOLOGY/TOXICOLOGY REVIEW

INTRODUCTION AND DRUG HISTORY

NDA number: 22-081
Review number: 01
Date received: 12/18/06
Sponsor and/or agent: Gilead Science, Inc.
Manufacturer for drug substance: Patheon, Inc.

Reviewer name: William T. Link, Ph.D.
Division name: Div. CardioRenal Products
HFD #: 110
Review completion date: 5/1/07

Drug:
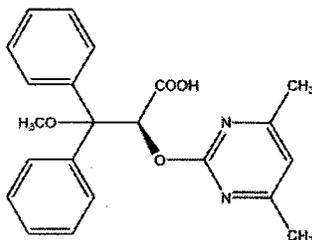
      Trade name: LETAIRIS™
      Generic name: ambrisentan
      Code name: LU 208075, BSF 208075
      Chemical name: (*S*)-2-(4,6-dimethylpyrimidin-2-yloxy)-3-methoxy- 3,3-
                     diphenylpropanoic acid
      CAS registry number: 177036-94-1
      Molecular formula/molecular weight: $C_{22}H_{22}N2O_4$, MW 378.42
      Structure:

Relevant INDs/NDAs/DMFs: ▬▬▬▬▬ ; IND 64,915

Drug class: Endothelin receptor antagonist (ET_A selective)

Indication: Pulmonary hypertension

Clinical formulation: Tablets of two dosage strengths as follows:

Component	Function	Quality Standard	5 mg Tablet (mg/tablet)	10 mg Tablet (mg/tablet)
Core Tablet				
Ambrisentan	Active Ingredient	In-house	5[1]	10[1]
Croscarmellose Sodium				
Lactose Monohydrate				
Magnesium Stearate				
Microcrystalline Cellulose				
Total Tablet Weight			147.0	147.0

[1]The quantity used is adjusted on the basis of purity (e.g.. drug content factor) of each batch of ambrisentan with a concomitant decrease in lactose monohydrate
[2]Approximate quantity, batch formula includes ▇ overage
[3]See Table 3 for composition of ▇▇▇ ilm coating material
[4]Film coated to a target weight gain of ▇ using a ▇▇ w/w aqueous suspension ▇▇▇▇
[5]Removed during the manufacturing process

Route of administration: oral

Proposed use: The proposed indication for ambrisentan is the treatment of PAH (WHO Group 1) to improve exercise capacity, delay clinical worsening, and ▇▇▇▇▇▇▇ .

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Brief summary

The primary pharmacodynamics of ambrisentan were evaluated in rats and dogs. In a rat model of endothelin induced hypertension (MPF/FT 9901), oral ambrisentan at 1, 3, or 10 mg/kg reduced the increases in arterial pressure that occurred after Big ET-1 infusion. In normotensive rats (MPF/FT 9902), oral administration of 300 mg/kg or intravenous (iv) administration of 100 mg/kg caused initial increases followed by sustained reductions in arterial pressure and heart rate. In normotensive dogs (MPF/FE 9924), oral administration of 1, 10, and 100 mg/kg ambrisentan caused dose-dependent reductions in arterial pressure with no increases in heart rate.

Secondary pharmacodynamics were evaluated in 2 rat models of tissue ischemia. One was a preparation of ischemia-induced acute renal failure in which iv ambrisentan showed no consistent protective effects at 5 or 10 mg/kg/day, although creatinine clearance and protein excretion were somewhat improved (MPF/FG 9914-6). Also, in a rat Langendorff isolated heart study (MPF/FG 9914-9), oral pretreatment with ambrisentan at 10 or 20 mg/kg improved left ventricular function following ischemia/reperfusion injury suggesting a protective effect. The potential efficacy of ambrisentan was also examined in a pig model of percutaneous transluminal coronary angioplasty (PTCA) induced arterial damage (MPF/FE 9925). In this study, oral ambrisentan treatment (10 or 30 mg/kg/day) reduced neointimal hypertrophy following PTCA induced arterial damage demonstrating the antiproliferative effects of ambrisentan.

Primary pharmacodynamics

In Vitro Receptor Binding Activity

In Vitro Binding of Ambrisentan to Recombinant Human ETA and ETB Receptors (MPF/FG 9809 and MSR-0001 AMB)

These studies were performed using membrane preparations from Chinese hamster ovary (CHO) cells permanently expressing either human ET_A or ET_B receptors. Twenty-five picomolar concentrations of ^{125}I-endothelin-1 and ^{125}I-endothelin-3 were used as radioligands for ET_A and ET_B receptors, respectively. Non-specific binding was measured in the presence of 100 nM unlabelled endothelin-1. Inhibition of radioligand binding by ambrisentan was evaluated over a concentration range of 0.1 nM to 10 μM. Incubation time was 30 minutes. Ambrisentan showed a high affinity for ET_A receptors (Ki = 0.63 nM) and a lower affinity for ET_B receptors (Ki = 48.7 nM) resulting in a 77-fold selectivity for ET_A receptors versus ET_B receptors. Bosentan, an approved product for the treatment of PAH, and SB 209670 were found to be much less selective for ET_A receptors compared to ambrisentan. Two positive controls, BQ-123, an ET_A selective

antagonist, and sarafotoxin S6c, an ET_B selective binding ligand were used in this study (Table 2).

Table 2 Inhibition of Endothelin Binding to Recombinant Human ET_A and ET_B Endothelin Receptors by Ambrisentan and Reference Compounds

Substance	K_i (nM) ± SEM		Selectivity
	ET_A	ET_B	ET_B / ET_A
Ambrisentan	0.63 ± 0.174	48.7 ± 11.7	77
Bosentan	3.44 ± 0.72	19.1 ± 4.1	5.6
SB 209670	0.55 ± 0.168	1.53 ± 0.37	2.8
BQ-123	14.3 ± 3.2	4570 ± 650	320
Sarafotoxin S6c	31.1 ± 17.8	0.18 ± 0.032	0.006

SEM = Standard error of the mean

Source: MPF/FG 9809

Subsequent receptor binding experiments showed that displacement of ET-1 from ET_A receptors by ambrisentan requires approximately 4 hours to attain steady state (MSR-0001 AMB). The CHO cell experiments described above were repeated with CHO cells transiently expressing ET receptors, using 50 pM ^{125}I-endothelin-1 and a 4-hour incubation time. Ambrisentan was evaluated over a concentration range of 1 pM to 1 mM (Figure 1).

Figure 1 Competition of Ambrisentan for ^{125}[I]-ET-1 Binding in rhET_A or rhET_B Receptor Expressing CHO Membranes (Representative Experiment)
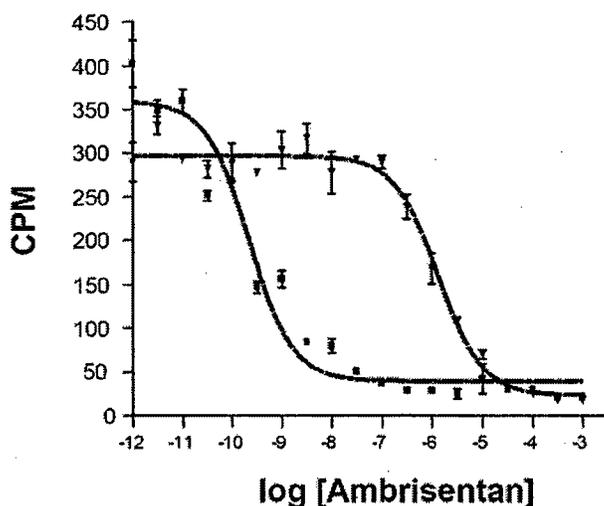
Values are mean ± SEM; n = 3 measurements per data point

rh = recombinant human; ■ = rh ET_A, ▼ = rh ET_B

Under steady-state conditions, ambrisentan exhibited high affinity for ET_A receptors (Ki = 0.049 nM) and a lower affinity for ET_B receptors (Ki = 148 nM), resulting in a >4000-fold selectivity for the ET_A relative to ET_B receptor. Identical experiments were performed with bosentan, BQ-123, and sitaxsentan (Table 3). These results show that ambrisentan has a high affinity for ETA receptors and high selectivity for ET_A relative to ET_B receptors.

Table 3 Inhibition of Endothelin Binding to Recombinant Human ET_A and ET_B Receptors by Ambrisentan and Bosentan Under Steady State Conditions

Substance	Ki (nM) ± SEM		Selectivity ET_B / ET_A
	ET_A	ET_B	
Ambrisentan	0.049 ± 0.026	148 ± 24	4347 ± 1237
Bosentan	0.117 ± 0.052	40.4 ± 15.8	367 ± 121
BQ-123	3.26 ± 1.09	18217 ± 4189	6053 ± 903
Sitaxsentan	6.15 ± 0.73	20153 ± 3025	3302 ± 365

SEM = standard error of the mean

Source: MSR-0001 AMB

In Vitro Binding of Ambrisentan to Human Cardiac ET_A and ET_B Receptors (MSR-0001 AMB)

The binding affinity of ambrisentan to native human ET receptors was examined using cellular membrane preparations obtained from the left ventricular myocardium of human hearts. The binding affinity (Ki) of ambrisentan to native human ET receptors in this preparation under steady state conditions (50 pM ^{125}I-endothelin-1, 4-hour incubation period) was approximately 0.011 nM for ET_A receptors and 40.9 nM for ET_B receptors. The resulting selectivity of ambrisentan for ET_A receptors was >4000-fold (Table 4). In this study, ambrisentan was found to be more potent and selective for the ET_A receptor than bosentan or sitaxsentan.

Table 4 Inhibition of Endothelin Binding to Human Myocardial ET_A and ET_B Receptors by Ambrisentan Under Non-steady and Steady State Conditions

Substance	K_i (nM)		Selectivity ET_B / ET_A
	ET_A	ET_B	
Ambrisentan	0.011 ± 0.003	40.9 ± 9.3	4675 ± 593
Bosentan	0.156 ± 0.051	23.4 ± 3.8	242 ± 49
Sitaxsentan	12.6 ± 3.0	12048 ± 2343	1154 ± 192
BQ-123	2.33 ± 0.7	6705 ± 2340	3302 ± 883

SD = standard deviation; SEM = standard error of the mean

Source: MSR-0001 AMB

In Vitro ET_A and ET_B Receptor Binding of Ambrisentan Metabolites (MPR/DB 0001)

Two cytochrome p450 metabolites of ambrisentan identified in nonclinical metabolism studies were evaluated for their affinity for human ET_A or ET_B receptors expressed in CHO cells (30-minute incubation). The 4-hydroxymethyl ambrisentan metabolite (M3; BSF 379912) was 64- and 84-fold less potent than ambrisentan for the ET_A and ET_B receptors, respectively (Table 5). The demethylated metabolite (O-demethyl ambrisentan; M4; BSF 4011587) was 35- and 11-fold less potent than ambrisentan at antagonizing ET_A and ET_B receptors, respectively. In human metabolism studies using radiolabeled [^{14}C]-ambrisentan, the O-demethyl ambrisentan (M4) was not detected in plasma. The 4-hydroxmethyl ambrisentan metabolite (M3) was detected and reached a maximum concentration of approximately 40-50 ng/mL (0.10-0.13 nM) in human plasma, which is 300- to 400-fold lower than the ET_A Ki determined for this metabolite (40.3 ± 6.06). Therefore, these metabolites of ambrisentan are not expected to contribute greatly to the pharmacologic effects of ambrisentan in humans.

Table 5 Inhibition of Endothelin Binding to Human ET_A and ET_B Endothelin Receptors by Ambrisentan and Metabolites

Substance	Ki (nM) ± SEM		Selectivity ET_B / ET_A
	ET_A	ET_B	
Ambrisentan	0.63 ± 0.174	48.7 ± 11.7	77
4-hydroxylmethyl-ambrisentan (M3;BSF 379912)	40.3 ± 6.06	4099 ± 322	102
O-demethyl ambrisentan (M4; BSF 4011587)	22.2 ± 4.2	556 ± 43.6	25

SEM = standard error of the mean

Source: MPR/DB 001

In Vitro Receptor Binding Profile of Ambrisentan (MPF/FG 9816E)

To further evaluate the specificity of ambrisentan for endothelin receptors, the receptor binding profile of the compound was evaluated in over 100 receptors and binding sites *in vitro*. The assays included the following receptor families: serotonin, glutamate, dopamine, histamine, growth factors, interleukin, leukotriene, acetylcholine, neurokinin, opiate, thromboxane/prostaglandin, purine, nuclear receptors, ion-channels, adenosine, adrenergic, angiotensin, bradykinin, cholecystokinin, .-aminobutyric acid, neuropeptide Y, atrial natriuretic peptide, and calcitonin gene-related peptide, insulin, galanin, glucagon, melatonin, ouabain, oxytocin and somatostatin. Ambrisentan was tested in each assay at 10 μM in duplicate.

Ambrisentan either did not inhibit or only weakly inhibited (<50%) specific radioligand binding to the receptors tested. These results suggested that the potential for any effects mediated by binding of ambrisentan to this set of receptors is limited.

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Primary Pharmacodynamics of Ambrisentan *In Vivo*

Pharmacodynamics of ambrisentan in a Big ET-1 induced hypertension model in anesthetized, normotensive rats (MPF/FT 9901)

The functional endothelin antagonist effects of ambrisentan were evaluated in anesthetized, normotensive, male Sprague-Dawley rats. In this study, animals were orally dosed by gavage with ambrisentan doses of 0, 1.0, 3.0, or 10 mg/kg body weight. The rats were then anesthetized with urethane (1780 mg/kg, intraperitoneally) 90 minutes (for a 2-hour pretreatment) or 5.5 hours (for a 6-hour pretreatment) after ambrisentan administration for implantation of carotid artery and jugular venous catheters to facilitate arterial pressure measurements and Big ET-1 administration, respectively.

Following a 15 minute equilibration period, Big ET-1 was injected intravenously at a dose of 20 µg/kg body weight. Big ET-1 is metabolized to active endothelin-1 and causes an increase in arterial pressure. Cardiovascular parameters [systolic arterial pressure (SAP), diastolic arterial pressure (DAP) and heart rate (HR)] were recorded over a 30-minute period. Administration of 20 µg/kg Big ET-1 to control animals caused rapid increases in SAP and DAP that lasted for the duration of the 30-minute recording period.

Figure 2 Effects of 2-Hour Oral Ambrisentan Pretreatment on Big ET-1 Induced Changes in Arterial Pressures and Heart Rate in Anesthetized Rats[1]
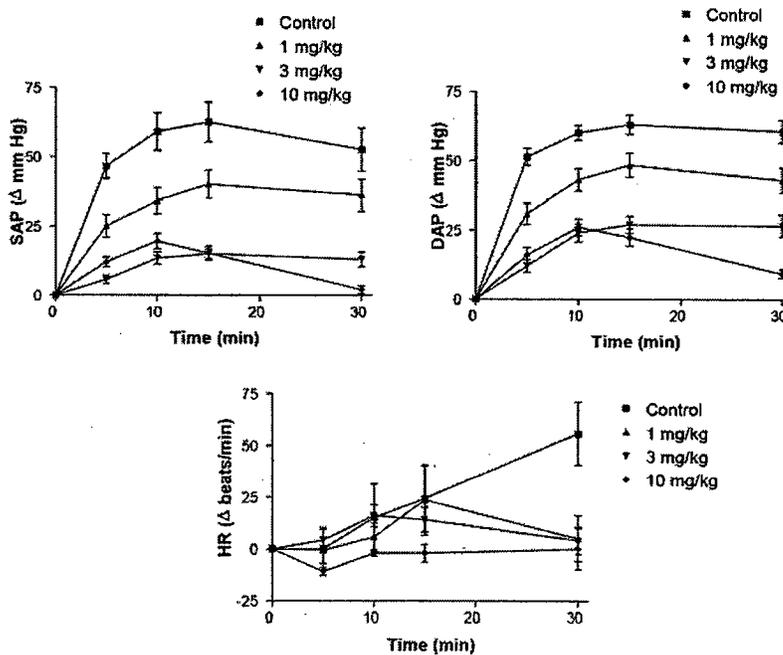
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[1]Values are mean ± SEM; n = 5-7 per treatment group

SAP = Systolic arterial pressure; DAP = Diastolic arterial pressure; HR = Heart rate

Figure 3 Effects of 6-Hour Ambrisentan Pretreatment on Big ET-1 Induced Changes in Arterial Pressures and Heart Rate in Anesthetized Rats[1]
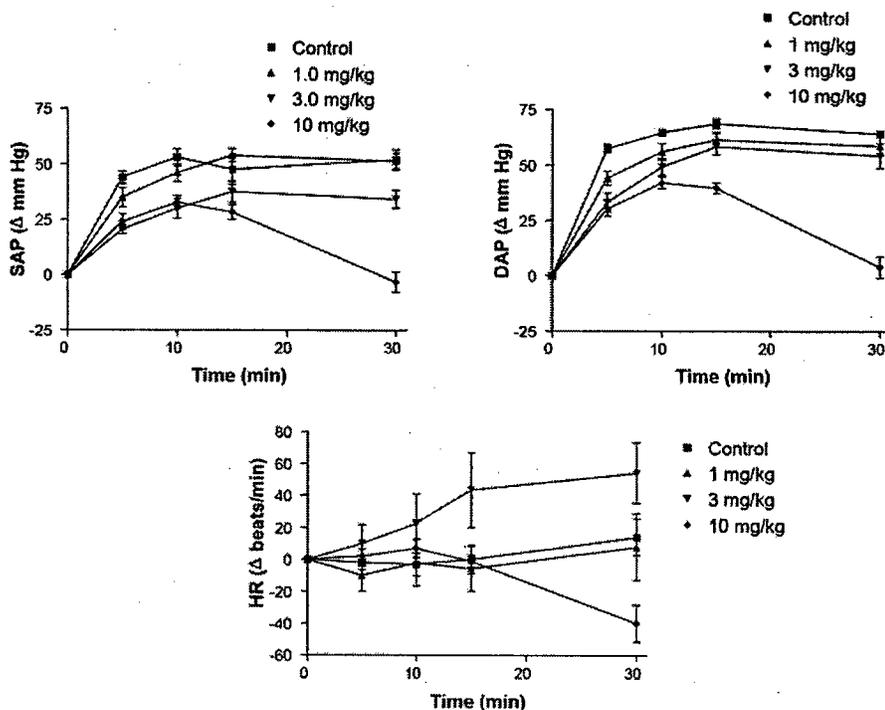
[1]Values are mean ± SEM; n = 5-7 per treatment group

SAP = Systolic arterial pressure; DAP = Diastolic arterial pressure; HR = Heart rate

Oral pretreatment with ambrisentan for 2 and 6 hours prior to infusion of Big ET-1 produced dose dependent reductions in the pressor response (diastolic and systolic pressures) to Big- ET-1. However the inhibition of Big-ET-1 effects were much greater after 2 hours of pretreatment compared to that observed after 6 hours of pretreatment. At 2 hours the highest dose on ambrisentan (10 mg/kg) was associated with an approximate 80% and 60% reduction in the systolic and diastolic pressor responses to Big-ET-1, respectively.

In this experiment, Big ET-1 administration was associated with an increase in heart rate. Oral pretreatment with ambrisentan for 2 hours appeared to block the heart rate effects of Big ET-1, although, in general, the heart rates measured in this study were highly variable. An ED_{50} for each pretreatment duration group at the 30-minute time point was calculated as 2 mg/kg (2 hour group) and 4.5 mg/kg (6 hour group).

Pharmacodynamics in chronically instrumented conscious, normotensive rats (MPF/FT 9902)

This rat study was designed to evaluate the effects of ambrisentan on arterial pressure and heart rate in normal rats. The cardiovascular effects of single oral (300 mg/kg) or iv (30 or 100 mg/kg) doses of ambrisentan were evaluated by telemetry in conscious, chronically-instrumented, freely moving, normotensive rats (Sprague-Dawley: 5 males, 5 females/dose). MAP, SAP, DAP, HR, pressure rate product (MAPxHR), locomotor activity (ACT), respiratory rate (RR), and body temperature (BT) were monitored. Measurements were taken over a 4-day control observation period to provide baseline data. The animals were then dosed and monitored for an additional 4 days.

Ambrisentan administration at 30 mg/kg iv produced no changes in any of the measured cardiovascular parameters from (MPF/FT 9902). Ambrisentan given 100 mg/kg iv and 300 mg/kg po caused a short, statistically significant, increase in arterial pressures that was followed after 30-60 minutes with a decline in SAP and DAP below baseline reaching a nadir by approximately 24 hours post-dosing (Figure 4). Thereafter, pressures partially recovered in rats orally dosed, but remained maximally depressed in the rats that received intravenous ambrisentan for 60 hours post-dosing. HR was elevated initially following ambrisentan administration by either dosing route, followed by a decline to baseline by ~8 hours. Beyond 8 hours, there was a continued decline in HR below baseline in orally treated rats, reaching a nadir of -80 beats/min at 24 hours post-dosing. In both dosing groups, a late phase decline of HR was observed between 40 and 60 hours post-dosing (Figure 4).

Ambrisentan produced increases in RR, BT, and locomotor activity during the first 1.5 hours after administration, but these variables were significantly lower than baseline at most time points during the remainder of the observation period (data not shown [MPF/FT 9902; Figure 2]).

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**Figure 4 Effects of 300 mg/kg Oral and 100 mg/kg Iv Ambrisentan on
Cardiovascular Parameters in Conscious, Normotensive Rats[1]**
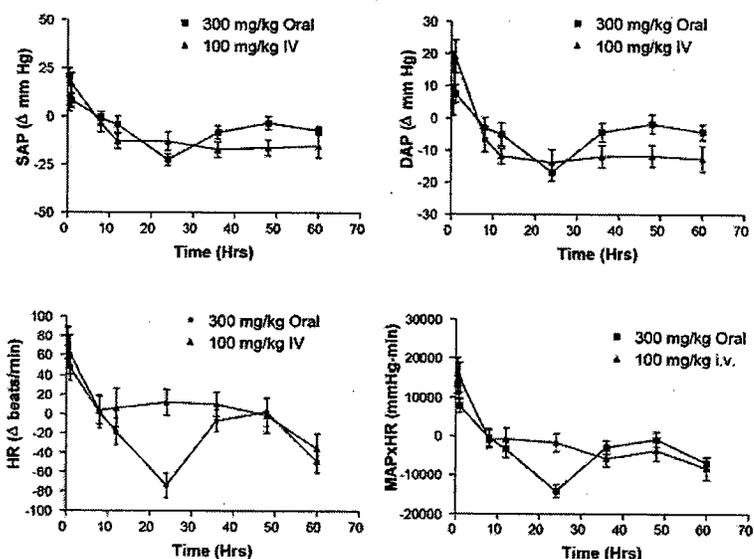
[1]Values are mean ± SEM; n = 10 per treatment group

These data indicate that the sustained ambrisentan administration at relatively high iv and oral doses results in a decrease in arterial pressure and heart rate. The early onset and transient hypertensive response observed was associated with increased heart rate, respiratory rate, body temperature, and locomotor activity, and may represent an acute adverse reaction to these high doses of the compound. The estimated ambrisentan Cmax values achieved in this study are several orders of magnitude higher than the expected Cmax in humans at the MHRD of 10 mg.

In Vivo Blood Pressure Lowering Effect of Ambrisentan in Conscious, Male, Normotensive Dogs (MPF/FE 9924)

The cardiovascular effects of single oral doses of ambrisentan were evaluated by telemetry in conscious, chronically-instrumented, normotensive male beagle dogs (6 males/group). At least 8 days before dosing, dogs were anesthetized and implanted with polyethylene catheters in the abdominal aorta and the left ventricle of the heart. SAP and DAP were measured in the abdominal aorta via a transducer. Heart rate was measured from the systolic peaks of the arterial pressure signal. Left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP) were measured with a transducer via the left ventricular catheter (left ventricular dP/dt, a measure of left ventricular contractility, was apparently not measured in this experiment). ECGs were recorded via chest leads and an ECG amplifier. On the day of dosing, ambrisentan was administered orally in gelatin capsules at doses of 0, 1, 10, or 100 mg/kg. Cardiovascular

parameters were measured continuously for 6 hours after drug administration. Table 6 lists the baseline values for all treatment groups, and Figure 5 summarizes the effects of ambrisentan on arterial pressures, heart rates, and QT intervals. QTc intervals were not calculated in this study as individual animal data were not available in the report to support a retrospective analysis of QTc.

Cardiovascular parameters were unchanged in the control group except for a reduction in heart rate that occurred late in the experiment (Figure 5).

Figure 5 Effects of Ambrisentan on SAP, DAP, HR, and QT Interval in Conscious, Normotensive Dogs[1]
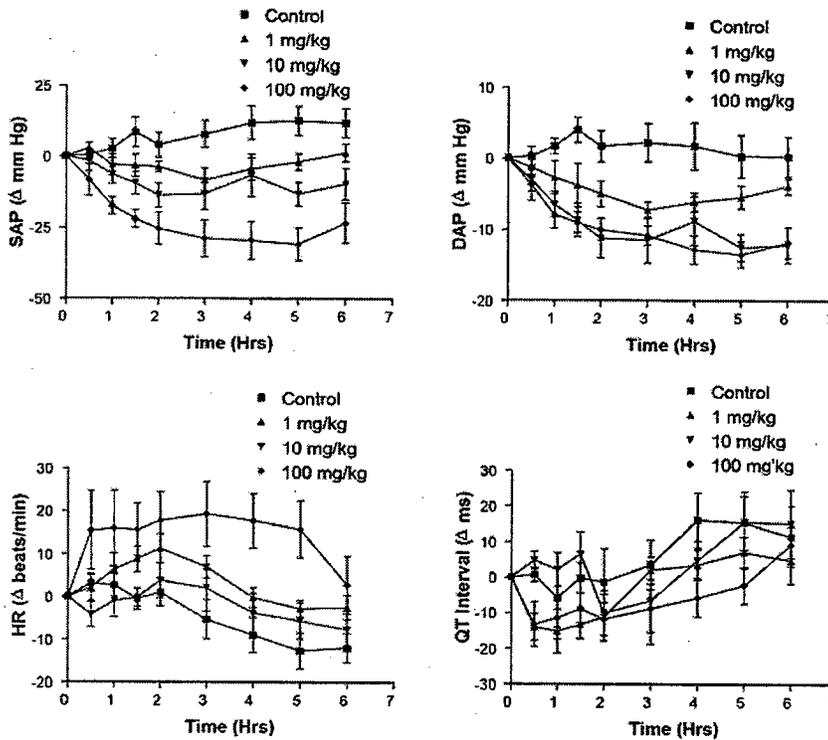
[1] Values are mean ± SEM; n = 6 per treatment group

SAP = Systolic arterial pressure; DAP = Diastolic arterial pressure; HR = Heart rate

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Table 6 Effects of Ambrisentan on Cardiovascular Parameters After a Single Oral Administration in Conscious, Normotensive Dogs[1]

Parameter	Initial value				Time Post Dose				
		30 min	60 min	90 min	2 h	3 h	4 h	5 h	6 h
					Change from Initial Value				
0 mg/kg ambrisentan, oral (Control)									
SAP (mmHg)	148 ± 3.7	0.5 ± 1.7	2.7 ± 3.3	8.5 ± 5.2	4.0 ± 4.4	7.8 ± 4.8	11.8 ± 5.9	12.5 ± 5.1	11.8 ± 5.1
DAP (mmHg)	78 ± 1.1	0.3 ± 1.3	1.7 ± 1.1	4.0 ± 1.8	1.7 ± 2.2	2.2 ± 2.7	1.7 ± 3.3	0.3 ± 3.0	0.2 ± 2.8
MAP (mmHg)	100 ± 1.7	0.7 ± 1.4	2.3 ± 1.6	5.2 ± 2.6	2.8 ± 2.5	4.2 ± 3.2	5.3 ± 3.7	4.5 ± 3.5	4.0 ± 3.4
HR (beats/min)	82 ± 6.8	3.2 ± 2.0	2.7 ± 2.6	-0.5 ± 2.5	1.0 ± 3.2	-5.3 ± 4.6	-9.0 ± 4.1	-12.7 ± 4.2*	-12 ± 3.4*
QT (ms)	252 ± 5	0.8 ± 2.0	-5.8 ± 8.3	-0.2 ± 4.2	-1.3 ± 9.3	3.5 ± 6.9	16 ± 7.7	15.2 ± 7.5	11.5 ± 8.4
1 mg/kg ambrisentan, oral									
SAP (mmHg)	144 ± 3.9	2.8 ± 2.0	-3.0 ± 3.8	-3.3 ± 3.9	-3.7 ± 1.9	-8.5 ± 4.4	-4.5 ± 3.9	-2.0 ± 2.9	1.3 ± 3.1
DAP (mmHg)	75 ± 1.7	-1.3 ± 1.1	-2.7 ± 2.3	-3.8 ± 3.1	-5.0 ± 1.8*	-7.3 ± 1.2*	-6.2 ± 1.3*	-5.5 ± 1.6*	-4.0 ± 1.1*
MAP (mmHg)	98 ± 2.0	-0.5 ± 1.0	-2.2 ± 2.5	-2.5 ± 3.3	-3.7 ± 1.6	-6.5 ± 2.1*	-4.8 ± 1.6*	-4.0 ± 1.6	-1.8 ± 0.9
HR (beats/min)	82 ± 4.5	2.2 ± 3.1	6.3 ± 3.8	8.8 ± 3.0*	11.3 ± 3.4*	7.0 ± 2.6	-0.2 ± 2.3	-2.8 ± 2.0	-2.5 ± 2.9
QT (ms)	258 ± 6.2	-14 ± 3.8*	-15.2 ± 6.2	-13.3 ± 3.9*	-11.3 ± 6.6	2.0 ± 2.6	3.5 ± 4.2	7.0 ± 4.4	4.8 ± 6.6
10 mg/kg ambrisentan, oral									
SAP (mmHg)	154 ± 6.1	-1.5 ± 3.8	-6.3 ± 3.5	-9.5 ± 4.0	-13.8 ± 4.2*	-13.2 ± 5.6	-6.7 ± 7.5	-13.3 ± 4.2*	-9.8 ± 5.5
DAP (mmHg)	79 ± 2.2	-3.0 ± 1.7	-6.5 ± 1.8*	-8.7 ± 2.3*	-11.2 ± 2.8*	-11.5 ± 3.2*	-8.8 ± 3.4	-12.5 ± 1.9*	-12.2 ± 2.5*
MAP (mmHg)	103 ± 3.1	-3.2 ± 2.0	-6.5 ± 2.2*	-8.5 ± 3.0*	-11.8 ± 3.3*	-12 ± 3.8*	-8.7 ± 4.5	-13 ± 2.4*	-12 ± 3.3*
HR (beats/min)	81 ± 3.7	-4.2 ± 2.9	-1.0 ± 3.8	-0.2 ± 2.1	3.8 ± 4.1	2.0 ± 5.5	-3.8 ± 4.6	-5.7 ± 4.2	-7.7 ± 4.4
QT (ms)	266 ± 11.5	4.8 ± 2.5	2.2 ± 4.7	6.6 ± 6.2	-10 ± 6.4	-6.6 ± 12.3	4.8 ± 5.4	15.4 ± 8.6	15 ± 9.5
100 mg/kg ambrisentan, oral									
SAP (mmHg)	147 ± 4	-8.3 ± 5.6	-17.5 ± 3.1*	-22 ± 3.0*	-25.3 ± 5.6*	-28.8 ± 6.5*	-29.5 ± 6.6*	-30.8 ± 5.7*	-23.3 ± 6.9*
DAP (mmHg)	76 ± 2.5	-3.7 ± 2.3	-8.0 ± 1.8*	-9.0 ± 1.4*	-10 ± 1.5*	-10.8 ± 1.3*	-12.8 ± 2.0*	-13.5 ± 1.8*	-11.8 ± 2.2*
MAP (mmHg)	99 ± 2.4	-4.7 ± 3.2	-10.7 ± 1.9*	-13.2 ± 1.4*	-14.2 ± 2.4*	-15.3 ± 2.5*	-16.5 ± 3.1*	-16.8 ± 2.2*	-14 ± 3.2*
HR (beats/min)	87 ± 4.9	15.5 ± 9.2	16 ± 8.8	15.7 ± 6.1	17.7 ± 6.7	19.3 ± 7.5	17.7 ± 6.3*	15.8 ± 6.6	2.8 ± 6.7
QT (ms)	251 ± 4.4	-13.2 ± 6.3	-11.5 ± 5.9	-8.7 ± 4.2	-11.8 ± 4.7	-8.7 ± 6.8	-5.7 ± 5.4	-2.3 ± 5.2	9.2 ± 5.0

[1] Values represent the mean ± SEM and are reported as change from the initial value.
HR = heart rate; SAP = systolic arterial pressure; DAP = diastolic arterial pressure; MAP = mean arterial pressure; QT = electrocardiogram interval that represents the duration of ventricular depolarization and subsequent repolarization
* p<0.05 versus initial value. Student's t-test for paired samples (2-sided); n = 6 per treatment group

Source: MPF/FE 9924

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Ambrisentan administration produced a dose-dependent reduction in arterial pressures and LVSP. LVEDP was not altered. Ambrisentan also produced a dose-dependent increases HR that partially recovered, compared to the vehicle control group by the end of the experiment. The QT interval was moderately elevated in all treatment groups, with no evidence for a treatment related effect.

The results in this study show that oral (gelatin capsule) administration of 1, 10, or 100 mg/kg of ambrisentan to normotensive, conscious dogs significantly reduced arterial pressures consistent with the pharmacology of the drug. The observed increase in heart rate observed with ambrisentan likely reflects a reflex response to the hypotensive effects of the drug.

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Summary

Ambrisentan was demonstrated in cloned human receptor expression systems and human myocardial membranes to potently and selectively antagonize the ET_A relative to ET_B receptors. Oral pretreatment with ambrisentan was shown in an intact, anesthetized rat model to antagonize an exogenous ET-1 pressor challenge. Oral, single dose ambrisentan demonstrated a sustained (hours) reduction in baseline arterial pressure when administered to conscious, instrumented rats and dogs.

Secondary pharmacodynamics

Effects of Ambrisentan in a Rat Acute Renal Failure Model (MPF/FG 9914-6)

In this study, the effects of ambrisentan were evaluated on the recovery of renal function in a rat model of ischemic acute renal failure (ARF). Male Sprague-Dawley rats were anesthetized (50-60 mg/kg, ip, pentobarbital) and a venous catheter was implanted into the right femoral vein for drug delivery at the onset of ischemia via an osmotic minipump. The kidneys were isolated, and the renal arteries clamped to induce ischemia. After 40 minutes, the kidneys were unclamped and reperfused. One-tenth of the daily treatment dose of 0, 1, 5, or 10 mg/kg/day ambrisentan was given as an iv bolus in the tail vein. This was followed by continuous infusion from the minipump. A sham group underwent the surgical procedure but without induction of renal ischemia. Over the 4-day post-ischemic period the following parameters were evaluated: urine excretion, body weight gain, food, and water consumption, and changes in serum creatinine, serum sodium, serum urea, urine creatinine, urine sodium, urine urea, and urine protein.

Acute renal failure resulted in an increase in serum creatinine concentration and a marked decrease in urinary excretion of creatinine compared to baseline (Figure 6 and Figure 7). There was a decrease in creatinine clearance in the control group on days 1, 2, and 4 post-ischemia. Treatment with 1 and 10 mg/kg/day ambrisentan had no effect on the decrease in creatinine clearance post-ischemia. Treatment with 5 mg/kg/day ambrisentan resulted in a significant increase in creatinine clearance compared to the control group (p<0.05) on days 2 and 4.

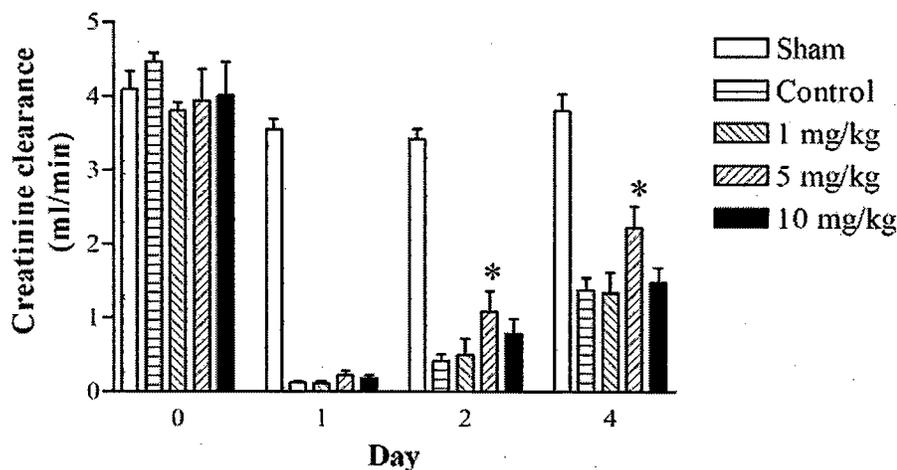Appears This Way
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Figure 6 Effects of Ambrisentan on Serum Creatinine Following Ischemia Induced Acute Renal Failure[1]
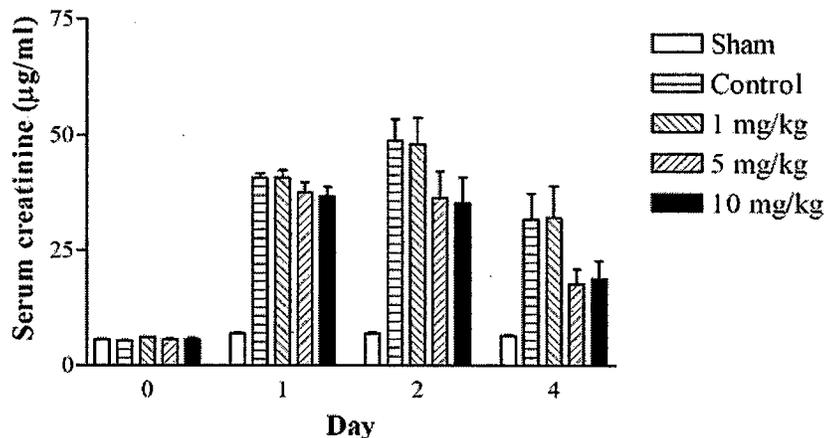
[1]Values are mean ± SEM: n = 10 (sham), n = 22 (control), n = 12 (1 mg/kg), n = 13 (5 mg/kg), n = 11 (10 mg/kg)
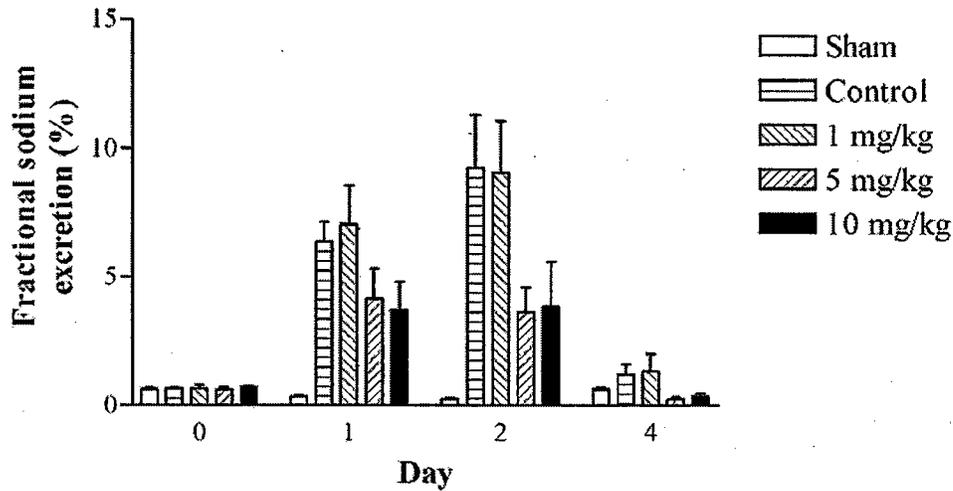
Figure 7 Effects on Ambrisentan on Urinary Creatinine Clearance Following Ischemia Induced Acute Renal Failure

Values are mean ± SEM; n = 10 (sham), n = 22 (control), n = 12 (1 mg/kg), n = 13 (5 mg/kg), n = 11 (10 mg/kg)
* = p≤0.05 versus control. Dunnett test

Serum sodium values were unchanged throughout the experiment for any treatment group (MPF/FG 9914-6). However, there was an increase in fractional sodium excretion after reperfusion in control rats on days 1 and 2, which by day 4 had largely disappeared (Figure 8). Ambrisentan treatment with 5 or 10 mg/kg/day appeared to attenuate the increase in fractional sodium excretion, but that trend was not statistically significant.

Figure 8 Effects of Ambrisentan on Fractional Sodium Excretion Following Ischemia Induced Acute Renal Failure[1]

[1]Values are mean ± SEM; n = 10 (sham), n = 22 (control), n = 12 (1 mg/kg), n = 13 (5 mg/kg), n = 11 (10 mg/kg)

Figure 9 Effects of Ambrisentan on Urine Protein Excretion Following Ischemia Induced Acute Renal Failure[1]
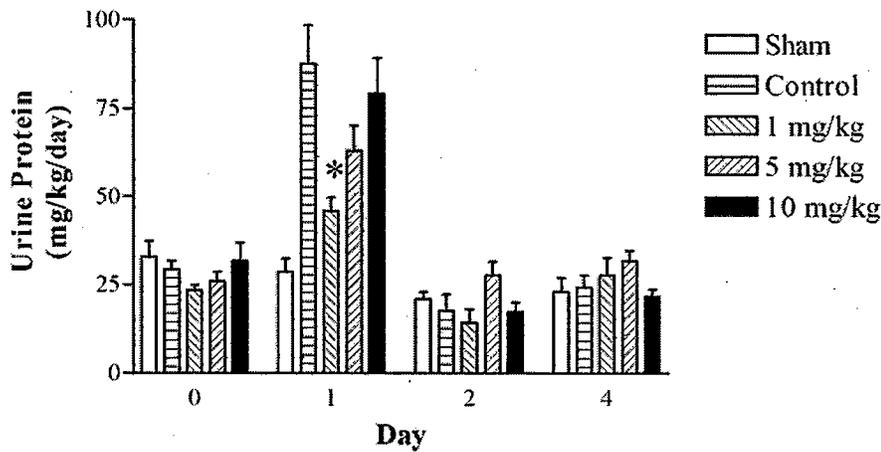
[1]Values are mean ± SEM; n = 10 (sham), n = 22 (control), n = 12 (1 mg/kg), n = 13 (5 mg/kg), n = 11 (10 mg/kg)

* = $p < 0.05$ versus control, Dunnett test

The results of this study show that ambrisentan had no consistent protective effect on renal function in a model of renal failure induced by ischemia/reperfusion.

Effects in the Perfused Langendorff Rat Heart (MPF/FG 9914-9)

The effects of ambrisentan in myocardial ischemia were evaluated in male Sprague-Dawley rats treated orally by gavage with 0, 5, 10, or 20 mg/kg ambrisentan. Two hours after ambrisentan pretreatment, the animals were sacrificed and explanted hearts were prepared for Langendorff perfusion. The isolated heart was allowed to stabilize for 20 minutes before undergoing global normothermic ischemia for 30 minutes. During the following 60-minute reperfusion period, sampling occurred at 5, 10, 15, 30, and 60 minutes. The following parameters were evaluated: left ventricular pressure (LVP), contractility (LV +dP/dtmax), left ventricular end diastolic pressure (LVEDP), creatine kinase release, and coronary flow rate.

LVP and LV +dP/dtmax of hearts from all treatment groups were both reduced by approximately 90% after 3 minutes of reperfusion. Pretreatment with 5-20 mg/kg ambrisentan dose-dependently attenuated the ischemia/reperfusion associated decrements in LVP and LV +dP/dtmax compared to control (p<0.05). No other protective effects were observed following ambrisentan pretreatment in this study.

Based on these results, ambrisentan may have a beneficial effect on myocardial ischemia/reperfusion injury as characterized by an improvement in left ventricular developed pressure and contractility.

Effects of Ambrisentan on Neointima Formation after PTCA in Pigs (MPF/FE 9925)

Animal studies have shown that the cellular injury occurring during percutaneous transluminal coronary angioplasty (PTCA) results in an upregulation of ET_A receptors on vascular smooth muscle cells. The effect of ambrisentan on the response to vascular injury was examined in a porcine PTCA restenosis model. The study included 2 arms. The objective of the first arm was to choose the lowest dose effective in the PTCA model. The objective of the second arm of the study was to further investigate the role of endothelin receptor antagonism for 6 weeks in a PTCA plus stent-induced restenosis model using the lowest effective dose identified in the first arm of the study.

Pigs were anesthetized (2 mg/kg stresnil, intramuscular; 4 mg/kg metomidate, iv) and a standard PTCA guide catheter was inserted into the left coronary artery via the aortic route. The balloon catheter was positioned in the first third of the left anterior descending artery using a 0.014 inch PTCA guide wire. The balloon was inflated twice to 10 atmospheres for 30 seconds. The balloon was then deflated, withdrawn, and an angiogram was prepared to verify the lesion. The animals were then randomly allocated to 3 different treatment groups that received 0, 10, and 30 mg/kg/day ambrisentan orally as a water/NaOH solution admixed with feed. After 4 weeks of treatment, coronary

arteries at the site of PTCA were histologically evaluated. The cross sectional area of each segment was determined using digital morphometry. Neointimal area was determined as the difference of the residual luminal area from the total area within the internal elastic lamina. Total area within the internal elastic lamina was considered to be the normal luminal area. Medial area was defined as that smooth muscle outside the the neointimal zone.

PTCA of normal coronary arteries had no effect on vessel outer diameter in all 3 groups (all groups diameter = 1.5 mm). The medial area was significantly increased in both ambrisentan treated groups compared to controls (control = 0.39 ± 0.03 mm2; 10 mg/kg/day = 0.60* ± 0.05 mm2; 30 mg/kg/day = 0.53* ± 0.04 mm2; * = significantly different from control p<0.05). A reduction in neointimal area was apparent in both treatment groups versus controls, but statistical significance occurred only in the 10 mg/kg/day group (control = 0.85 ± 0.15 mm2; 10 mg/kg/day = 0.55* ± 0.10 mm2; 30 mg/kg/day = 0.62 ± 0.08 mm2). Non-significant reductions of neointimal area in the treatment groups resulted in a trend toward increased in total luminal area (MPF/FE 9925).

Since the results were similar for the 10 and 30 mg/kg/day groups, the lower dose was chosen for the subsequent experiment in which PTCA was combined with stent implantation. Following PTCA and stent implantation, animals were treated orally for 6 weeks with 0 or 10 mg/kg/day ambrisentan in the feed. The coronary arteries were then excised and histologically evaluated.

PTCA with subsequent stent implantation had no effect on total coronary artery diameter. In contrast to the results obtained for animals treated with 10 mg/kg/day ambrisentan, medial area was not increased (control = 1.81 ± 0.15 mm2; 10 mg/kg/day = 1.91 ± 0.55 mm2). This lack of difference in medial area results between control and treatment may be related to the presence of the stent. A significant reduction in the neointimal area from control in the 10 mg/kg/day group (control = 4.6 ± 0.02 mm2; 10 mg/kg/day = 3.6* ± 0.35 mm2) was observed. This resulted in a statistically significant increase in total luminal area in the 10 mg/kg/day which was 66% larger than that of the control group.

The results of this study show that ambrisentan treatment reduces the neointimal formation that occurs following PTCA thereby increasing total luminal area.

Summary

Although the study suggests that ambrisentan may attenuate myocardial ischemia / reperfusion injury the rat myocardial ischemia, ambrisentan was not effective in a rat renal ischemia/reperfusion model. Ambrisentan was also effective in reducing coronary arterial balloon dilatation injury in the pig.

Safety pharmacology

Ambrisentan was examined in a series of 26 safety pharmacology studies performed *in vitro, ex vivo*, and *in vivo*; 24 of these studies were conducted under GLP conditions. The safety pharmacology studies were all conducted with the same batch of ambrisentan (Lot L0002155), which was comparable in purity to drug substance batches used in clinical trials. The majority of these studies are reviewed in the initial submission ▬▬▬▬▬ ▬▬▬▬▬ which can be found in DFS.

Single dose effects of ambrisentan on the central nervous system were evaluated in mice and rats. In mice (MPF/FG 9901E, MPF/FG 9906E, MPF/FG 9916E and MPF/FG 9917E), ambrisentan (10 to 100 mg/kg, iv; 100 and 300 mg/kg, oral) had no significant effects on locomotor activity, motor coordination, behavior, or hexobarbital-induced sleep. In a rat model of spontaneous vigilance (MPF/FG 9907E and MPF/FG 9908E), ambrisentan (30 and 100 mg/kg, iv; 300 mg/kg, oral) had no effect on electroencephalogram (EEG) parameters.

Effects of ambrisentan on cardiovascular and respiratory systems were evaluated *in vitro* and *in vivo*. An *in vitro* study (MPF/FS 9808) showed that 0.1, 1.0 and 10 μM ambrisentan had no effect on the electrophysiological properties of guinea pig heart papillary muscle or on the phrenic nerve-diaphragm neuromuscular junction. When administered to anesthetized dogs at 100 mg/kg iv, ambrisentan caused transient, significant decreases in arterial pressure that recovered within 10 to 20 minutes (MPF/FG 9910E, review appended). Intravenous administration had no significant effects on electrocardiogram (ECG) parameters or respiration (MPF/FG 9910E). Intraduodenal administration of 100 mg/kg ambrisentan in dogs had no consistent effects on arterial pressure, arterial blood velocity, cardiac output, ECG, or respiratory parameters (MPF/FG 9911E).

Gastrointestinal effects of ambrisentan were evaluated *in vitro* and *in vivo*, in rats. Ambrisentan at concentrations of 0.1, 1.0 and 10 μM had no effect on the histamine induced spasmolytic activity of isolated guinea pig ileum segments (MPF/FG 9922E). In the intact rat, ambrisentan had no significant effects on intestinal motility after iv (30 and 100 mg/kg; MPF/FG 9914E) or oral (100 or 300 mg/kg; MPF/FG 9915E) administration. However, gastric emptying of the stomach to the small intestine was reduced by administration of iv ambrisentan (100 mg/kg) and oral ambrisentan (300 mg/kg). Intravenous administration of 30 mg/kg and 100 mg/kg ambrisentan to rats induced dose-dependent increases in gastric acid secretion (MPF/FG 9918E). Intraduodenal administration of 100 or 300 mg/kg in the rat had no effect on gastric secretion (MPF/FG 9919E). Intravenous administration of 10 and 30 mg/kg (MPF/FG 9920E) and intraduodenal administration of 30 mg/kg (MPF/FG 9921E) ambrisentan caused increases in bile secretion in the rat.

The effects of ambrisentan on renal function were evaluated in rats. When administered intravenously (10, 30, or 100 mg/kg), dose-dependent reductions in sodium and chloride

excretion occurred in males and females (MPF/FG 9912E). Oral administration of 100 or 300 mg/kg also reduced sodium and chloride excretion in both males and females (MPF/FG 9913E). Calcium excretion was also reduced following oral administration of ambrisentan but reached statistical significance in females only.

In an *in vitro* model of oxytocin induced uterine spasmolytic activity (MPF/FG 9923E), ambrisentan showed marginal inhibitory effects at high doses (10 µM) in combination with low doses of oxytocin (2 and 6 mIU/ml).

Ambrisentan had no effects on blood coagulation endpoints nor caused hemolysis at concentrations of 0.1, 1, and 10 µM (MPF/FG 9924E) in *in vitro* studies. No mitogenic effects were observed in an *in vitro* study using human blood at ambrisentan concentrations of 10 pM to 10 µM (MPF/FG 9984).

Pharmacodynamic drug interactions

No pharmacodynamic drug interaction studies were performed.

PHARMACOKINETICS/TOXICOKINETICS

Brief summary

The absorption, distribution, metabolism, and excretion (ADME) profile of ambrisentan has been assessed in the mouse, rat, rabbit, and dog, the main species used in the nonclinical safety evaluation of the drug. The studies were conducted following single dose administration of either non-radiolabeled or [^{14}C]-labeled ambrisentan by oral and/or intravenous (iv) routes. The doses used in ADME studies were generally in the range used in toxicology studies, and the formulations were also similar to those used in the toxicology studies. The pharmacokinetic/toxicokinetic characteristics of ambrisentan following repeat dosing were assessed during the conduct of the toxicology studies. Single and repeat dose pharmacokinetic/toxicokinetic parameters were obtained using validated analytical methods for determination of ambrisentan in plasma. The absorption, metabolism and excretion of [^{14}C]-ambrisentan have also been determined in healthy male human subjects, and these data have been used to determine the validity of the species used for toxicology evaluations. All of these studies have been conducted in compliance with Organizational Standard Operating Procedures and Policies or in general accordance with the principles of Good Laboratory Practice (GLP).

Ambrisentan was well absorbed following oral administration, with little or no first pass metabolism, resulting in high absolute oral bioavailability both in rats (85%) and dogs (72%). Pharmacokinetic studies following iv dosing showed that ambrisentan has low plasma clearance (2.6 and 3.0 mL/min/kg in rats and dogs, respectively) and low to moderate steady-state volume of distribution in rats (0.37 L/kg) and dogs (0.91 L/kg). In

toxicology studies, there were differences in exposure to ambrisentan between male and female animals. In mice and dogs, repeat ambrisentan dosing resulted in greater exposures in females than in males. In contrast, repeat dosing in rats resulted in greater exposure in males than in females. Escalation in dose generally resulted in an approximate proportional increase in the plasma AUC and Cmax in males and females in all species.

Ambrisentan was highly bound to plasma proteins with somewhat higher binding in humans (98.9%) compared to that in nonclinical species (91.8 - 97.2%) at concentrations up to 20 µg/mL. The binding occurred primarily to serum albumin. Following oral administration of [^{14}C]-ambrisentan, the drug-related material was distributed widely, with the highest concentrations observed in the gastrointestinal tract, followed by the liver, plasma, lungs, and kidneys. There was very limited penetration of drug-related material into brain, and there was no preferential binding to melanin. The radioactivity levels declined with time, and there was no evidence of retention in any tissue.

Figure1 provides the chemical structure of ambrisentan and the sites of metabolism. Enzymes that metabolize ambrisentan were evaluated in microsomes prepared from a human lymphoblastoid cell line or baculovirus-infected insect cells expressing individual human CYPs and UGT enzymes. The results indicate that ambrisentan was glucuronidated by UGT1A9S, UGT2B7S, and UGT1A3S, and was hydroxylated at the 4-methyl position of the pyrimidine, primarily by CYP3A4, 3A5 and 2C19, at relatively low turnover rates. CYP3A4 appears to be the primary enzyme responsible for oxidative metabolism of ambrisentan because of its greater abundance in the liver and its relatively higher turnover rate. CYP3A5 and CYP2C19 could also contribute to metabolism of ambrisentan to lesser extent. A number of other CYPs also appear to metabolize ambrisentan to the 4-hydroxymethyl ambrisentan (M3) metabolite, although, to a much lesser extent.

Figure 1 Ambrisentan Chemical Structure and Sites of Metabolism
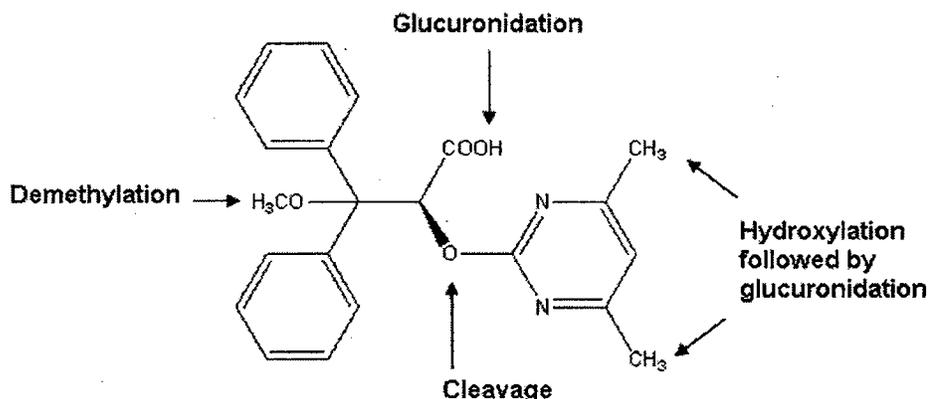
Metabolism studies using [^{14}C]-ambrisentan have been conducted in the mouse, rat, rabbit, dog, and humans. The structural sites on the ambrisentan molecule at which metabolism occurs is shown in Figure 1. The extent of metabolism and predominant routes observed varied somewhat among species, but generally in all species, the parent drug was the predominant species in plasma. In the mouse, metabolism was more extensive than in the rat and rabbit, with the least extent of metabolism observed in the dog.

In the mouse, the extent of metabolism was highest with up to 6 separate radio-components detected in plasma. In a [^{14}C]-ambrisentan study completed in humans (AMB-107), the parent drug was also the predominant circulating species with 4-hydroxymethyl ambrisentan and glucuronides ambrisentan glucuronide and 4-hydroxymethyl ambrisentan glucuronide representing minor metabolites. A cleavage product, 4,6-dimethyl-2-hydroxypyrimidine, was observed in mouse, rat, rabbit and dog plasma, as a minor metabolite, but not in human plasma. In the rat, O-demethylation (O-demethyl ambrisentan) and a combined product of O-demethylation and hydroxylation (O-demethyl 4-hydroxymethyl ambrisentan) represented minor drug-related components in plasma. Circulating ambrisentan glucuronide was observed in appreciable amounts (up to 32% of total radioactivity) in dog plasma and to a lesser extent in the mouse, but it was not observed in rat plasma.

The three metabolites observed in human plasma, urine, and feces were ambrisentan glucuronide, 4-hydroxymethyl ambrisentan, and 4-hydroxymethyl ambrisentan glucuronide and were also observed in one or more nonclinical species.

In vitro, ambrisentan did not inhibit human cytochrome p450 enzymes (CYPs) and UDP-glucuronosyltransferases (UDP-GT) at concentrations of at least up to 100 µM. *In vivo*, ambrisentan had no marked effect on the selected CYPs, UDP-GT, and glutathione-S-transferase (GST) activities in intact rats dosed up to 400 mg/kg/day for 4 weeks. In dogs, daily ambrisentan administration for 4 weeks resulted in only mild induction (<5-fold) of CYP2B and CYP3A enzymes at doses of 100 to 1000 mg/kg. In an *in vitro* study using human liver microsomes, 23 of the 24 potential concomitant medications had no effect on the metabolism of ambrisentan. In Caco-2 cells, ambrisentan was observed to be a substrate for Pgp mediated efflux, as its transport was inhibited by cyclosporine A. However, ambrisentan was not found to be an inhibitor of Pgp transporter in MDCK cells. Additional *in vitro* studies using rat and human hepatocyte sandwich cell cultures showed that ambrisentan did not inhibit or induce the hepatic transporters, NTCP, OATP, BSEP and MRP2. These results suggest that the potential for drug-drug interactions with ambrisentan is low.

Studies using [^{14}C]-ambrisentan demonstrated that the predominant route of elimination was feces (69-91%) with urine representing a minor route (7-23%) in mice, rats, and dogs. The majority (=85%) of administered radioactivity was excreted during the first 24 hours in all species. Similar results were obtained in humans, where fecal and urinary excretion accounted for 66% and 23% of the dose, respectively. Studies conducted in bile duct-cannulated animals showed that a major portion of the intraduodenal dose was

secreted in bile in both rats (95%) and dogs (54%), suggesting that a significant proportion of the dose excreted in feces was likely due to parent drug and metabolites eliminated in bile.

Overall, the ADME characteristics of ambrisentan were generally consistent in mice, rats, and dogs and were similar to those observed in humans. These results support the use of these species as the principal species for assessment of nonclinical safety of ambrisentan.

METHODS OF ANALYSIS

Ambrisentan concentration in plasma was determined by using validated High Performance: ~~_____~~) or High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) methods. The plasma sample (0.1 to 1 mL) was adjusted to pH ~2 with a buffer, spiked with an internal standard, and then extracted with an ~~_____~~ . The ~~___~~ phase was ~~_____~~ reconstituted in mobile phase and injected into ~~____~~ or HPLC-MS/MS : ~~_____~~). The ambrisentan calibration curves ranged 10-5000 ng/ml for HPCE and 20-1000 ng/ml for HPLC-MS/MS. The methods also included quality control (QC) standards representing the low, medium and high concentrations. The linearity as measured by r^2 was ~~__~~ or greater. The extraction efficiency was greater than ~~__~~ . The precision, as measured by the coefficient of variance (CV%) was = ~~__~~ for all concentrations, and the intra-series accuracy of the QC samples was within ±20% of the target concentration. The assay was reproducible when repeated over several days, with an overall CV% of 15% or less. Lower limit of quantitation under optimal assay conditions was 10 ng/mL for ~~_____~~ ' and 10-50 ng/ml for the HPLC-MS/MS assays, depending on the sample volume. There was no significant interference from endogenous or compound-related materials with the quantitation of ambrisentan in plasma.

The results obtained during validation studies showed that ambrisentan in rat and dog plasma was stable up to 48 hours at 25°C. Similarly, rat and dog plasma QC samples subjected to three freeze thaw cycles at -20°C were found to be within 12% of their nominal concentrations. Ambrisentan was also found to be stable at -20°C for 9 months in rat and dog plasma.

The validation of the ~~_____~~ and HPLC-MS/MS assays showed that the results were suitable for the determination of ambrisentan in plasma samples from pharmacokinetic and toxicokinetic studies. These assays were also found to be generally suitable for quantitation of ambrisentan in other biological matrices for ADME evaluation.

Mass balance, tissue distribution, metabolism and excretion studies in animals were conducted with [^{14}C]-ambrisentan, with the [^{14}C]-radiolabel placed in the 4 position of the pyrimidine ring (Figure 2a). For the human ADME study, the label was incorporated (Figure 2b) in the 4-methyl group of the pyrimidine ring. Both positions were considered to be metabolically stable, such that the [^{14}C]-label was associated with metabolites in all species. Although, [^{2}H6] labeled ambrisentan (Figure 2c) was also synthesized and mixed

with [^{14}C]- and unlabelled ambrisentan to achieve desired specific activity for the human study, the [^{2}H6] label was not utilized for tracking metabolites. The radioactivity in biological samples was quantitated using appropriate methods including liquid scintillation counting (LSC), HPLC- ▬▬▬▬ detection, or whole body autoradiography.

Figure 2 [^{14}C]-radiolabeled Ambrisentan (a) Used in Animal Studies and (b) Used in Human Studies, (c) [^{2}H$_6$]-labeled Ambrisentan
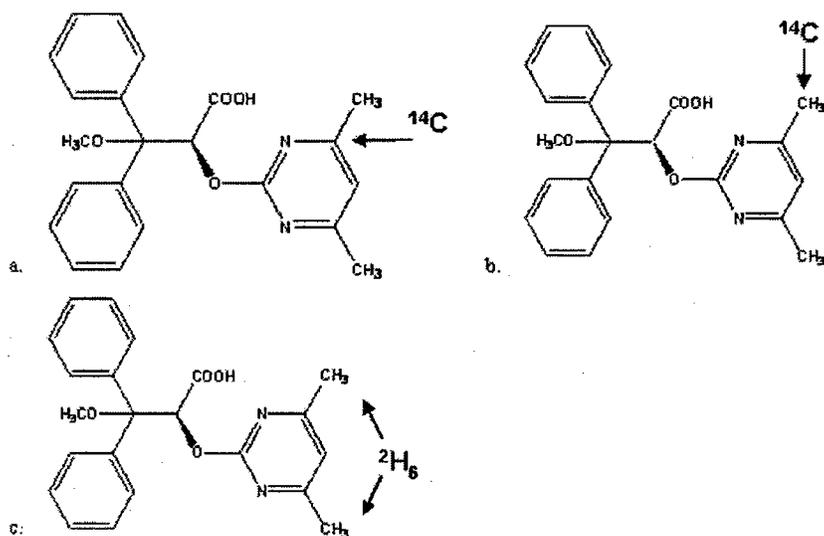
Absorption

Single Dose Pharmacokinetic Studies

Single-dose pharmacokinetic characteristics of ambrisentan were assessed in rats, and dogs following oral and iv dosing, and in humans following oral dosing. Male Wistar rats were given a single dose of 9.8 mg/kg by iv or a 30 mg/kg by oral gavage (MPF/EK 9805; Table 2). In another study, male Wistar rats were administered 800 and 2000 mg/kg by oral gavage (MPF/DT 9862E). Similarly, male beagle dogs received ambrisentan as a single iv dose of 10 mg/kg (MPF/DDB 9866) or as a single oral dose (hard gelatin capsule) of 30 mg/kg (MPF/EBK 9808; Table 2). In humans, a single oral dose (tablets) of 1, 5, 10, 15, 20, 50, and 100 mg ambrisentan were administered to healthy male volunteers (EE- 001; Table 2). The results are summarized in Table 3.

Table 2 List of Single Dose Pharmacokinetic Studies Performed with Ambrisentan (BSF 208075) Wistar Rats, Beagle Dogs and Humans

Type of Study	Species (strain)	Sex	Mode of Administration	Dose (mg/kg)	Plasma Sampling Occasions (hr)	GLP Status	Testing Facility[1]	Report No. (Study No.)
Single dose pharmacokinetic study	Rat / Wistar	M	iv	9.8	0, 0.033, 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 32, 48	GLP		MPF/EBK 9805
			Oral (gavage)	30	0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 32, 48			
Single dose pharmacokinetic study	Rat / Wistar	M	Oral (gavage)	800 2000	0, 1, 2, 4, 8, 24	GLP		MPF/DT 9862E
Single dose pharmacokinetic study	Dog / Beagle	M	iv	10	0, 0.033, 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 30, 48, 72	GLP		MPF/DDB 9866
			Oral (Capsule)	30	0, 0.17, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 30, 48, 54, 72			MPF/EBK 9808
Single dose pharmacokinetic study	Humans	M	Oral (Tablet)	1, 5, 10, 15, 25, 50 and 100 mg	0, 0.17, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 30, 48, 54, 72	GLP		EE-001

Testing Facility ▬▬▬▬▬▬▬▬▶

In the single dose (9.8 mg/kg) iv studies in Wistar rats and beagle dogs, ambrisentan exhibited multi-phasic plasma concentration vs. time curves. In general, all rat and dog plasma concentrations were adequately described by a two compartmental model. The steady state volume of distribution was 0.37 L/kg in rats and 0.91 L/kg in dogs. Ambrisentan was rapidly cleared from the central compartment in both rats and dogs with 1st elimination half-lives of 28 and 27 minutes, respectively. The terminal elimination half-life values for the iv dose were 4.7 hours in rats and 8.8 hours in dogs. The mean residence time was approximately 2 hours in rats and 4 hours in dogs. Based on the comparison of AUCs after oral and iv dosing, ambrisentan had high absolute oral bioavailability of 85% in rats and 72% in dogs.

In the single oral dose studies, ambrisentan was rapidly absorbed in both rats and dogs. In rats, after administration of 30 mg/kg of ambrisentan by gavage, a mean maximum plasma concentration (Cmax) of 32.4 µg/mL was achieved at 1 hour (tmax) (Table 3). In dogs, after a 30 mg/kg dose given in capsule, Cmax was 43.6 µg/mL with tmax between 0.5 and 2 hours. The half-life ($t_{1/2}$) was determined to be 5.7 hours in rats and 7.9 hours in dogs. Systemic exposure (AUC and Cmax) increased in rats with escalation of the oral dose.

In humans, after a single oral dose of 1, 5, 10, 15, 20, 50, and 100 mg of ambrisentan to healthy male volunteers, (EE-001), the drug was rapidly absorbed into the systemic circulation, with peak plasma concentrations attained after 1.5 hours post dose (tmax) with a range of 1 to 2 hours in fasting subjects and 1.5 to 2 hours for fed subjects. The Cmax was linear with dose, increasing from 0.057 µg/mL at 1 mg to 4.52 µg/mL at 100 mg. Similarly, the AUC increased with dose in a linear manner, from 0.34 µg·hr/mL at 1 mg to 37.15 µg·hr/mL at 100 mg. The elimination half life for ambrisentan ranged from 5.75 to 12.71 hrs. The Cmax, AUC and $t_{1/2}$ values for the maximum therapeutic dose of 10 mg were 0.67 µg/mL, 3.62 µg·hr/mL and 9.4 hrs, respectively. In general, the single

dose PK characteristics of ambrisentan in humans were similar to those observed in animal studies.

Table 3 Single Dose PK parameters for Wistar Rats, Beagle Dogs and Humans Following Single Oral and/or iv Administration of Ambrisentan (BSF 208075)

Single Dose PK parameters	Wistar Rat				Beagle Dog		Human						
	Oral 30 mg/kg	iv 9.8 mg/kg	Oral 800 mg/kg	Oral 2000 mg/kg	Oral 30 mg/kg	iv 9.8 mg/kg	Oral 1 mg	Oral 5 mg	Oral 10 mg	Oral 15 mg	Oral 20 mg	Oral 50 mg	Oral 100 mg
t_{max} (hr)	1	0.03	2	4	0.5-2	0.033	1.5	1.0	1.5	1.5	1.5	1.5	2.0
C_{max} (µg/mL)	32.4	106.3	185.4	252.5	43.6	104.5	0.057	0.35	0.67	0.915	1.40	3.23	4.52
AUC (µg·hr/mL)	161.1	61.9	2084.2	4858.0	115.6	55.4	0.34	2.04	3.62	6.52	9.72	19.22	37.15
Time for calculation (hr)	0-48	0-32	0-∞	0-∞	0-24 & 0-72	0-30 & 0-48	0-48	0-48	0-48	0-48	0-48	0-48	0-48
Vss (L/kg)	nd	0.37	nd	nd	nd	0.91	nd	nd	nd	nd	nd	nd	nd
$t_{1/2}$ (hr)	5.7	4.7	nd	nd	7.9	8.8	5.75	8.9	9.4	12.0	11.1	12.7	10.7
F (%)	85.0	nd	nd	nd	71.5	nd	nd	nd	nd	nd	nd	nd	nd
Cl (mL/min·kg)	nd	2.6	nd	nd	nd	3.0	nd	nd	nd	nd	nd	nd	nd

nd = not determined

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Repeat Dose Pharmacokinetic / Toxicokinetic Studies

Repeat dose oral pharmacokinetic characteristics of ambrisentan were assessed in mice, rats, rabbits and dogs as part of the toxicology studies (Table 4). Repeat oral pharmacokinetic characteristics of ambrisentan were assessed in pregnant or nursing rats or rabbits as part of the toxicology studies (Table 5).

Table 4 List of Repeat Dose Studies Performed with Ambrisentan (BSF-208075)

Study Duration	Species (strain)	Sex	Mode of Administration	Dosage (mg/kg/day)	Plasma Sampling Occasions (hr)	GLP	Testing Facility[1]	Report No. (Study No.)
4 Week	Mouse / NMRI	M/F	Oral (feed mix)	0, 250, 500, 1000, 2000	Days 2/3 and 24/25 0, 1, 2, 4, 6, 8, 10, 12, 18, 24	Non-GLP		MPF/DT 2600
13 Week	Mouse / CD1	M/F	Oral (feed mix)	0, 60, 150, 500, 1250	Week 11 0, 1, 2, 4, 6, 8, 10, 12, 18, 24	GLP		MPF/PT 0115E
104 Week	Mouse / CD1	M/F	Oral (feed mix)	0, 50, 100, 250/150	Week 2 and 52 0, 4, 8, 12, 16, 20, 24, and Week 104 at 0 and 4	GLP		‑851466
4 Week	Rat / Wistar	M/F	Oral (gavage)	0, 1, 4, 16, 40	Days 1/2 and 28/29 0.5, 1, 2, 4, and 24	GLP		MPF/DT 0033
4 Week	Rat / Wistar	M/F	Oral (gavage)	0, 40, 125, 400	Dose 1 and Dose 10 0.5, 1, 2, 4, and 24	GLP		MPF/DDB 9953
6 Week	Rat / Wistar	M/F	Oral (feed mix)	0, 250, 500, 1000, 2000	Days 2/3 and 24/25 0, 1, 2, 4, 6, 8, 10, 12, 18, 24	Non-GLP		MPF/DT 2500E
13 Week	Rat / Wistar	M/F	Oral (feed mix)	0, 10, 100, 500, 2000	Days 2/3, 29/30 and 91/92 8, 12, 20, 24 / 4, 8	GLP		MPF/DT 0032E
15 Week	Rat / Wistar	M	Oral (feed mix)	0 and 300	Day 2 and 29 0, 4, 12, 16, 20, 24	GLP		AMB 001
26 Week	Rat / Wistar	M/F	Oral (feed mix)	0, 5, 100, 500	Days 2/3, 85/86 and 175/176 0, 1, 2, 4, 6, 8, 10, 12, 18, 24	GLP		‑800313
104 Week	Rat / Wistar	M/F	Oral (feed mix)	0, 10, 30/20, 60/40	Week 2, 52, 65 and 104 at 0, 4, 8, 12, 16, 20, 24	GLP		‑851465
4 Week	Dog / Beagle	M/F	Oral (powder in capsule)	0, 80, 200, 500	Day 1 and 28 0, 1.5, 3, 6, 24	GLP		MPF/DDB 9907

Table 4 List of Repeat Dose Studies Performed with Ambrisentan (BSF-208075) (continued)

Study Duration	Species (strain)	Sex	Mode of Administration	Dosage (mg/kg/day)	Plasma Sampling Occasions (hr)	GLP	Testing Facility[1]	Report No. (Study No.)
4 Week	Dog / Beagle	M/F	Oral (gavage)	0,100, 300,1000,15	Day 1 and 27 0, 0.5, 1, 3, 6, 24	GLP	----	MPF/PT 0031E
4 Week	Dog / Beagle	M/F	Oral (powder in capsule)	0, 500, 1000	Day 1 and 28 0, 1.5, 3, 6, 24	Non-GLP		MPF/DT 1500
13 Week	Dog / Beagle	M/F	Oral (gavage)	0, 100, 300, 1000	Day 1, 28 and 91 0, 0.5, 1, 3, 6, 24	GLP		MPF/PT 0035E
26 Week	Dog / Beagle	M/F	Oral (gavage)	0, 100, 300, 900	Day 1, 90 and 174/177 0, 0.5, 1, 3, 6, 24	GLP		MPF/PT 0111E
39 Week	Dog / Beagle	M/F	Oral (gavage)	0,30, 300, 600	Day 1, 91, 182 and 273 0, 0.5, 1, 3, 6, 24	GLP		2002-6032
10 days	Humans	M	Oral (gavage)	5, 7.5, 10	Day 1 and 10 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 16	GLP		EE-002
Repeat dose	Humans	M / F	Oral (capsule)	1, 2.5, 5, 10 mg	Day 1 and Week 12	GLP	Myogen	AMB-220 and AMB-320/321-E

Testing Facility[1]:

Table 5 List of Repeat Dose Studies Performed in Pregnant or Nursing Animals with Ambrisentan (BSF 208075)

Type of Study	Species (strain)	Sex	Mode of Administration	Dosage (mg/kg/day)	Sampling Occasions	GLP	Testing Facility[1]	Report No. (Study No.)
Repeat dose Pharmacokinetic study	Rat / Wistar Pregnant	F	Oral (gavage)	0, 15, 47, 150	Day 6 and day 17 post coitus 1, 2, 4, 8, 24, hrs	GLP		MPF/DDR 0011E
Repeat dose Pharmacokinetic study	Rabbit, Chinchilla Pregnant	F	Oral (gavage)	150, 300, 600	Day 6 and day 18 post coitus 2 and 24 hrs	GLP		MPF/DT 0599E
Repeat dose Pharmacokinetic study	Rabbit, Chinchilla Pregnant	F	Oral (gavage)	7, 21, 63	Day 6 and day 18 post coitus 0, 0.5, 2, 4, 8, 24 hrs	GLP		MPF/DT 9926E

Testing Facility[1]:

Mice

Male and female NMRI mice were orally dosed in feed mix at a target dose of 250 to 2000 mg/kg/day for 4 weeks (MPF/DT 2600). These mice showed an increase in AUC and Cmax with increasing dose. However, AUC decreased in males (250, 500, and 1000 mg/kg/day) by 25-86% and in females (500, 1000 and 2000 mg/kg/day) by 15-40% on day 24/25 compared to day 2/3. In a dose range finding study, CD1 mice were administered ambrisentan at target doses of 0, 60, 150, 500, and 1250 mg/kg/day in the feed for 13 weeks. Systemic exposure, as assessed by the AUC of parent drug in plasma samples during week 11, increased with increasing dose. The exposure was higher in female CD1 mice compared to males. AUCs declined in both males and females on day 24/25 relative to day 2/3.

In a 104 week study, male and female CD1 mice were orally dosed in feed mix at target doses of 50, 100 and 250/150 mg/kg/day. These mice showed an increase in AUC and Cmax with increasing dose. Total body exposure was higher in females than in males. Males and females in the high dose group showed a decrease in exposure at week 52 relative to week 2.

Rats

Ambrisentan was administered for 4 weeks by gavage to male and female Wistar rats at doses of 0, 1, 4, 16, and 40 mg/kg/day in one study (Table 4; MPF/DT 0033) and 0, 40, 125, or 400 mg/kg/day in the second study (MPF/DBB 9953). Blood samples were drawn on days 1 and 28 in the first study and on days 1 and 10 in the second study.

In both studies, ambrisentan was rapidly absorbed, and peak plasma concentrations were reached between 0.5 and 1 hour after dosing in both males and females and AUC and Cmax values showed a dose proportional increase. Exposure in males was slightly higher than in females. No marked differences were observed in AUCs following repeated exposure, suggesting a lack of systemic accumulation in rats.

Similarly, Wistar rats dosed orally from 250 to 2000 mg/kg/day for 6 weeks (MPF/DT 2500E), 10 to 2000 mg/kg/day for 13 weeks (MPF/DT 0032E), and from 5 to 500 mg/kg/day for 26 weeks (R&D 03/022), all showed an increase in systemic exposure with an increase in dose. No marked differences in exposure between sexes were observed in all three studies. Male and female rats in the high dose groups of the 13 week study showed an increase in exposure as the study progressed, suggesting possible systemic accumulation.

The effect of ambrisentan on fertility in male Wistar rats was evaluated following 15 weeks of repeat dosing at a target concentration of 300 mg/kg in feed mix (Table 4; AMB-001). Ambrisentan plasma $AUC_{(0\text{-}24hrs)}$ values in male rats on day 2 and 29 were similar, 712 and 584 $\mu g \cdot hr/mL$, respectively. Ambrisentan concentrations in plasma reached a constant plateau on day 2 and 29, indicating sufficient and uniform systemic exposure.

Ambrisentan exposure in pregnant Wistar rats was assessed following oral gavage administration of 15 to 150 mg/kg/day from day 6 through 17 post coitus (Table 5; MPF/DDT 9926E). Plasma samples were taken on days 6 and 17 and assayed for systemic exposure. The results showed that AUC and Cmax increased with an increase in dose. No marked differences in AUC or Cmax were seen in pregnant rats after repeated exposure between day 6 and day 17. No fetal toxicokinetic evaluations were conducted.

In a 104 week study, male and female Wistar rats were orally dosed in feed mix at a target doses of 10, 30/20 and 60/40 mg/kg/day. These rats showed a dose proportional increase in $AUC_{(0\text{-}24)}$ with increasing dose. The AUC of females was lower than in males after 2 weeks, with a ratio of 0.59 to 0.69. However, after 52 weeks the trend was reversed, with AUC ratios of 1.2 to 1.8. From week 62 up to week 104, no clear differences could be found (0.71 to 1.3). The AUC tended to increase in both males and female rats after repeated oral administration of ambrisentan from week 2 to week 65 for the highest dose and 104 weeks for the low and mid dose groups.

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Rabbits

Ambrisentan exposure in pregnant rabbits for 18 days at 7-63 mg/kg/day was investigated (Table 5; MPF/DT 9926E). Plasma samples were taken on days 6 and 18 post-coitus and assayed for systemic exposure. Both AUC and Cmax values increased in a dose proportional manner. Pregnant rabbits showed a two-fold increase in AUC on day 18 relative to day 6. The Cmax values in the rabbits were slightly elevated on day 18 relative to day 6. No fetal toxicokinetic evaluations were conducted. The preliminary rabbit study (MPF/DT 0599E) was performed as a range finder for the above study.

Dogs

Ambrisentan toxicokinetic evaluation in beagle dogs, were conducted in several repeat dose toxicology studies (Table 4). In a 4-week repeat dose study, dogs received ambrisentan at doses of 0, 80, 200, and 500 mg/kg/day in gelatin capsules (MPF/DDB 9907) and systemic exposure was assessed on day 1 and day 28. Increasing dose resulted in increases in exposures (AUC) and Cmax, that were approximately proportional to the dose. However, there was a noticeable decrease in both Cmax and AUC in both males and female dogs on day 28 when compared to the first dose.

The effects of repeated oral administration (gavage) on the pharmacokinetic profile was assessed in beagle dogs receiving ambrisentan for 4 weeks at doses of 0, 100, 300, 1000, and 1500 mg/kg/day (MPR/PT0031E). Blood samples were collected on day 1 and day 27. The toxicokinetic results showed that ambrisentan was quickly absorbed and, in most animals, the Cmax was reached within 1 hour after administration. Exposure to ambrisentan, as represented by the AUC and Cmax values, appeared to be generally independent of treatment duration or the sex of the animals at 100 and 300 mg/kg/day. At 1000 and 1500 mg/kg/day, the Cmax and AUC were approximately 2- fold greater in females compared with males. The exposure increased with the dosage; however, this increase appeared less than proportional, especially at dose levels above 300 mg/kg/day.

The pharmacokinetic profile of ambrisentan was also assessed in beagle dogs following twice-daily oral administration (gelatin capsule; 2-hour intervals) of 0, 500 (2 x 250), or 1000 (2 x 500) mg/kg/day ambrisentan for 4 weeks (Table 4; MPF/DT 1500). The 2-hour interval between doses was chosen such that the second daily dose was administered at the anticipated Cmax of the first dose. For the determination of plasma concentrations, blood samples were collected at 0, 1.5, 3, 6, and 24 hours after dosing on day 1 and day 28.

After single and repeat dosing, the AUC_{0-24h} increased in a dose-proportional manner in male dogs. For females, an increase in AUC occurred with dose but was less than proportional. There were no consistent differences in the Cmax and AUC values observed between the male and female dogs. The exposure to ambrisentan was higher after the first dose than after repeat dosing suggesting an increase in clearance or a decrease in absorption or both.

Additional toxicokinetic assessments were also included in toxicology studies for repeat dose durations of 13, 26, and 39 weeks were conducted in male and female dogs (Table 4; MPR/PT 0035E). Dogs were administered 100, 300, and 1000 mg/kg/day by oral gavage for 13 weeks and serial blood samples were withdrawn on days 1, 28, and 91 to assess systemic exposure. Similarly, dogs were dosed at 100, 300, and 900 mg/kg/day for 26 weeks by oral gavage and serial blood samples taken on day 1, 90, and 174 of 177 to assess systemic exposure (MPR/PT 0111E) In a 39-week study in male and female dogs were orally dosed at 30, 300 and 600 mg/kg/day, and serial blood samples were obtained on days 1, 91, 182, and 273 (2002-6032).

The results from all three studies showed that AUC and Cmax increased with increasing dose. No notable differences in exposure were observed between the male and female animals. There was a moderate increase in exposure on day 91 and 182 relative to day 1 in both sexes, indicating possible systemic accumulation. There were no marked differences in exposure between sexes observed with time.

Humans

In humans, repeat oral dose pharmacokinetic characteristics of ambrisentan were investigated following administration of 5, 7.5 and 10 mg in healthy volunteers (EE-002) and 1, 2.5, 5 and 10 mg to pulmonary arterial hypertension (PAH) patients (AMB-220, and AMB-320/321-E).

In healthy volunteers (EE-002), ambrisentan was rapidly absorbed into the systemic circulation, with peak plasma concentrations (tmax) being attained within 1.2 to 2.1 hours post dose. Cmax increased linearly and proportional with dose after the day 1 and day 10 dose. The mean Cmax values differed by less than 2% between the two doses. Similarly, the mean $AUC_{(0-\infty)}$ and $AUC_{(216-240)}$ also increased in a linear dose proportional manner, ranging from 2242 to 4655 ng·hr/mL. The mean AUC for day 10 increased by less than 5% compared to the day 1 AUC. The small variability in both AUC and Cmax indicate that there was no accumulation of ambrisentan following repeat dosing. Steady state concentrations were reached by day 4. The mean terminal elimination half-life ($t_{1/2}$) ranged from 13.6 to 16.5 hours across all dose groups.

In PAH patients (AMB-220 and AMB-320/321-E), tmax-ss (steady state) ranged from 1.66 to 1.94 hrs for 1 to 5 mg doses. The tmax-ss was longer for the 10 mg dose, ranging from 2.68 to 3.33 hrs. The Cmax at steady state was linear with dose, ranging from 111.0 to 1223.3 ng/mL for the 1 to 10 mg dose, respectively. Similarly, the steady state $AUC_{(0-last)}$ was linear with the dose, ranging from 777 to 14839 ng·hr/mL. The mean terminal elimination half-life, t1/2, ranged from 12.9 to 17.9 hours across all dose groups.

In general, the pharmacokinetic characteristics of ambrisentan, including rapid absorption and dose linear exposure, were observed in animals and humans following repeat dose administration (Figure 3). Overall, there was a linear relationship between AUC and dose observed across the animal species and humans; however, exposures in humans tended to be larger than those in animals for a given dose. Also, ambrisentan AUCs in patients with

PAH were consistently higher compared to healthy volunteers for a given dose. Reduction in metabolic capacity of the liver as a consequence of PAH by either hepatic congestion or hypoperfusion may alter the systemic clearance of ambrisentan in PAH patients (AMB-220 and AMB-320/321-E).

Distribution

Tissue distribution and plasma protein binding studies for ambrisentan are listed in Table 6.

Table 6 List of Distribution Studies Performed With Ambrisentan (BSF 208075)

Type of Study	Species (strain)	Sex	Mode of Administration	Dosage (mg/kg)	Sampling Occasions	GLP	Testing Facility[1]	Report No. (Study No.)
Single dose Tissue Distribution study	Rat / Wistar and Long Evans	M	Oral (gavage) [14]C Radiolabelled	32 4.6 x 10³ Bq/mg	1, 8, 24, 48 and 72 hrs	GLP		MPF/DDK 9906
Plasma Protein and Erythrocytes binding	CD-1 Mouse. Wistar Rat, Chinchilla rabbit, Beagle Dog and Human	M/F	in vitro	0.2, 20 and 200 µg/mL	5 hr equilibrium dialysis	Non-GLP		48563 MPF/DDK 9912
Human Plasma Protein binding	Human	M/F	in vitro	0, 10, 20 µg/mL of Ambrisentan 10 µg/mL Warfarin	5 hr equilibrium dialysis	Non-GLP		MPR/PKD0105

[1]Knoll = BASF Knoll, Germany; ■■■■■■■■

Tissue Distribution

The tissue distribution of radioactivity was examined in male Wistar rats and pigmented Long Evans rats, following a single oral administration of [^{14}C]-ambrisentan (Table 6; MPF/DDK 9906). Tissue distribution of [^{14}C]-ambrisentan was assessed by whole body autoradiography and in individual excised organs by liquid scintillation counting. The maximum concentration was observed in the gastrointestinal tract (GI), with $t_{1/2}$ values ranging from 6 hours (wall of the stomach) to 14 hours (wall of the small intestine). Other organs with high concentrations of radioactivity (in decreasing order) included liver, plasma, blood, lungs, and kidneys. The $t_{1/2}$ values in these tissues were 17, 11, 13, 14, and 21 hours, respectively. The concentrations in the brain were very low at 1 hour and declined rapidly. Drug concentrations in the eyes were below the limits of quantitation. At 72 hours, small but measurable concentrations of radioactivity remained in the GI tract. The [^{14}C]-ambrisentan distribution pattern in the pigmented Long Evans rats was similar to Wistar rats. Therefore, ambrisentan does not appear to preferentially bind to melanin.

Plasma protein binding

In vitro, ambrisentan was highly bound to plasma proteins of mice, rats, rabbits, dogs and human, with mean values of 91.8%, 97.2%, 96.8%, 96.4% and 98.8%, respectively (Table 6; 48563 and MPF/DDK 9912). Ambrisentan binding to human plasma proteins was greater than in animals. Binding was independent of the ambrisentan concentration

up to 20 μg/mL. However, the ambrisentan binding to plasma proteins was substantially decreased at 200 μg/mL in tested species (rat, rabbit, and dog). These results suggest saturation of binding sites occurs at concentrations that are approximately 200-fold higher than normally seen in the human circulation. No sex-dependent differences in rabbit, dog and human plasma protein binding were observed.

Ambrisentan was bound to human albumin to a much greater extent (96.5%) than to α1-acid glycoprotein (15.4%), indicating albumin is the primary binding protein in plasma. The distribution of ambrisentan between erythrocytes and plasma in the blood from rats, rabbits, dogs, and humans were also examined in this study. The results showed that ambrisentan was mainly present in the plasma fraction, between 90.2 and 95.9%, with only a small amount associated with erythrocytes in all species.

Human plasma protein binding interaction between ambrisentan and warfarin was evaluated *in vitro* (Table 6; MPR/PKD 0105). Ambrisentan was incubated in male or female human plasma in the presence of warfarin and vice versa. The results showed both ambrisentan and warfarin were highly bound to human plasma proteins ranging from 98.7 to 98.9%. Neither warfarin nor ambrisentan displaced each other from their respective binding sites up to the highest concentrations.

Metabolism

In vitro and *in vivo* ambrisentan metabolism studies are listed in Table 7.

Table 7 List of In Vitro, In Vivo and In Situ Metabolism Studies Performed with Ambrisentan (BSF-208075)

Type of Study	Species (strain)	Sex	Mode of Administration	Dosage (μM, or mg/kg, or MBq/mmol of ^{14}C)	Sampling Occasions (hr)	GLP	Testing Facility[1]	Report No. (Study No.)
In Vitro								
Metabolism in Hepatocyte	Rat, Wistar, Dog / Beagle, Human	M/F	Cell culture	5, 10 μM (663 MBq/mmol)	4, 8, 24 hrs	Non-GLP		MPF/DDM 0010
Reaction Phenotyping	Microsomes, cDNA expressed CYPs	NA	in vitro	50 μM (663 MBq/mmol)	1 hr	Non-GLP		MPF/DDM 0010
In Situ								
Single Dose Metabolism Study	Rat / Wistar	M	Perfusion, Isolated liver	3, 10, 30 mg/kg (663 MBq/mmol)	Perfusate, 6 hrs Bile, 6 hrs	Non-GLP		MPF/DDM 9913
In Vivo								
Single dose metabolism study	Mouse / NMRI	M	Oral (gavage)	30 (734 MBq/mmol)	Plasma 1, 2 and 3 Urine 0-24 Feces 0-24	GLP		MPF/DDM 9958
Single dose metabolism study	Rat / Wistar	M/F	iv	3 (734 MBq/mmol) 3 (663 MBq/mmol)	Plasma 2 and 4 Urine 0-72	GLP		MPF/DDM 0009
			Oral (gavage)		Feces 0-72			MPF/DDM 9916
Single dose metabolism study	Rat / Wistar	M	Oral (gavage)	30 (663 MBq/mmol)	Plasma 2 and 4 Urine 0-24 Feces 0-24	GLP		MPF/DDM 9950
4 week Repeat Dose Metabolic Induction Study	Rat / Wistar	M/F	Oral (gavage)	0, 40, 125, 400 mg/kg	Liver, after final dose	GLP		MPF/DT 6199

Table 7 List of In Vitro, In Vivo and In Situ Metabolism Studies Performed with Ambrisentan (BSF-208075) (continued)

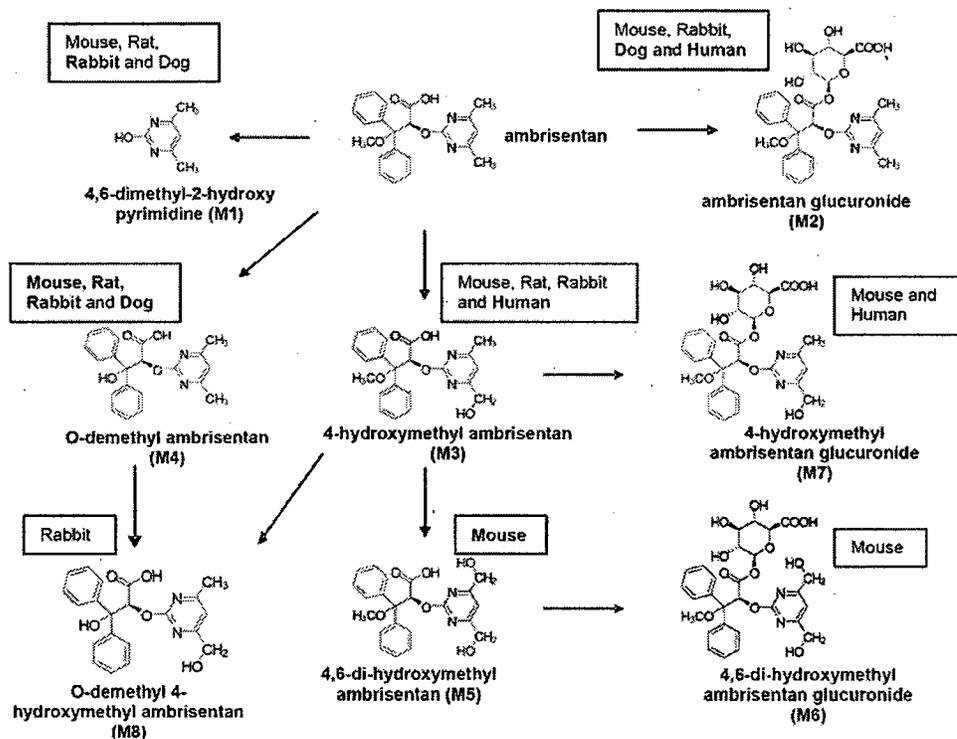| Type of Study	Species (strain)	Sex	Mode of Administration	Dosage (µM, or mg/kg, or MBq/mmol of ^{14}C)	Sampling Occasions (hr)	GLP	Testing Facility[1]	Report No. (Study No.)
Single dose metabolism	Rat / Wistar	M	Intraduodenal	Donor 33 mg/kg Acceptor 20 mg/kg (734 MBq/mmol)	Donor Bile 0-24 hrs Acceptor Bile 0-24 hr	Non-GLP	--	MPF/DDK 9945
Single dose metabolism study	Rabbit, New Zealand	F	Oral (gavage)	30 (734 MBq/mmol)	Plasma 2 and 4 hrs Urine 0-48 hrs Feces 0-48 hrs	GLP		MPF/DDM 9959
Single dose metabolism study	Dog / Beagle	M	Oral (Capsule)	3 (663 MBq/mmol)	Plasma 1 and 4 hrs Urine 0-72 hrs Feces 0-72 hrs	GLP		MPF/DDM 9940
Single dose metabolism study	Dog / Beagle	M	Oral (gavage)	30 (663 MBq/mmol)	Plasma 2 and 4 Urine 0-72 Feces 0-72	GLP	......	MPF/DDM 9951
Single dose metabolism study	Dog / Beagle	M/F	Intraduodenal	10 mg/kg (734 MBq/mmol)	Bile 0-8 hr	Non-GLP		MPF/DDM 0022
4 Week repeat dose induction study	Dog / Beagle	M	Oral (gavage)	0, 100, 300, 1000	Liver, after final dose	GLP		MPR/PT 3301E
Single dose metabolism study	Human	M/F	Oral [³H, ¹⁴C] ambrisentan blend in capsule	10 (100 µCi)	Plasma 0-168 Urine 0-168 Feces 0-168	GLP	Myogen	AMB-107
In vitro enzyme inhibition	Microsomes, cDNA expressed	NA	in vitro	0,0.1, 0.3, 1, 3, 10, 30, 100, 300 µM	1 hr	Non-GLP	...	MPF/DDM 0025 E

NA – not applicable
Testing Facility[1] ████████

Metabolic Pathways

The potential metabolic pathways and metabolites in mouse, rat, rabbit, dog, and human are provided in Figure 4.

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Figure 4 Ambrisentan Metabolic Pathways in Mouse, Rat, Rabbit, Dog and Human.
(Bold letters denote major pathway for that animal species)

Mouse, Rat, Rabbit and Dog

4,6-dimethyl-2-hydroxy pyrimidine (M1)

Mouse, Rabbit, Dog and Human

ambrisentan

ambrisentan glucuronide (M2)

Mouse, Rat, Rabbit and Dog

O-demethyl ambrisentan (M4)

Mouse, Rat, Rabbit and Human

4-hydroxymethyl ambrisentan (M3)

Mouse and Human

4-hydroxymethyl ambrisentan glucuronide (M7)

Rabbit

O-demethyl 4-hydroxymethyl ambrisentan (M8)

Mouse

4,6-di-hydroxymethyl ambrisentan (M5)

Mouse

4,6-di-hydroxymethyl ambrisentan glucuronide (M6)

Source: MPF/DDM 9958, MPF/DDM 9950, MPF/DDM 9959, MPF/DDM 9951 and AMB-107

In Vitro metabolism of Ambrisentan

The *in vitro* metabolism of ambrisentan was investigated in liver microsomes and hepatocytes of rats, dogs, and humans (Table 7; MPF/DDM 0010). In rat hepatocytes, glucuronidation and hydroxylation were the main routes of metabolism. In contrast, glucuronidation was the preferred route of metabolism in dog and human hepatocytes, along with minor amounts of oxidative metabolism. Ambrisentan conversion rates were 15% to 25% over 24 hours in all three species. The primary metabolite identified was ambrisentan glucuronide (M2), with only trace amounts (<1%) of hydroxylated metabolite (M3) detected in dogs and humans (Figure 4). Rats appear to form the glucuronide and 4- hydroxymethyl ambrisentan (M3) metabolites to equal extent.

Reaction Phenotyping

Enzymes that metabolize ambrisentan were evaluated in microsomes prepared from a human lymphoblastoid cell line or baculovirus-infected insect cells expressing individual human CYPs and UGT enzymes (Table 7; MPF/DDM 0010). The results indicate that

ambrisentan glucuronide was formed by UGT1A9S, UGT2B7S, and UGT1A3S. Ambrisentan was hydroxylated at the 4-methyl position of the pyrimidine, primarily by CYP3A4, CYP3A5 and CYP2C19, at a relatively low overall turnover rates. Among the CYPs evaluated CYP3A4, CYP3A5 and CYP2C19 exhibit highest turnover rates in metabolizing Ambrisentan. However, CYP3A4 appears to be the primary oxidative enzyme because of its high affinity and turnover rate for ambrisentan; and its relatively large abundance in the liver. CYP3A5 and CYP2C19 could also contribute to metabolism of ambrisentan to a lesser extent as indicated by a lower turnover rate. A number of other CYPs also appear to metabolize ambrisentan to the 4-hydroxymethyl ambrisentan (M3) metabolite, although, to a much lesser extent.

In situ Liver Perfusion Study in Rats

The metabolic pathways of ambrisentan in male Wistar rats were investigated using the isolated perfused rat liver model (Table 7; MPF/DDM 9913). [^{14}C]- labeled ambrisentan was perfused at doses of 3, 10, and 30 mg/kg. The livers were removed from 2 animals in each dose group and perfused in a recirculating system for 6 hours, and the metabolite pattern of the perfusion medium and collected bile was determined.

Approximately 25-31% of the dose was recovered in the bile for the three dose groups. The cumulative excretion of total radioactivity in the bile was 25% (3 mg/kg dose), 30% (10 mg/kg dose), and 31% (30 mg/kg dose). Metabolic pathways included metabolites of C-oxidation (M3, Figure 4) and conjugation with glucuronic acid (M2, Figure 4). Saturation of the C-oxidation reaction did not occur. The metabolite pattern was similar in the perfusate and bile, with 4 peaks that were identified as 1) unchanged parent compound, 2) ambrisentan glucuronide (M2), 3) a hydroxylated derivative of the parent compound (4-hydroxymethyl ambrisentan [M3]), and 4) a glucuronide of the hydroxylated metabolite (4-hydroxymethyl ambrisentan glucuronide [M7]). The parent ambrisentan was the predominant peak at all doses tested in the perfusate and in the bile. Additionally, traces of 2 more oxidative metabolites were detected in the bile.

In vivo metabolism studies

In vivo metabolism studies in NMRI mouse, Wistar rat, New Zealand rabbit and beagle dogs were completed following oral and/or iv administration of [^{14}C]-ambrisentan. In humans, metabolism was investigated following administration of a 10 mg dose of a blend comprised of [^{14}C]-ambrisentan and [^{2}H6]-ambrisentan and unlabeled ambrisentan.

In all species, the ambrisentan parent was the most prominent drug-related species in plasma along with relatively low concentrations of metabolites observed in the systemic circulation. Table 8 illustrates the ambrisentan metabolic products identified and their relative amounts (as % of total radioactivity concentrations) in plasma for mouse, rat, rabbit, and dog as well as humans.

Table 8 Ambrisentan and Its Metabolites in the Plasma of Nonclinical Species and Humans as a Percentage of Total Radioactivity

	ambrisentan	4, 6-dimethyl-2-hydroxy-pyrimidine	4-hydroxymethyl ambrisentan	O-demethyl-ambrisentan	4, 6-dihydroxymethyl ambrisentan	O-demethyl-4-hydroxymethyl ambrisentan	ambrisentan glucuronide	4,6-dihydroxymethyl ambrisentan glucuronide	4-hydroxymethyl ambrisentan glucuronide
			Phase I metabolites				Glucuronide conjugates		
Plasma	Parent	M1	M3	M4	M5	M8	M2	M6	M7
Mouse[a,b] (1, 2, 3hrs)	28.2	9	12.9	18.2	25.3	nd	nd	6.5	nd
Rat[b,c] (2, 4 hrs)	74.2	3.3	3.4	17.1	nd	nd	2.1	nd	nd
Rabbit[c] (4 hrs)	56.1	20.9	2.1	15.3	nd	2.3	2.3	nd	nd
Dog[a,b] (2, 4 hrs)	53.4[f]	7.6	nd	10.6	nd	nd	28.4[f]	nd	nd
Human[b] (2-8 hrs)	79.1	nd	2.81	nd	nd	nd	6.34	nd	1.93
			Plasma AUC[d]						
Human[e] AUC(0-infinity) (ng*hr/g)	5202		1515.5				228.6		43.8
AUC %	74.8%		21.3%				3.2%		0.7%

a-d = 30 mg/kg [14C]-ambrisentan oral dose; e = 10 mg [3H,13C,14C] ambrisentan blend in capsule; f = plasma AUCs for ambrisentan and its metabolite were not determined for animals; g = average values; h = pooled sample value.

Although the overall metabolic pathways, such as, hydroxylation, O-demethylation, pyrimidine ring cleavage and glucuronidation in all animal species tested appear to be similar, there were species differences in the extent and type of metabolites formed (Figure 4).

NMRI mice

(Table 7; MPF/DDM 9958): Ambrisentan undergoes extensive metabolism involving mono-hydroxylation (M3), di-hydroxylation (M5), O-demethylation (M4) and pyrimidine ring cleavage (M1). The dihydroxy metabolite (M5) was unique to NMRI mice. In general glucuronidation appeared to be a minor metabolic pathway in NMRI mice.

Wistar rats

(Table 7; MPF/DDM 9950): Parent drug was the main component in circulation and O-demethylation (M4) appeared to be the predominant oxidative pathway. Mono-hydroxylation (M3), pyrimidine ring cleavage (M1) and glucuronidation were minor metabolic pathways in this species.

New Zealand rabbits

(Table 7; MPF/DDM 9959): Ambrisentan was the major circulating component except in this species. Cleavage of the molecule to its pyrimidine ring (M1) occurred to a greater extent than in all other species. O-demethylation (M4) appears to be another preferred oxidative metabolic pathway. Similar to the mouse and rat, New Zealand rabbits were also found to produce the least amount of glucuronide conjugates.

Beagle dogs

(Table 7; MPF/DDM 9951 and MPF/DDM 0022): Ambrisentan was the major circulating drug-related component. In this species, ambrisentan glucuronide appeared to a greater extent than all other species. However, cleavage product (M1) and O-demethylation product (M4) were also detected as minor metabolic products.

Acyl glucuronides are ester-structured compounds that are chemically unstable in aqueous solution due to the susceptibility of the acyl group to undergo nucleophilic attack. The resulting labile isomeric acyl glucuronides may have a tendency to react with proteins and form covalent adducts that may be immunogenic *in vivo*. To assess the potential risk of covalent adducts formation, the stability of ambrisentan glucuronide, which specifically is a 1-O-acylgluronide metabolite of ambrisentan, was assessed in dog plasma (Table 7; MPF/DDM 0022). Isomerization of the acyl glucuronide in plasma was very slow with only 10% isomerization at pH 7.4 over 24 hours. This indicates that ambrisentan-1-O-acylglucuronide has high stability, with an estimated half-life of >24 hours. Plasma concentrations of ambrisentan-1-O-acylglucuronide are very low in humans and it is likely that its elimination rate is markedly greater than its isomerization rate. In addition, extraction of radioactivity from plasma samples from both human and animal radiolabeled studies was generally high (>95%), indicating a lack of any significant covalent binding. These results combined with low levels of ambrisentan glucuronide in human plasma, suggest the potential for covalent binding and a subsequent immunogenic response may be low following ambrisentan administration.

Humans

(Table 7; AMB-107): Ambrisentan was the most prominent drug-related species in human plasma, along with three metabolites which were present at relatively low concentrations. The metabolites were 4-hydroxymethyl ambrisentan (M3), the 4-hydroxymethyl ambrisentan glucuronide metabolite (M7); and ambrisentan glucuronide (M2) (Table 8; Figure 5). No pyrimidine cleavage product or O-demethylated metabolites were observed in human plasma. The only phase I oxidative metabolite observed in humans, 4-hydroxymethyl ambrisentan, was also present in mice, rats, rabbits at varying concentrations. (Table 8).

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Figure 5 Ambrisentan Metabolites in Humans*
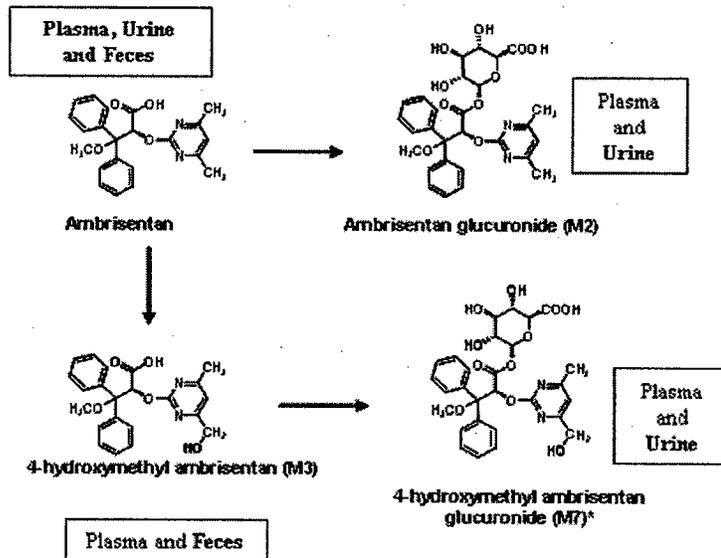

bold designates the higher concentration.

M7* - is identified as Metabolite 1 (M1) in the AMB-107 report.

Figure 6 shows the human plasma concentration time profile of ambrisentan and its metabolites following radio-HPLC analysis. The parent drug was the predominant radiolabeled product with 75% relative $AUC_{(0-last)}$ percent of total, and relatively low amounts of M2 (3.2%), M3 (21.3%), and M7 (0.7%) (Figure 6 and Table 8). Metabolite M7, the 4-hydroxymethyl-ambrisentan glucuronide is identified as Metabolite 1 in the AMB-107, the human mass balance study. Ambrisentan metabolite M3 was low in concentration (<40 ng/mL) but persisted up to 96 hours. The long persistence could be in part due to enterohepatic recirculation.

In terms of number of phase I metabolites formed, plasma results indicate mouse had the highest extent of metabolism followed by rabbit, rat, dog, and human (in decreasing order). Although, the extent of metabolism varied among animal species, the only phase I metabolite (M3) observed in humans was also present in moue, rat and rabbit. Based on plasma profiles, rat is most closely approximated the metabolite pattern seen in the humans.

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Figure 6 Plasma Concentration vs. Time Profile of Ambrisentan and Its Metabolite Following a Single 10 mg Oral Dose of [^{14}C]-Ambrisentan in Humans
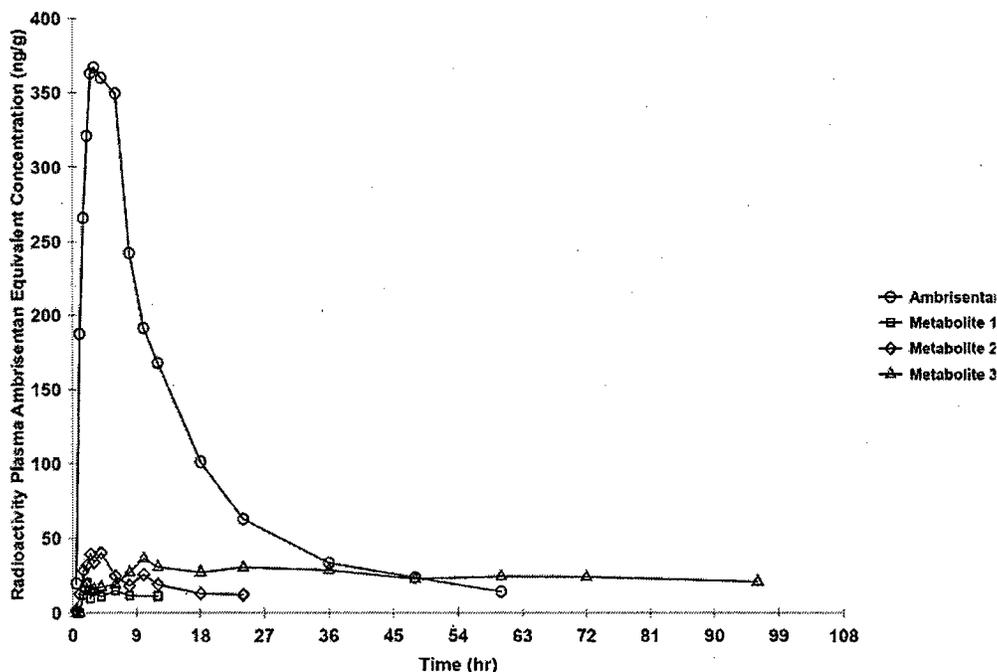
Metabolite 1: 4-hydroxymethyl ambrisentan glucuronide is identified as M7 in animal studies.

Metabolite 2: ambrisentan glucuronide is also M2 in the animal studies; Metabolite 3: 4-hydroxymethyl ambrisentan is also M3 in the animal studies

Metabolism profiles in bile

In bile duct-cannulated rats and dogs (Table 7), the vast majority of drug-related material was secreted into bile following intraduodenal administration of radiolabeled [^{14}C]-ambrisentan (MPF/DDK 9945 and MPF/DDM 0022). Within 24 hours of dosing in rats, 94.8% of total administered radioactivity was recovered from the bile mainly as parent drug (~70%), with the remaining appearing as several minor metabolites. Similar studies in dogs showed that approximately 50% of the dose was excreted into the bile within 8 hours of administration and that that the major drug component in bile was the ambrisentan glucuronide (76%-81%), along with minor amounts of parent drug and four other metabolites.

The ambrisentan glucuronide was the predominant metabolite in dog bile. However, dog feces contained large amounts of the parent and very small amounts of this metabolite. These data suggest that in dogs, the glucuronide is hydrolyzed in the gut by microflora, thus releasing the parent drug. In contrast to the dog, rat bile contained approximately

95% of the radioactivity that was attributed to the unchanged ambrisentan, and ~80% of the radioactivity recovered in rat feces was parent compound.

5.7 Metabolism profiles in urine and feces

Table 9 Ambrisentan Metabolites in Mouse, Rat, Rabbit, Dog and Human Urine and Feces Following Oral Administration

Species	Sample	Number	Sampling Duration (hr)	% of Dose	% of compound in sample								
					Phase I metabolites[1]						Conjugated metabolites[1]		
					Parent	(M1)	(M3)	(M4)	(M5)	(M8)	(M2)	(M6)	(M7)
Mouse[a]	Urine	18 M	0-24	7	11	7	6	2	13	nd	32	16	13
	Feces	18 M	0-24	67	35	nd	28	3	33	nd	nd	nd	nd
Rat[b]	Urine	6 M	0-24	7	71	19	6	nd	nd	nd	4	nd	nd
	Feces	6 M	0-24	66	74	nd	22	4	nd	nd	2	nd	nd
Rabbit[c]	Urine	3 F	0-48	44	47	16	5	12	nd	3	14*	nd	nd
	Feces	3 F	0-48	24	76	nd	20	2	nd	1	2*	nd	nd
Dog[d]	Urine	3 M	0-48	7	34	40	nd	2	nd	nd	21	nd	nd
	Feces	3 M	0-48	76	93	nd	1	2	nd	nd	2	nd	nd
Human[e]	Urine	8 M	0-168	23	20	nd	nd	nd	nd	nd	57	nd	24
	Feces	8 M	0-168	66	62	nd	32	nd	nd	nd	nd	nd	nd

[1]Compounds described in Figure 3
a-d -30 mg/kg oral dose; e-10 mg capsule.
*glucuronide isomers also detected
nd = not detected

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Metabolism profiles in urine and feces

Mouse urine contained the largest number (6) of phase I metabolites, followed by rabbit (4), rat and dog (2). 4-hydroxymethyl ambrisentan (M3) was the only phase I metabolite observed in human feces and was observed in urine and feces of mice, rats and rabbits in approximately same proportions. This metabolite was not observed in dog urine, however, small amounts were present in the feces. The pyrimidine cleavage product (4,6-dimethyl-2- hydroxypyrimidine; M1) appeared to be excreted solely in the urine of mice, rats, rabbits, and dogs. 4,6-dimethyl-2-hydroxypyrimidine was not observed in human excreta. Typically, urine from all species contained greater concentrations of the glucuronide conjugates (M2, M7), while the feces contained little or none. Unchanged ambrisentan was observed in the urine of all species, 11% in mice, 71% in rat, 47% in rabbit, 34% in dogs, and 20% in humans. With the exception of the NMRI mice, feces from all other animal species contained unchanged ambrisentan as the largest component.

Human urine contained approximately 20% parent drug, 57% ambrisentan glucuronide (M2) and 24% 4-hydroxymethyl ambrisentan glucuronide (M7) (Table 9; AMB-107). Human feces contained 62% ambrisentan and 32% 4-hydroxymethyl ambrisentan (M3). Glucuronide conjugates of parent and this metabolite are present in urine but not in feces. High concentrations of ambrisentan and 4-hydroxymethyl ambrisentan in feces suggest there is deconjugation of ambrisentan glucuronide (M2) and 4-hydroxymethyl ambrisentan glucuronide (M7) as observed in rats and dogs. This also suggests that parent

drug and 4- hydroxymethyl ambrisentan may undergo enterohepatic circulation in humans as indicated in rats.

Ambrisentan glucuronide and 4-hydroxymethyl ambrisentan, the metabolites seen in humans were also seen in the excreta of all animal species. 4-hydroxymethyl ambrisentan glucuronide observed in human urine, was only observed in the urine of the mouse. The systemic exposure of 4-hydroxymethyl ambrisentan in humans was 21% when assessed from plasma and 26% (M3+M7) when calculated from urine and fecal excretion. The total amount of 4-hydroxymethyl ambrisentan excreted in humans was similar to the amounts measured in the excreta of mice (34%), rats (28%) and rabbits (25%).

Enzyme Induction

Ambrisentan effects to induce hepatic phase I and II enzymes were examined in rats and dogs (Table 7), after oral dosing for 4 weeks at 100, 300, and 1000 mg/kg/day (MPF/DT 6199 and MPR/PT 3301). Ambrisentan administration produced mild induction in rat liver metabolizing enzymes. CYP2B enzyme activity was increased 3-fold at all doses tested; however, the lowest dose in the dog at which CYP2B enzyme induction was determined corresponds to approximately 50-fold higher ambrisentan exposure compared to the human exposure achieved at the highest clinical dose of 10 mg (0.14 mg/kg). The liver enzyme induction study results in rats and dogs suggest that ambrisentan is unlikely to have any significant hepatic phase I or II metabolizing enzyme induction potential at clinically relevant doses.

In Vitro Enzyme Inhibition

The potential of ambrisentan to inhibit various hepatic CYP and glucuronyltransferase enzymes was evaluated in microsomes prepared from a human lymphoblastoid cell line or baculovirus-infected insect cells expressing individual CYPs (Table 7; MPF/DDM 0025 E). The specific enzymes examined were: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, UGT1A1, UGT1A6, UGT1A9, and UGT2B7. Inhibition of these enzymes was measured using model substrates.

With the exception of a slight inhibition of CYP2A6 and CYP2C8 (13-25% at 300 µM), there was essentially no inhibition of CYP enzymes. Ambrisentan inhibited UGT1A1, UGT1A6, UGT1A9, and UGT2B7 by 10-30% at 300 µM. Inhibition did not exceed 50% for any enzyme at the highest concentration (300 µM) of ambrisentan tested. In a Phase 2 dose-ranging study (AMB-220), the Cmax for ambrisentan in the highest dose group (10 mg) was 3.2 µM. These results suggest a low potential for drug-drug interaction based on inhibition of CYP and UGT enzymes.

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Excretion

Studies that determined ambrisentan excretion in mice, rats, rabbits, dogs and humans are listed in Table 10.

Table 10 List of Excretion Studies Performed With Ambrisentan (BSF-208075)

| Type of Study	Species (strain)	Sex	Mode of Administration	Dosage (mg/kg) ^{14}C	Sampling Occasions (hr)	GLP	Testing Facility[1]	Report No. (Study No.)
Single dose Excretion and Metabolism study	Mouse / NMRI	M	Oral (gavage)	30 (734 MBq/mmol)	Urine 0-24 Feces 0-24	GLP		MPF/DDM 9958
Single dose Excretion and Metabolism study	Rat / Wistar	M/F	iv Oral (gavage)	3 (734 MBq/mmol) 3 (663 MBq/mmol)	Urine 0-72 Feces 0-72	GLP		MPF/DDM 0009 MPF/DDM 9916
Single dose Excretion and Metabolism study	Rat / Wistar	M	Oral (gavage)	30 (663 MBq/mmol)	Urine 0-24 Feces 0-24	GLP		MPF/DDM 9950
Single dose Excretion and Metabolism study	Rabbit, New Zealand	F	Oral (gavage)	30 (734 MBq/mmol)	Urine 0-48 Feces 0-48	GLP		MPF/DDM 9959
Single dose Excretion and Metabolism study	Dog / Beagle	M	Oral (Capsule)	3 (663 MBq/mmol)	Urine 0-72 Feces 0-72	GLP		MPF/DDM 9940
Single dose Excretion and Metabolism study	Dog / Beagle	M	Oral (gavage)	30 (663 MBq/mmol)	Urine 0-24 Feces 0-72	GLP		MPF/DDM 9951
Single dose Excretion and Metabolism study	Human	M / F	Oral (capsules)	10 (100 µCi)	Urine 0-168 Feces 0-168	GLP	Myogen	AMB-107

Excretion in Urine and Feces

The excretion of ambrisentan and its metabolites was assessed in NMRI mice dosed orally at 30 mg/kg (Table 10; MPF/DDM 9958), in rats following iv administration of a single 3 or 10 mg/kg doses of ambrisentan (MPF/DDM 0009 and 9904) and oral doses of 3 or 30 mg/kg (MPF/DDM 9916 or MPF/DDM 9950), and in dogs following administration of a single 3 or 30 mg/kg oral dose of ambrisentan (MPF/DDM 9940 and MPF/DDM 9951). The feces were the primary route of excretion of parent drug and metabolites in all species except the rabbit. In humans, mice, rat and dogs, the fecal recovery accounted for 66%-76% of the dose.

Total radioactivity recovered in urine and feces following oral administration of ambrisentan was 89% in NMRI mice, 94% in Wistar rats, 88% in New Zealand rabbits, 96% in beagle dogs and 89% in humans. With the exception of rabbit, approximately 66-76% of the dose was recovered in mouse, rat, dog, and human feces, while approximately 7% of the dose was recovered in mice, rat, and dog urine (Figure 7). Rabbit and human urine contained 44% and 23% of dose, respectively. Unchanged ambrisentan was the major drug component recovered in feces and urine, except that mouse and human urine contained more ambrisentan glucuronide. The total amount of parent recovered from urine and feces ranged from 24% in mice, 54% in rats, 39% in rabbits, and 73% in dogs (Table 11). In humans, approximately 45% of the dose was excreted as unchanged drug,

mostly in the feces. Both oxidative and conjugated metabolites made up the remainder. Table 11 lists all the metabolites and their amounts as a percent of dose for all species.

Figure 7 Urine and Fecal Excretion of Ambrisentan Following a Single 30 mg/kg Oral Dose in Mice, Rats, Rabbits and Dogs and 10 mg Dose in Humans
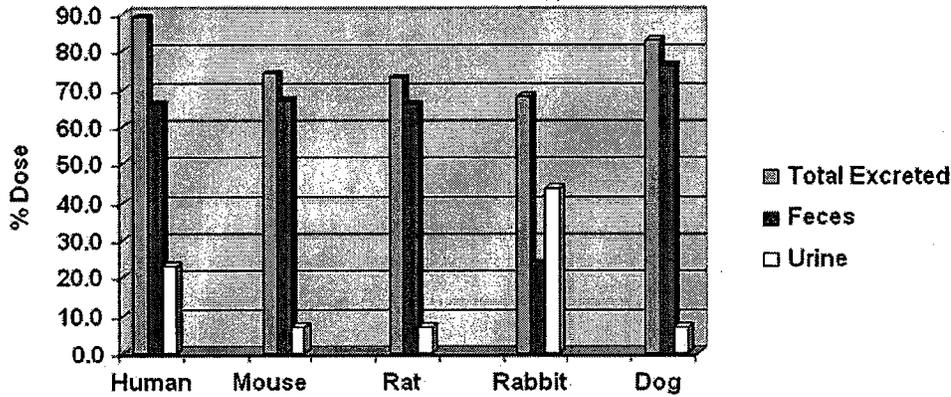
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Source: Reports in Table 10 above

Table 11 Interspecies Comparison - Urine and Fecal Excretion of Ambrisentan and Its Metabolites in NMRI Mouse, Wistar Rat, New Zealand Rabbits, Beagle Dogs and Humans

	ambrisentan BSF 208075	4,6-dimethyl-2-hydroxy pyrimidine	4-hydroxymethyl ambrisentan	O-demethyl-ambrisentan	4,6-dihydroxymethyl ambrisentan	O-demethyl-4-hydroxymethyl ambrisentan	ambrisentan glucuronide	4,6-dihydroxymeth yl ambrisentan glucuronide	4-hydroxymethyl ambrisentan glucuronide
				Phase I metabolites				Glucuronide Conjugates	
	Parent	M1	M3	M4	M5	M8	M2	M6	M7
% Dose									
Mouse									
Urine (7%)	0.8	0.5	0.4	0.1	0.9	nd	2.2	1.1	0.9
Feces (67%)	23.5	nd	18.8	2.1	22	nd	nd	nd	nd
Total (74%)	24.3	0.5	19.2	2.2	22.9	nd	2.2	1.1	0.9
Rat									
Urine (7%)	5	1.3	0.4	nd	nd	nd	0.5	nd	nd
Feces (66%)	48.8	nd	14.5	2.6	nd	nd	1.3	nd	nd
Total (73%)	53.8	1.3	14.9	2.6	nd	nd	1.8	nd	nd
Rabbit									
Urine (44%)	20.7	7.0	2.2	5.3	nd	1.3	6.2	nd	nd
Feces (24%)	18.2	nd	4.8	0.5	nd	0.2	0.5	nd	nd
Total (68%)	38.9	7.0	7.0	5.8	5.8	1.5	6.7	nd	nd

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Table 11 Interspecies Comparison - Urine and Fecal Excretion of Ambrisentan and Its Metabolites in NMRI Mouse, Wistar Rat, New Zealand Rabbits, Beagle Dogs and Humans (continued)

% Dose	ambrisentan (BSF 208075)	4,6-dimethyl-2-hydroxy pyrimidine	4-hydroxymethyl ambrisentan	O-demethyl ambrisentan	4,6-dihydroxymethyl ambrisentan	O-demethyl-4-hydroxymethyl ambrisentan	ambrisentan glucuronide	4,6-dihydroxymethyl ambrisentan glucuronide	4-hydroxymethyl ambrisentan glucuronide
				Phase 1 metabolites				Glucuronide Conjugates	
	Parent	M1	M3	M4	M5	M8	M2	M6	M7
Dog									
Urine (7%)	2.4	2.8	nd	0.1	nd	nd	1.4	nd	nd
Feces (76%)	70.7	nd	0.8	1.5	nd	nd	1.5	nd	nd
Total (83%)	73.1	2.8	0.8	1.6	nd	nd	2.9	nd	nd
Human									
Urine (23%)	4.6	nd	nd	nd	nd	nd	13.1	nd	5.5
Feces (66%)	40.9	nd	21.1	nd	nd	nd	nd	nd	nd
Total (89%)	45.5	nd	21.1	nd	nd	nd	13.1	nd	5.5

Quantitative distribution of metabolites in urine and feces after a single oral dose of Ambrisentan in mice, rats, rabbits, and dogs received 30 mg/kg. The human dose was 10 mg. Data are presented as the percent of dose.
nd = not detected
a Study MPT DOM 9953, Table A1, b Study MPT DOM 9956, Table A1, c Study MPT DOM 9954, Table A2, d Study MPT DOM 9953, Table A2, e Study AMB-197, f Approximately 82% of the total radioactivity was recovered during the HPLC analysis.

Total amount of 4-hydroxymethyl ambrisentan (M3) excreted as a percent of dose was similar in mice, rats and humans, and it was excreted to a lesser extent in rabbit and dogs. The glucuronide conjugate of 4-hydroxymethyl ambrisentan (M7) was only observed in human and mouse urine. The total amount of 4-hydroxymethyl ambrisentan excreted as M3 and M7 was 26.6% in humans, 20.1% in mice, 14.9% in rats, 7% in rabbits and 0.8% in dogs (Table 11).

The systemic exposure of 4-hydroxymethyl ambrisentan (M3) based on plasma AUC was not determined in animals; therefore, systemic exposure in animals was assessed from urine and fecal excretion, as described in the FDA draft guidance *Safety Testing of Drug Metabolites* [1]. The combined relative systemic exposure of M3 plus 4-hydroxymethyl ambrisentan glucuronide (M7) after normalizing for body weight and dose between humans and animals following single oral administration is shown in Figure 8. These results show that the exposure of 4-hydroxymethyl ambrisentan in animals is sufficiently higher in animals compared to humans alleviating any need for further toxicity testing.

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Figure 8 Relative Exposure of 4-hydroxymethyl Ambrisentan (M3 and M7) Across Species
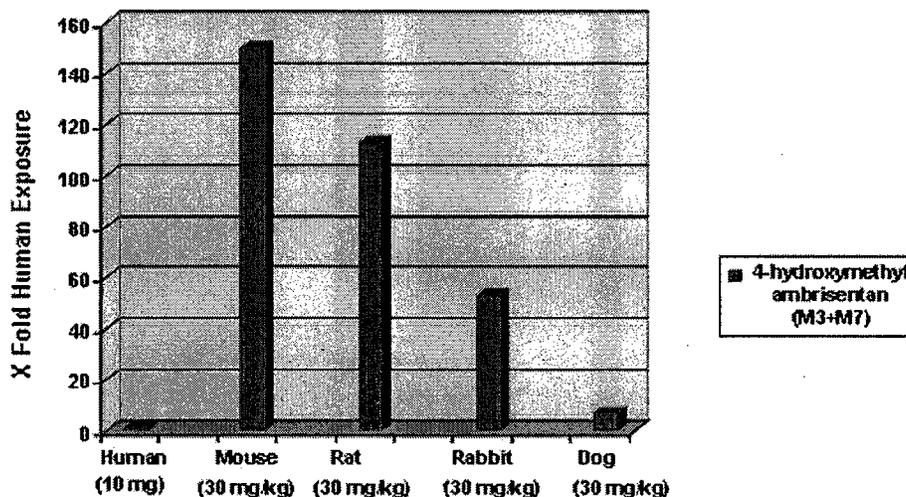
Source: Table 11 and FDA draft guidance-*Safety Testing of Drug Metabolites*

Biliary Excretion

The biliary excretion and enterohepatic circulation of ambrisentan and its metabolites was assessed in anesthetized male rats after intraduodenal administration of radiolabeled [^{14}C]- ambrisentan (Table 7; DPF/DDK 9945). Within 24 hours of dosing, 94.8% of total radioactivity was recovered from the bile, and 1.5% from the urine. To assess enterohepatic circulation, a second set of rats (acceptor rats) was intraduodenally dosed with pooled bile (0-12 hour period) from the first set of rats (donor rats), and bile and urine were again collected from the acceptor rats over a 24-hour period. A large portion (76.5-94.8%) of total radioactivity from the donor rats' bile was recovered in the bile of acceptor rats, indicating a high extent of enterohepatic circulation of ambrisentan and/or its metabolites. The metabolites identified in bile from donor and acceptor rats were similar (M2, M3, and M4).

Radiolabeled [^{14}C, ^{2}H] ambrisentan (10 mg/kg) was administered to dogs intraduodenally to assess the extent of biliary excretion and to identify ambrisentan metabolites in the bile (Table 7; MPF/DDM 0022). Eight hours post-administration, 54% and 47% of total radioactivity was excreted into the bile in males and females, respectively. Ambrisentan glucuronide was the predominant metabolite, representing 77%-81% of the total radioactivity.

Although biliary excretion and enterohepatic recirculation of ambrisentan were not directly observed in humans, the similarity in ADME profiles between humans and animals suggests that biliary excretion is likely to play a predominant role in humans as well.

Pharmacokinetic interactions

The *in vitro* drug interaction and transport studies performed with ambrisentan are listed in the following table (Table 12).

Table 12 In Vitro Drug Interaction Studies Performed with Ambrisentan (BSF-208075)

Type of Study In Vitro	Species (strain)	Sex	Mode of Administration	Dosage	Sampling Occasions (hr)	GLP	Testing Facility[1]	Report No. (Study No.)
In vitro Drug-Drug Interaction study	Human Microsomes	M /F	In vitro	223 µM 0.1x, 1x, 10x C$_{max}$, 24 DDI test compounds	1 hr	Non-GLP		50495
In vitro permeability – Pgp transport	CACO-2 cells	NA	Cell culture	10 µM, 10 µM cyclosporine A	2 hrs	Non-GLP	1	4MYOGP1S1 4MYOGP2
In vitro permeability – Pgp transport	MDCK II-MDR1 cells	NA	Cell culture	0, 0.3, 1, 3, 10, 30, 100 µM, 30 nM digoxin	1.5 hrs	Non-GLP		06DDM108
In vitro Hepatobiliary Transport Inhibition	Rat and human Hepatocytes	NA	Cell culture	2, 20, 100 µM	10 min	Non-GLP		MG-1001 MG-1002
In vitro Hepatobiliary Transport Induction	Rat and human Hepatocytes	NA	Cell culture	2, 100 µM	48 and 96 hrs	Non-GLP		MG-1003
In vitro Hepatobiliary Transport Substrate	Rat Hepatocytes	NA	Cell culture	2 µM	10 min	Non-GLP		MG-1004

NA - not applicable

Drug-Drug Interaction (DDI) Study

The inhibitory effects of several drugs that are used in the treatment of PAH patients as concomitant medications were evaluated *in vitro* using pooled human liver microsomes (Table 12; 50495). The list of drugs was selected from the concomitant medication data listings from the Phase 2 and 3 clinical studies (AMB-220, AMB-320, and AMB-321) that were reported for >10% of patients in any study. These drugs were: acenocoumarol, acetaminophen, amlodipine, amoxicillin, aspirin, atorvastatin, carvedilol, cyclosporine A, digoxin, diltiazem, enalapril, epoprostenol, furosemide, levothyroxine, metoprolol, nifedipine, omeprazole, pravastatin, prednisone, sildenafil, simvastatin, spironolactone, and warfarin. The inhibitory potential of these medications were examined over a wide range of concentrations, from 0.1-fold to 10-fold of their respective Cmax values. Probenecid, a potent inhibitor of UDPGT was included as a positive control. [^{14}C]-labeled ambrisentan concentration was fixed at its Km value of 223 µM. The results showed that of the 24 drugs, only probenecid, the positive control, strongly inhibited ambrisentan glucuronidation by 59%. Nifedipine inhibited ambrisentan glucuronidation by 39% at 0.23 µM, but inhibition was only 30% at 2.3 µM, which suggests a non-concentration related inhibition. Atorvastatin and warfarin were weak inhibitors (19 - 20%) only at 10-fold their human Cmax concentrations. These results suggest that the concomitant medications that are likely to be administered with ambrisentan are unlikely to affect the glucuronidation of ambrisentan *in vivo*.

Drug Transporter Effects

The bidirectional permeability of ambrisentan across Caco-2 cell monolayers was investigated in the presence and absence of Pgp inhibitor, cyclosporine A (Table 12; 4MYOGP1S1 and 4MYOGP2). The results show that ambrisentan is a medium to high permeability drug with an apparent permeability (Papp) from basolateral to apical direction (B to A) of 0.57 -1.21 x10-6 cm/sec and a significant efflux potential Papp from B to A of 4.34x10-6 cm/sec. Cyclosporine A increased the Papp of ambrisentan from A to B slightly (1.94x10-6 cm/sec) and decreased the efflux transport by 56% of Papp from B to A to 1.97x10-6 cm/sec. This study indicates that ambrisentan has a high absorption potential by passive diffusion but it may also be a substrate for Pgp-mediated efflux.

The inhibitory potential of ambrisentan on human efflux transporter Pgp was investigated in MDCKII cells transfected with human MDR1 gene to produce Pgp protein (Table 12; 06DDM108). The Pgp mediated transport of [^{3}H]-digoxin from B to A was determined in the absence and presence of ambrisentan at concentrations of 0.3 - 100 µM. GF120918, a potent inhibitor of Pgp was included as a positive control. The results show that ambrisentan (0.3 -100 µM) had no inhibitory effect on the Pgp efflux of digoxin, while the positive control compound GF120918 decreased digoxin transport by 77.6%.

Hepatic Uptake and Efflux Transporters

Ambrisentan was examined for inhibition and induction of selected biliary/xenobiotic uptake or efflux hepatic transporters in an *in vitro* sandwich-cultured system composed of rat or human hepatocytes (Table 12; MG-1001, MG-1002, MG-1003). The rat culture system was also used to assess the potential for ambrisentan to be a substrate for hepatic transporters (Table 12; MG-1004). Bosentan and sitaxsentan were studied in the same culture systems for comparison with ambrisentan.

Ambrisentan did not inhibit the following rat or human liver transporters: the uptake sodium-taurocholate co-transporter (NTCP), the uptake organic anion export pump (OATP), the efflux bile salt export pump (BSEP) and the efflux multi-drug resistance protein isoform- 2 (MRP2) (effects on MRP2 were not determined in human cultures). Both bosentan and sitaxsentan demonstrated a significant concentration-dependent inhibition of all four transporters in rat hepatocytes. Bosentan and sitaxsentan inhibited NTCP and BSEP in the human hepatocytes at the highest concentration of 100 µM, but only sitaxsentan inhibited OATP. None of the three agents had induction effects on transporter proteins BSEP, MRP2, and Pgp (efflux) in rat hepatocytes.

Ambrisentan was found to be a possible substrate for OATP, whereas bosentan and sitaxsentan were demonstrated to be potential substrates for BSEP, OATP, and MRP2 in rat hepatocytes.

DISCUSSION AND CONCLUSIONS

Extensive characterization of the ADME properties of ambrisentan in mice, rats, and dogs, the three main species used for the nonclinical safety assessment of the drug has been completed. The results showed that the disposition characteristics were generally consistent across species and there were no marked gender-related differences. The pharmacokinetic and metabolic disposition of ambrisentan has also been characterized in humans. The ADME characteristics in animal species and humans showed many similarities and when differences were observed, they were mostly quantitative in nature. Results were obtained in several *in vitro* studies to determine the potential of ambrisentan for drug-drug interactions with other concomitant medications during the clinical use of the drug.

The pharmacokinetic parameters obtained following iv administration showed that ambrisentan is a low clearance compound with low to moderate volume of distribution in all nonclinical species. The elimination was characterized as multiphasic, with a terminal half-life of 4.7 hours in rats and 8.8 hours in dogs. In patients, the terminal half-life following oral administration was determined to be 12.9-17.9 hours. Ambrisentan is well absorbed following oral administration which is consistent with the high passive membrane permeability observed in CACO-2 cells *in vitro*. Ambrisentan also showed high absolute oral bioavailability in nonclinical species (72 – 85%), indicating that the drug undergoes little or no hepatic first pass metabolism. In the toxicology studies, toxicokinetic results demonstrated that escalation in dose generally resulted in a proportional increase in systemic exposure to the drug. In several repeat dose studies in mice and dogs, there was evidence for greater systemic exposure in females than in males. In contrast, with repeated exposure in rats, there was evidence for greater systemic exposure in males than in females.

In vitro results showed that ambrisentan binds to plasma proteins to a greater extent in plasma of humans (98.9%) than in nonclinical species (91.8-97.2%). Ambrisentan primarily binds to albumin in human plasma. An *in vitro* protein binding displacement study showed that ambrisentan did not displace warfarin, a highly protein bound drug which has a narrow therapeutic window. Co-administration of warfarin and ambrisentan in healthy human subjects also showed no significant changes in their respective Cmax and AUC values indicating no interaction between the two drugs.

The results of a rat tissue distribution study with [^{14}C]-ambrisentan indicated a wide distribution of drug into tissues but it was predominantly present in the gastrointestinal tract. A majority of the radiolabeled ambrisentan was eliminated from major organs relatively rapidly. There was no evidence of retention in any tissues, including melanin-containing tissues. These data predicted that ambrisentan was not likely to accumulate in tissues, which has been confirmed by the lack of increase in Cmax and AUC levels in humans following repeated daily exposure. It is also noteworthy that the penetration of radiolabeled drug into brain was very limited.

The metabolism data obtained following administration of $[^{14}C]$-ambrisentan showed that human metabolic pathways and the metabolites formed were also observed in multiple species. The metabolites identified for ambrisentan include 4,6 dimethyl-2-hydroxypryimidine (M1), ambrisentan glucuronide (M2), 4-hydroxymethyl ambrisentan (M3), O-demethyl ambrisentan (M4), 4,6-dihydroxymethyl ambrisentan (M5), 4,6-dihydroxymethyl ambrisentan glucuronide (M6), 4-hydroxymethyl ambrisentan glucuronide (M7), and O-demethyl-4-hydroxymethyl ambrisentan (M8). The metabolism of ambrisentan was most extensive in the mouse, followed by rabbit, rat, dog, and human. In humans, only three metabolites (M2, M3 and M7) were present at relatively low concentrations in plasma.

Although, the extent of metabolism and predominant pathway varied between animal species, the parent drug was the prominent drug-related component in the plasma at Cmax of rat, rabbit, and dog. At Cmax, parent drug was also the predominant circulating species in humans, accounting for about 79.1% of the total radioactivity. The remaining radioactivity in human plasma was accounted for by three metabolites, M2 (6.3%), M3 (2.8%) and M7 (1.9%). In terms of AUC in humans, parent drug was the predominant radiolabeled product comprising 75% of total $AUC_{(0-last)}$, and the remainder was 3.2% for ambrisentan glucuronide, 21.3% 4-hydroxymethyl ambrisentan, and 0.7% for 4-hydroxymethyl ambrisentan glucuronide. The ambrisentan metabolite, 4-hydroxymethyl ambrisentan (M3), appeared to have low (<40 ng/mL) but more persistent levels up to 96 hours, which may be due to enterohepatic recirculation. 4-hydroxymethyl ambrisentan (M3), was also seen in the mouse, rat, and rabbit plasma, but not in dog plasma. Ambrisentan glucuronide (M2) was a substantial metabolite in dog plasma accounting for 21-28% of radioactivity, and a minor metabolite (approximately 5% radioactivity) in mouse, rat, and rabbit.

The formation of acyl glucuronide metabolites has been associated with the potential to form covalent binding to proteins and result in idiosyncratic toxicity. This can occur when these glucuronides undergo acyl migration and form labile isomeric glucuronides. These species have a tendency to react with proteins to form covalent adducts that may be immunogenic. However, in a study designed to assess the risk of covalent adduct formation due to the formation of ambrisentan glucuronide (an acyl glucuronide), it was found that in dog plasma, this glucuronide was stable and isomerization was slow. In addition, extraction of radioactivity from human and animal plasma samples from radiolabeled studies was high (>95%), indicating the lack of any significant covalent binding. These results combined with the fact that the levels of ambrisentan glucuronide were low in human plasma, provide assurance that the likelihood of covalent binding and the potential for an immunogenic response is very low with ambrisentan.

In humans, of the 66% of oral dose excreted in feces, about two-thirds (62% of fecal radioactivity) appeared as parent drug and 32% was characterized as 4-hydroxymethyl ambrisentan (M3). In urine, which represented 23% of the dose, parent drug represented 20% of the urinary radioactivity with most of the remaining radioactivity accounted for by ambrisentan glucuronide (M2) and 4-hydroxymethyl ambrisentan glucuronide (M7). Since M7 was observed at relatively low concentrations in circulation, it is possible that

this metabolite was rapidly filtered by the kidney and excreted in urine. All three metabolites, M2, M3, and M7, observed in human excreta were also observed in nonclinical species, either in plasma, urine, or feces, indicating that these human metabolic pathways for ambrisentan were also similar in animal species.

Based on disposition studies conducted with [^{14}C]-ambrisentan, the primary route of excretion of drug-related species was feces in humans and all nonclinical species, except the rabbit. About 66% of the dose was recovered in human feces, and the fecal recovery in animals generally accounted for 69%-91% of the dose. With the exception of rabbit, urinary excretion was a minor route of elimination in animals (7-23%) as well as in humans (23%). Studies conducted in bile duct-cannulated rats and dogs suggested that in large part, fecal radioactivity likely represented absorbed dose that was secreted in bile. A rat biliary excretion study shows a substantial enterohepatic recirculation of ambrisentan and ambrisentan glucuronide. The collective evidence indicates that both humans and test animal species primarily excrete parent drug and metabolites via the feces.

In vitro studies showed that ambrisentan did not inhibit major human CYP or UGT enzymes at concentrations up to 100 μM. Administration of ambrisentan did not result in marked induction of CYP enzymes, UPD-GT, and GST in rats at doses up to the 400 mg/kg/day given daily for 4 weeks. Also, upon incubation of ambrisentan with liver microsomes, the formation of ambrisentan glucuronide was unaffected by the presence of 23 of the 24 potential concomitant drugs at concentrations up to 10-fold their maximal therapeutic levels expected in humans. Only nifedipine appears to inhibit ambrisentan glucuronidation *in vitro*.

Ambrisentan did not inhibit human Pgp-mediated transport of digoxin in MDCK cells at concentrations up to 100 μM, but it was found to be a relatively weak substrate for efflux transport (most likely by Pgp) in CACO-2 cells. In studies using the rat or human hepatocyte sandwich cell culture model, it was found that ambrisentan had no inhibitory effect on the transporters investigated. Under the same conditions, concentration-dependent inhibition of these hepatic transporters was observed with two sulfonamide-class endothelin receptor antagonists, bosentan and sitaxsentan, that are structurally distinct from ambrisentan. The inhibitory effect of bosentan on the bile acid transporter has been advanced as a possible mechanism for hepatic toxicity associated with this drug. Ambrisentan, bosentan and sitaxsentan did not induce these hepatic transporters as indicated by no change in the protein levels of Pgp, BSEP or MRP2 in the rat hepatocyte preparations. The above results suggest that ambrisentan is unlikely to be involved in hepatic drug-drug interactions by metabolic or transporter mechanisms. Ambrisentan has a distinctly different profile of interaction with hepatic transporters compared to bosentan and sitaxsentan.

In summary, the ADME characteristics of ambrisentan appear to be predictable and generally consistent across various animal species. The metabolic disposition of ambrisentan in mice, rats, and dogs was qualitatively similar to that in humans and thus, support the choice of these species for nonclinical safety evaluation of the drug. All metabolites observed in humans were also seen in one or more animal species at levels

comparable to or greater than those observed in humans. Experimental results also suggested that ambrisentan has low potential for metabolism or transporter based drug-drug interactions.

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TOXICOLOGY

Brief Summary

The toxicological profile of ambrisentan has been evaluated in single-dose, repeat-dose, carcinogenicity, and reproductive/developmental toxicity studies in mice, rats, rabbits, and dogs and genotoxicity studies *in vitro* (mammalian and bacterial cells) and *in vivo* (rats). Doses administered *in vivo* ranged from 1 to 2000 mg/kg. The repeat-dose studies ranged from 4 weeks to 39 weeks of treatment, and the carcinogenicity studies were 2 years in duration, with the exception of female mice (see Carcinogenicity section below). Individual reviews of these studies are available in DFS '   IND 64,915; for toxicology studies up to 13 weeks, safety pharmacology, genotoxicity and embryo-fetal toxicity). The remaining studies: 26-week rat and dog, 39-week dog, carcinogenicity in rats and mice, fertility, and pre- and post-natal reproductive toxicology studies are appended in this document. A listing of these toxicology studies is provided in the Table below.

Type of Study	Duration	Route	Species
Single-dose	1 dose; 14 day observation	oral and iv	mouse and rat
Repeat-dose	4 weeks	oral	rat and dog
	6 weeks	oral	mouse and rat
	13 weeks	oral	mouse, rat and dog
	26 weeks	oral	rat and dog
	39 weeks	oral	dog
Carcinogenicity	104 weeks	oral	mouse and rat
DART[1]	Fertility, early embryonic	oral	rat
	Embryo-fetal	oral	rat and rabbit
	Pre- and post- natal	oral	rat
Genotoxicity	Bacterial revertants	In vitro	*S. typhimurium*
	Chromosome aberration	In vitro	human lymphocytes
	Unscheduled DNA synthesis (UDS)	In vivo	rat
	Micronucleus formation	In vivo	rat

[1]DART = Developmental and Reproductive Toxicity

Single-dose toxicity

In single-dose toxicity studies in mice and rats, the maximum non-lethal doses for orally administered ambrisentan were 1000 and 2150 mg/kg in the mouse (female and male, respectively) and 3160 mg/kg in the rat (male and female). The maximum non-lethal doses for intravenously administered ambrisentan were 511 and 619 mg/kg in the mouse

(male and female, respectively) and 464 mg/kg in the rat (male and female). The clinical signs observed at toxic doses included lassitude, forced respiration, prone position, partial palpebral closure, and convulsions. Necropsy findings of the animals that died spontaneously revealed congestion in the lungs, liver and kidneys.

Repeat-dose toxicity

<u>Mice</u>

In repeat-dose studies in mice, ambrisentan was administered orally by diet admixture for 6 or 13 weeks at doses of 60-2000 mg/kg/day. Overt clinical signs of toxicity, observed at doses greater than 500 mg/kg/day, included respiratory effects, rough coat, gastrointestinal disturbances, and emaciation. Toxicokinetic evaluation indicated adequate, dose-proportional exposures with no evidence of accumulation, but females had up to 2-fold higher exposures than the males. There were statistically significant increases in red blood cells, hemoglobin, and hematocrit values and significant decreases in total numbers of white blood cells and platelets for males receiving 1250 mg/kg/day. Statistically significant increases in serum ALT and AST (2-3 x levels in concurrent controls) were seen in females receiving 500 mg/kg/day and 1250 mg/kg/day. The absolute organ weights of the brain, heart, liver, kidneys, and prostate were reduced in males receiving 1250 mg/kg/day at the end of treatment. Histopathological examination showed treatment-related increases in nasal cavity findings (inclusions and inflammation) at doses of 150 mg/kg/day or more for both sexes and increases in focal testicular tubular atrophy in males at 500 and 1250 mg/kg/day. The NOAEL reported in the 13 week mouse study was 60 mg/kg/day for males and females, corresponding to $AUC_{0-24hrs}$ of 7.2 and 13.5 ($\mu g \cdot hr$)/mL in males and females, respectively. This NOAEL was based on nasal cavity findings and testicular atrophy. This dose represents 3.6- and 6.73-fold the calculated human exposure in PAH patients [based on free (unbound) drug at the MRHD of 10 mg] for male and female mice, respectively.

<u>Rats</u>

In rats, repeat-dose toxicity studies of oral ambrisentan ranged from 4 to 26 weeks of treatment with doses of 1-2000 mg/kg/day. In these studies, the target organs were the gastrointestinal tract (distension and dilation), nasal cavity (osseous hyperplasia) and testes (testicular tubular atrophy). Mortality was observed at doses greater than 300 mg/kg/day. In longer duration studies (≥13 weeks) and at the highest doses tested (500 or 2000 mg/kg/day), elevations in liver enzymes (alkaline phosphatase [AP or ALP], serum alanine aminotransferase [ALT or ALAT], or serum aspartate aminotransferase [AST or ASAT]) were observed. These elevated values were less than 2-fold concurrent control values and were considered within normal ranges for the laboratory. When histopathology revealed changes in the liver, the changes were hypertrophy not necrosis of the liver and did not correlate with liver enzyme elevations.

Osseous hyperplasia of the nasal turbinates associated with dose-dependent delayed-onset morbidity and mortality was observed following >13 weeks of treatment. This finding was present in shorter duration studies (4-6 weeks), but not with the morbidity associated with the longer studies. Clinical signs included hunched posture, rapid labored breathing, distended gastrointestinal tract (gas), rales (defined as audible breathing), and peripheral cyanosis. These signs were associated with a greater incidence and severity of nasal turbinate findings upon histological evaluation, and evidence of profound polycythemia. This constellation of findings was indicative of upper airway obstruction resulting in hypoxia, secondary erythrocytosis, and death due to suffocation in rats, which are obligate nose-breathing animals.

There was an apparent dose-related increase in the incidence of focal/multifocal testicular tubular atrophy in the survivors in the 100 and 500 mg/kg/day groups at the end of the treatment period, 55% and 100%, respectively. At the end of the recovery period, the incidence of these lesions in these groups (40% and 50%, respectively) was still higher than that in the concurrent control (17%) or the 5 mg/kg/day (33%) groups. One male in the 5 mg/kg/day group exhibited bilateral diffuse testicular tubular atrophy at the end of the treatment period. No animals in any treatment groups exhibited this diffuse lesion at the end of the recovery period. However, in all but one of the male decedents in the 500 mg/kg/day group, diffuse testicular tubular atrophy was observed, along with other histology findings supporting general systemic toxicity responses such as general system/organ congestion.

The NOAEL dose in the 26-week study was 5 mg/kg/day for males and females based on osseous hyperplasia of the nasal turbinates. This dose corresponds to $AUC_{0-24hrs}$ of 9.6 and 8.0 ($\mu g \cdot hr$)/mL for males and females, respectively and Cmax values of 0.5 and 0.4 μg/mL, respectively. Calculated exposure ratios are 1.9- and 1.6-fold for males and females, respectively.

Dogs

In dogs, repeat-dose studies of oral ambrisentan (capsule or gavage) ranged in duration from 4 to 39 weeks of treatment with doses of 30-1500 mg/kg/day. The target organs were the gastrointestinal tract, kidneys, and heart at doses ≥1000 mg/kg/day. Mortality was observed at 1500 mg/kg/day in a 4-week study. While minimal purulent inflammation was observed in 3 male dogs at 900 mg/kg/day in the 26-week study, the profound upper airway findings associated with chronic ambrisentan administration in rats were not observed. The NOAEL in the 39-week dog study was 300 mg/kg for males and females based on the weight decreases and decreased food consumptions at higher dose. The dose corresponds to AUC_{total} of 476.8 and 674.7 ($\mu g \cdot hr$)/mL, and Cmax of 257.1 and 260.3 μg/mL for males and females, respectively. It should be noted that testicular atrophy was reported in the 30 mg/kg male group, but not at the higher doses. Calculated exposure ratios are 104- and 147-fold for males and females, respectively.

Genetic toxicology

Reverse mutation

The genotoxic potential of ambrisentan was assessed in the following assays: Ames test using *S. typhimurium* strains; an *in vitro* chromosome aberration assay; a test for bone marrow micronucleus formation in the rat after oral administration; and an evaluation of unscheduled DNA synthesis (UDS) in the rat after oral administration.

Ambrisentan was evaluated in standard *Salmonella typhimurium* reverse mutation assays (i.e., Ames test). In the pilot study (TXP 97082), S. typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 were cultured with ambrisentan at concentrations (doubling concentrations up to the maximum recommended 5000 µg/plate) of 0, 313, 625, 1250, 2500, and 5000 µg/plate for 72 hours, in the presence or absence of rat liver S-9 fraction activation (Arochlor-1254-induced S-9; 2 % v/v). Appropriate positive controls were included for each bacterial strain. There were no revertants in any strain at any concentration of ambrisentan tested.

In the second study (MPF/ET 9812E), ambrisentan was again tested for its ability to induce revertants in the standard battery of S. typhimurium strains. Five strains of *S. typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) were cultured with ambrisentan at concentrations of 0, 8, 40, 200, 1000, and 5000 µg/plate (3 replicate cultures per concentration) for 72 hours at 37°C. The ambrisentan concentrations were selected in a small range finder experiment with strain TA100. Appropriate positive control compounds were used with each strain to ensure that revertants would be detected. The metabolizing system was Arochlor-1254-induced rat liver S-9 fraction in a culture concentration of 10%. The positive controls provided the expected results, inducing revertants in their corresponding strains in the presence or absence of S-9 activation. There were no revertants in any S. typhimurium strain at any concentration of ambrisentan tested.

In the second assay in this study, the same conditions were employed using ambrisentan concentrations of 0, 312.5, 625, 1250, 2500, and 5000 µg/plate. These concentrations were selected to provide a number of higher concentrations to maximize the potential for detecting revertants. Again, the positive control compounds induced the expected revertants, whereas none of the ambrisentan concentrations induced revertants, with or without S-9 activation.

Chromosome aberration

Ambrisentan induced chromosome aberrations in the *in vitro* chromosome aberration assay using human lymphocytes. The concentrations of ambrisentan evaluated ranged from 208 to 2025 µg/mL. Structural chromosome aberrations were seen in male donors in a specific pulse-chase scheme: 4-hour culture with ambrisentan in the presence or absence of S-9 activation, followed by 16 hour with culture medium alone. The ambrisentan concentrations that yielded significant increases in structural aberrations

were 1300 and 1600 µg/mL in the presence of S-9 mixture and 1600 µg/mL or more in the absence of S-9 mixture. Ambrisentan induced structural chromosome aberrations, but not numerical aberrations, in concentration-dependent fashion, in this *in vitro* assay.

Rat micronucleus

Ambrisentan was evaluated in a rat bone marrow micronucleus test to determine its ability to induce chromosome damage when administered in vivo. In the main study (Table 10; MPF/ET 9809E), groups of 8 male Han Wistar rats received ambrisentan by oral gavage at doses of 0, 500, 1000, and 2000 mg/kg. Evidence of exposure was the clinical observation of brown perianal staining at the 2000 mg/kg dose. Rats were euthanized 24 or 48 hours after dosing. The results can be seen in Table 2.6.7.9-A. The positive control agent, cyclophosphamide, was given at a dose of 25 mg/kg to 4 rats that were euthanized 24 hours after dosing. Femoral bone marrow smears were prepared from each animal. Two thousand (2000) cells were examined from each rat and the percent micronuclei per 2000 PCE (MNPCE) was determined. The PCE:NCE ratios were determined for each rat from at least 500 erythrocytes.

Smears from the rats treated with the positive control (cyclophosphamide) exhibited mean MNPCE of 1.45% and a PCE:NCE ratio of 0.79. The rats that received vehicle exhibited mean MNPCE values of 0.11% and 0.09% at 24 and 48 hours, respectively, with corresponding PCE:NCE ratios of 1.01 and 1.12. The ambrisentan-treated rats exhibited MNPCE values of 0.05-0.16% at 24 hours and 0.08-0.16% at 48 hours. Corresponding PCE:NCE ratios were 0.99-1.06 at 24 hours and 1.02-1.14 at 48 hours. In summary, there was no evidence of ambrisentan-induced bone marrow toxicity.

Unscheduled DNA synthesis

For the assessment of unscheduled DNA synthesis, groups of 5 male Han Wistar rats received single oral doses (gavage) of ambrisentan at 0, 800, or 2000 mg/kg. One negative (water) and 2 positive (2-acetamidofluorene and dimethylnitrosamine) control groups were included in the study. Study evaluations included unscheduled DNA synthesis and plasma level determinations on satellite animals (3 per dose group). In this assay, a compound is considered positive if the group mean net grains/nucleus (NNG) is >0 and >20% of the cells examined are in repair (NNG>5).

Rats were euthanized 2-4 or 12-14 hours after dosing and cultures of hepatocytes were established and treated with [^{3}H]-thymidine for 4 hours followed by overnight culture without radioactivity. The slides were developed using standard autoradiography techniques and the grains of the emulsion present in the cells in various cell locations were determined. Blood samples for toxicokinetic evaluation were drawn from the satellite animals at 1, 2, 4, 8, and 24 hours after dosing.

The toxicokinetic evaluation revealed adequate exposure (Cmax was 185.4 µg/mL and 252.5 µg/mL at 800 and 2000 mg/kg, respectively). The positive control compounds (2-acetamidofluorene at 75 mg/kg and dimethylnitrosamine at 10 mg/kg) gave NNG of 10.4

and 13.0, respectively, with 88-89% of the cells in repair. In contrast, ambrisentan at 800 and 2000 mg/kg gave NNG of less than 1 with less than 4% of the cells in repair. The vehicle control (water) yielded NNG of less than 1 with less than 1% of the cells in repair. Based on the criteria for a positive response in this assay, ambrisentan did not induce unscheduled DNA synthesis in rat liver under the experimental conditions employed.

Carcinogenicity

Carcinogenic potential was evaluated in the rat and mouse. In the rat study, ambrisentan was administered daily for 104 weeks as a diet admixture in doses of 0, 10, 30, and 60 mg/kg/day to rats. Body weight gain and food consumption were dose-dependently reduced for both sexes at the mid and high dose levels ($p < 0.01$), and hunched posture, labored respiration, rales and emaciation were evident in these groups before the end of the first year of dosing. The high and mid-dose male and female groups had their doses lowered to 40 and 20 mg/kg/day, respectively, in week 51. The high dose males and females were taken off drug completely in weeks 69 and 93, respectively. Effects on survival of these groups became evident within the first 6 months. With continued mortality, ambrisentan administration to the high dose rat group was stopped in an attempt to retain as many of these animals as possible for scheduled termination. The only evidence of ambrisentan-related carcinogenicity was a positive trend ($p < 0.025$) for the combined incidence of benign basal cell tumor and basal cell carcinoma of skin/subcutis in male rats when the high dose group was eliminated from the analysis (1 animal with each tumor at the mid-dose, none in any other group, $p < 0.025$) and the occurrence of mammary fibroadenomas in male rats of that same high dose group (4 animals with the tumor in that group, none in any other male group, $p < 0.05$, pairwise comparison with controls).

In a second carcinogenicity study in mice 0, 50, 100, and 250 mg/kg/day was administered. Increased incidences of hunched posture, emaciation and rales were observed in high and mid dose males and high dose females. The high dose male and female groups had their dose lowered to 150 mg/kg/day in week 39 and were taken off drug completely in week 96 (males) or week 76 (females). Effects on survival became evident within the first six months in males and females. Only 11 high dose males, compared with at least 25 in each of the other main study male control and treated groups, survived to scheduled sacrifice at 24 months. None of the main study high dose females survived to 24 months as all 9 surviving members of this group were sacrificed at 84 weeks. Only 14 mid dose females and 18 low dose females survived to 24 months compared with at least 24 females in each of the concurrent control groups. Statistical analysis revealed no evidence of drug-related tumorigenesis, whether or not the high dose groups were included.

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Reproductive and developmental toxicology

Ambrisentan was evaluated in all three segments of reproductive and developmental toxicity. To assess the effects on fertility and early embryonic development, ambrisentan was tested in a rat study in which males were treated for 70 days prior to mating with treated females and then mating with untreated females. The females were euthanized on post-coitum day 21 and the fetuses were examined histologically. A supplemental study examined the presence and severity of testicular tubular atrophy, the effect of these lesions on functional fertility, and the extent of recovery of functional fertility and/or histopathological findings. To study the effect of ambrisentan on embryo/fetal development, a total of 4 studies were performed (2 studies in rats and 2 studies in rabbits). The 2 studies in rats included a dose range-finding study in 5 pregnant females/group followed by a comprehensive study with 22 pregnant rats/dose group. Similarly, the 2 studies in rabbits also included a dose range-finding study in 5 pregnant rabbits/dose group followed by a comprehensive study with 20 pregnant rabbits/dose group. In both studies, the pregnant rats or rabbits were treated with ambrisentan from post-coitum day 6 through post-coitum day 17 (rats) or day 18 (rabbits). The fetuses were examined after Caesarean-section (C-section) on post-coitum day 21 (rats) or day 28 (rabbits). Ambrisentan was also evaluated in a pre/postnatal and maternal function study in rats wherein pregnant rats were given drug from post-coitum day 15 through postpartum day 21, and the offspring were evaluated for developmental hallmarks and fertility.

Fertility and early embryonic development

Ambrisentan was administered by once-daily oral gavage at doses of 0, 10, 30, 100, or 300 mg/kg/day. Groups of 22 male Han Wistar rats received ambrisentan during a 70-day pre-pairing period and through 2 pairing periods of 2 weeks in duration, until they had been dosed for 14 weeks. Groups of 22 female Han Wistar rats received the same doses of ambrisentan for 14 days prior to pairing, through a 2-week pairing period, and up to day 6 post-coitum. Following the first pairing phase (i.e., treated males and treated females), groups of 22 untreated females were paired with the treated males for another 2-week period. After the second pairing phase (a total of 14 weeks of treatment), half of the males were killed (n=11) and the other half were allowed a 13-week drug-free recovery period. All females were sacrificed on day 21 post-coitum. Study evaluations included mortality, clinical signs, body weights, food consumption, paternal parameters (fertility, histopathology and semen analysis), maternal parameters (gross pathology and reproductive data), and fetal assessments (weight, sex, live vs. dead, visceral and skeletal anomalies). No toxicokinetic evaluation was done in this study.

A total of 4 males that received 300 mg/kg/day were either found dead or were euthanized in extremis between days 7 and 12 of treatment. No clinical signs were observed for the other males or any of the females throughout the study. Food consumption for males receiving 300 mg/kg/day was significantly reduced during the 70-day pre-pairing period with some reduction in body weight gain. Food consumption and

body weight gain for all other males and all females in all treatment groups were similar to controls.

Ambrisentan had an effect on male fertility at 300 mg/kg/day, as evidenced by a reduced fertility index in this group (77.8% with treated females and 83.3% with untreated females) as compared to 100% of the control group for both treated and untreated females. C-section results for treated females showed an increase in pre-implantation loss at 300 mg/kg/day (11.7%) as compared to 5.9% for control females with treated males. C-section results for untreated females showed increased post-implantation loss of 6.3-8.1% when mated with treated males compared to 4% when mated with untreated males.

Semen evaluation demonstrated a slight but significant decrease in the percentage of morphologically normal sperm at 300 mg/kg/day at the end of treatment and a similar, although not significant, reduction at the end of the recovery period. However, these values were still above 90% normal sperm. There was no effect on sperm motility at any dose.

The development of focal/multifocal testicular tubular atrophy was present in all treatment and in control groups. None of the 22 males in the control group developed diffuse testicular tubular atrophy. Overall, 7 treated males exhibited this type of lesion. One male that received 10 mg/kg/day exhibited bilateral moderate diffuse testicular atrophy plus bilateral aspermia at the end of the recovery period. One male that received 30 mg/kg/day had bilateral massive diffuse testicular atrophy plus bilateral aspermia at the end of the recovery period. Two males that received 100 mg/kg/day displayed diffuse testicular atrophy plus aspermia; 1 at the end of treatment and 1 at the end of the recovery period. Two males that received 300 mg/kg/day exhibited bilateral massive diffuse testicular atrophy; 1 at the end of treatment and 1 at the end of the recovery period. A third male that received 300 mg/kg/day exhibited unilateral diffuse testicular atrophy at the end of treatment.

All fertility assessments were based upon pairing periods at the end of the treatment period. All males (100%) in the control group were fertile. One of the 22 males that received 10 mg/kg/day of ambrisentan was infertile at the end of treatment, and exhibited bilateral diffuse testicular atrophy when necropsied at the end of the recovery period. All males (100%) that received ambrisentan at 30 or 100 mg/kg/day were fertile, including the 2 animals that exhibited bilateral diffuse testicular atrophy at the end of recovery. Three of the 18 surviving animals that received 300 mg/kg/day of ambrisentan were infertile. One exhibited bilateral massive diffuse testicular atrophy at the end of treatment and one exhibited bilateral moderate diffuse testicular atrophy at the end of the recovery period. The third infertile animal did not exhibit any testicular or sperm abnormalities at the end of treatment that would explain the observed infertility. In the 300 mg/kg/day group, the third male that had massive bilateral diffuse testicular atrophy at the end of treatment was fertile.

There was no treatment-related effect on fetuses. For both pairing phases, there were no treatment-related effects on fetal weight, sex ratio, or on the incidences of external,

visceral or skeletal findings. There was no effect on the fetuses at the time of C-section whether the females were directly exposed to ambrisentan (up to gestation day 6), or indirectly exposed through mating with treated males.

The NOAEL for paternal toxicity was reported as less than 10 mg/kg/day due to occurrence of diffuse testicular tubular atrophy and infertility. This was comparable to the NOAEL in repeat dose toxicology studies. The NOAEL for maternal toxicity was reported as 100 mg/kg/day due to increased post-implantation loss at 300 mg/kg. The NOAEL for early embryonic development was reported as 300 mg/kg/day, but since early embryonic development happens within a female, the NOAEL is more precisely defined as 100 mg/kg/day, the level for the pregnant female.

A supplemental study (AMB-001) was designed to examine the presence and severity of histologically-graded testicular tubular atrophy, the effect of these lesions on functional fertility, and the extent of recovery of functional fertility and/or histopathological findings after chronic ambrisentan administration. Ambrisentan was administered for 15 weeks (300 mg/kg/day as a diet admixture) followed by a drug-free recovery period of 20 weeks.

Groups of 50 male Han Wistar rats received ambrisentan as a diet admixture at doses of 0 or 300 mg/kg/day for 15 weeks. During the last 2 weeks of treatment, half of the males in each group (n = 25) were paired with untreated females. The other 25 males in each group completed their 15 weeks of treatment and then were allowed a 20-week drug-free recovery period prior to pairing. All females were euthanized at postcoitum day 14 and evaluated for reproductive parameters. Each male remained with a female partner for up to 2 weeks. If mating or pregnancy did not occur, the male was placed with another female 6 weeks later (2nd pairing period). Drug was not administered past the first 2-week mating period. If mating/pregnancy still did not occur, the male was placed with 2 untreated females 6 weeks later (3rd pairing period). Once a successful mating (pregnancy) was achieved, male animals were euthanized for histopathological examination of the testes. Study evaluations included mortality, body weight, food consumption, toxicokinetics, reproductive processes/parameters, histopathology, and semen analysis.

All animals survived until their scheduled termination. Toxicokinetic results revealed adequate systemic exposure that was slightly higher at day 2 than at day 29. There was a significant reduction in body weight of the males receiving 300 mg/kg/day compared to the control rats after 13 and 15 weeks of treatment. There was no reduction in body weight by the end of the recovery period.

A small increase in the frequency of histologically-graded testicular tubular atrophy was associated with ambrisentan administration. At the end of the treatment period, 21 males in the control group and 24 males in the treated group exhibited focal/multifocal testicular tubular atrophy. At the end of the recovery period, 16 males in the control group and 21 males in the treated group exhibited this same lesion. One rat that received 300 mg/kg/day presented with moderate bilateral diffuse testicular tubular atrophy at the

end of the treatment period. There was no incidence of diffuse testicular tubular atrophy at the end of the recovery period. There were statistically significant decreases in total sperm counts and in the percent of morphologically normal sperm in the testes of males receiving 300 mg/kg/day at the end of the treatment period, but not at the end of the recovery period.

All males were functionally fertile. At the end of the treatment period, 3 males in the control group and 4 in the 300 mg/kg/day group required a second pairing period; 1 male in the 300 mg/kg/day group required a third pairing period. At the end of the recovery period, 3 males in the control group and 1 in the 300 mg/kg/day group required a second pairing period. The one treated rat that exhibited bilateral diffuse testicular tubular atrophy at the end of the treatment period was fertile and required only 1 pairing period for successful impregnation.

These studies demonstrate a potential for testicular injury which, although variable and also present in control rats, is consistently seen at higher incidence in treated rats. These results are consistent with findings in the majority of compounds in this class. To this reviewer, it appears that at least two effects are apparent. There appears to be an exacerbation of the _focal_ tubular atrophy which is a commonly observed, age-related phenomena in rats (more so in Sprague Dawley than Wistar). This exacerbation appears somewhat reversible. In addition, there is a treatment-related increase in _diffuse_ atrophy, where recovery is less complete, that is distinct from the age-related lesion. The effects seem dose-related, however, the majority of treated rats do not exhibit the lesions. The decrease in sperm counts and the percent of morphologically normal sperm represent a functional correlate to the observed histopathology.

The smaller effects on fertility of male rats could simply be a result of the high inherent fertility in this species and reflect a general insensitivity of rodent fertility assays. The possibility remains that even a small level of testicular pathology in a species of borderline infertility (such as man), could result in much larger effects on fertility.

Embryofetal development

The effects of oral (gavage) ambrisentan on pregnant females and on embryo-fetal development were examined in both rats and rabbits. Ambrisentan administration to the pregnant females was timed to include implantation to the closure of the hard palate. The doses for the full study in each species were selected from the corresponding dose-range finding study.

Rats:

In the dose-range finding study for rats (MPF/DT 0499E), groups of 5 pregnant female Han Wistar rats received ambrisentan at doses of 0, 150, 300, or 600 mg/kg/day by once-daily oral gavage from post-coitum days 6 through 17 (post-coitum day 0 = sperm positive vaginal smear or presence of a copulation plug). Females were euthanized on post-coitum day 21 and the fetuses were removed by C-section. Study evaluations

included maternal mortality, clinical signs, body weight, food consumption, and reproductive processes/parameters, and the fetuses were examined for sex ratio, body weights, and gross malformations.

All animals survived until their scheduled termination. The only clinical signs observed were ruffled fur in 4 out of 5 females in each of the 300 and 600 mg/kg/day groups during the last few days of pregnancy after the dosing had stopped. One female receiving 600 mg/kg/day exhibited vaginal bleeding during the last 2 days of treatment, but at no other time during the remainder of the pregnancy. A reduction in body weight gain and food consumption was seen at doses =150 mg/kg/day. There was an increase in post-implantation loss at doses =300 mg/kg/day (>20% vs. 6% for control females). Ambrisentan had no effect on fetal sex ratios. At doses =150 mg/kg/day, fetal body weights were reduced and there was an increase incidence of malformations of the lower jaw and tongue. The highest dose proposed for the main study in rats was 150 mg/kg/day.

In the main study conducted to assess the effects of ambrisentan on embryo-fetal development in rats (MPF/DT 9925E, reviewed in ━━━━━━━━━━━ ι, groups of 22 pregnant Han Wistar rats received ambrisentan by once-daily oral gavage at doses of 0, 15, 47, and 150 mg/kg/day. A satellite group of 9 pregnant females per dose group was used for toxicokinetic evaluation. All females were treated from post-coitum day 6 through post-coitum day 17 (post-coitum day 0 = sperm positive vaginal smear or presence of a copulation plug). The main study groups were euthanized on post-coitum day 21 and the fetuses removed by C-section; whereas, the satellite toxicokinetic animals were euthanized on post-coitum day 18. Study evaluations included maternal clinical signs, body weights, food consumption, reproductive parameters, gross pathology, fetal body weight, sex ratios, external, visceral, and skeletal examinations. Blood was drawn for toxicokinetics at 0, 1, 2, 4, 8, and 24 hours post dosing on post-coitum days 6 and 17.

All animals survived until their scheduled termination. Toxicokinetic evaluation revealed adequate, possibly less than proportional systemic exposures, with no indication of accumulation or changes in clearance over the duration of treatment. There were no treatment-related effects on body weight, food consumption, pregnancy rates, mean number of corpora lutea or mean number of implantations. There was a significant increase in pre-implantation losses at the 15 mg/kg/day dose level, but a decrease in this parameter at the 150 mg/kg/day dose level. Both values were within the ranges of historical controls. In the satellite animals receiving 150 mg/kg/day, there were significant post-implantation losses (22.5% vs. 4% in satellite animals for the control) that were not seen in the animals in the main study.

There were no dead fetuses in any of the litters. There were no differences between the treatment groups in litter sizes or in fetal sex ratios. The mean fetal body weight in litters from dams receiving 15 mg/kg/day were larger than those in the controls and the mean fetal body weight in litters from dams receiving 47 or 150 mg/kg/day were lower than those in controls; however, these differences were within the range of historical values and were not considered to be of biological significance. There were treatment- and dose-related increases in the incidence of lower jaw, tongue, soft and hard palate, blood vessel,

and thymus abnormalities in fetuses from dams receiving ambrisentan, indicating a teratogenic effect. All fetuses from the 150 mg/kg/day group and a majority of the fetuses from the 15 and 47 mg/kg/day groups had grossly-evident lower jaw abnormalities.

The NOAEL for maternal toxicity was reported as 150 mg/kg/day due to no observable effects; whereas, the NOAEL for embryo-fetal toxicity was reported as less than 15 mg/kg/day due to the presence of fetal abnormalities in all treatment groups these levels correspond to maternal $AUC_{0-24hrs}$ at post-coitum day 17 of 459.5 and less than 51.7 (μg·hr)/mL, respectively.

Rabbits:

In the dose-range finding study for rabbits (MPF/DT 0599E, reviewed in ⎯⎯⎯⎯⎯⎯⎯ ⎯⎯⎯⎯), groups of 5 pregnant female Chinchilla rabbits received ambrisentan at doses of 0, 150, 300, or 600 mg/kg/day by once daily oral gavage from post-coitum day 6 through day 18 (post-coitum day 0 = sperm positive vaginal smear or presence of a copulation plug). Females were euthanized on post-coitum day 28 and the fetuses were removed by C-section. Study evaluations included maternal mortality, clinical signs, body weight, food consumption, toxicokinetics, and reproductive processes/parameters. The fetuses were examined for sex ratio, body weights, and gross malformations. Blood was drawn for toxicokinetics at 2 hours (approximate Cmax) and 24 hours (approximate Cmin) after the first and last doses (post-coitum days 6 and 18).

These doses of ambrisentan were highly toxic to Chinchilla rabbits. Three out of 5 does that received 600 mg/kg/day and 1 out 5 that received 300 mg/kg/day died during treatment. In the survivors, food consumption and body weight were significantly reduced in all treatment groups. Two does that received 300 mg/kg/day and 1 doe that received 150 mg/kg/day aborted. There was total resorption of litters in the groups receiving 300 or 600 mg/kg/day and greater than 90% post-implantation losses in recipients of 150 mg/kg/day. Toxicokinetic evaluation indicated possible accumulation since the plasma levels were higher after the last dose than after the first. The exposures ($AUC_{0-24 hrs}$) on post-coitum day 18 were 48.6, 201.3, and 473.6 (μg·hr)/mL for doses of 150, 300, and 600 mg/kg/day, respectively.

In total, of the fetuses from the treated females, only 6 fetuses from the 150 mg/kg/day group were available for examination. These fetuses all exhibited malformations of the lower jaw and tongue. The fetuses from the control group exhibited no abnormalities. Based on the high degree of toxicity and teratogenicity seen in this study, a NOAEL was not reported. The doses to be used the in the main study were proposed to be 0, 7, 21, and 63 mg/kg/day.

In the main study conducted to assess the effects of ambrisentan on embryo-fetal development in rabbits (MPF/DT 9926E, reviewed in ⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯⎯), groups of 20 pregnant Chinchilla rabbits received ambrisentan by once-daily oral gavage at doses of 0, 7, 21, and 63 mg/kg/day. A satellite group of 3 pregnant females per dose group was used for toxicokinetic evaluation. All females were treated from post-coitum

day 6 through post-coitum day 18 (post-coitum day 0 = sperm positive vaginal smear or presence of a copulation plug), and were euthanized on post-coitum day 28, at which time fetuses were removed by C-section. Study evaluations included maternal clinical signs, body weights, food consumption, reproductive parameters, gross pathology, fetal body weight, sex ratios, and external, visceral, and skeletal examinations. Blood was drawn for toxicokinetics at 0, 0.5, 2, 4, 8, and 24 hours post dosing on post-coitum days 6 and 18.

All animals survived until their scheduled termination. Toxicokinetic evaluation showed an adequate and dose-proportional exposure that was higher on post-coitum day 18 than on post-coitum day 6 for all doses, indicating a potential for accumulation in pregnant Chinchilla rabbits. There were no treatment-related effects on maternal body weight, food consumption, clinical signs, gross pathology, pregnancy rates, mean number of corpora lutea, mean number of implantation sites, or mean % pre-implantation loss between the groups.

There was a significant increase in post-implantation losses at 63 mg/kg/day in both the main study and the toxicokinetic satellite animals. In the 63 mg/kg/day group, 3 of the pregnant does had total resorption of their litters and 1 doe in the 21 mg/kg/day toxicokinetic satellite group had total resorption of her litter. There were no dead fetuses in any of the litters. There were no differences between the treatment groups in sizes of litters, mean fetal body weight or in fetal sex ratios. There was a treatment- and dose-related increase in the incidence of lower jaw, soft and hard palate, blood vessel, and thymus abnormalities in fetuses from does receiving ambrisentan, indicating a teratogenic effect.

The NOAEL for maternal toxicity was reported as 21 mg/kg/day due to abortion/resorption of litters at 63 mg/kg/day, whereas the NOAEL for embryo-fetal toxicity was reported as less than 7 mg/kg/day due to the occurrence of fetal abnormalities in all dose groups. These levels correspond to $AUC_{0-24hrs}$ at post-coitum day 18 of 71.7 and less than 24.7 ($\mu g \cdot hr$)/mL, respectively.

Prenatal and postnatal development

A study was conducted in rats to assess the effect of ambrisentan on pre- and post-natal development and maternal function (A 38654). Groups of 22 pregnant Han Wistar rats received daily administrations of ambrisentan by oral gavage at doses of 0, 15, 45, and 150 mg/kg/day drug from post-coitum day 15 through postpartum day 21. Dosing during post-coitum days 6-15 had already been shown to be teratogenic and was therefore not included in this study. A pilot study (included as an appendix to the main study report) was done to determine an acceptable starting day for dosing and gestation day 15 was selected as the earliest day showing no adverse fetal effects. The dams and their offspring were evaluated for maternal clinical signs, body weights, food consumption, reproductive parameters, and gross pathology. The offspring were evaluated for developmental and behavioral hallmarks and fertility as they reached sexual maturity. Toxicokinetic determinations were not done in this study. Evidence of exposure was provided by the responses to drug administration.

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All dams survived to their scheduled termination. Fertility rates were 90-95% and comparable across groups. There was an increase in body weight in females receiving 150 mg/kg/day at the end of gestation as compared to control animals; however, food consumption was unaffected. There were no clinical signs observed in the dams during gestation and no necropsy findings at the end of weaning. The mean duration of gestation, mean number of implantations, mean percentage post-implantation loss, mean litter size, and litter sex ratios were comparable across groups.

There was a significant decrease in pup survival from birth to day 4 in the 45 and 150 mg/kg groups compared to the other groups. This included a dam that lost her entire litter on day 2. The clinical signs and necropsy findings in the pups during the pre-weaning period were limited to lack of milk in the stomach of pups in 16 litters in the 150 mg/kg/day group and 6 litters in the 45 mg/kg/day group, indicating that maternal nursing behavior, or the nursing instincts of the pups, was affected by ambrisentan administration.

After weaning, there were very few differences in any of the developmental hallmarks between groups. Eye opening was earlier in pups in the 45 and 150 mg/kg/day groups at 14.1 and 14.3 days, respectively compared to 14.9 days for controls. Vaginal opening was slightly delayed in pups from the 150 mg/kg/day group (33.5 days as compared to 32.3 days for controls). None of the other developmental hallmarks and none of the behavioral tests showed any differences between the groups.

Once the behavioral tests were completed at approximately day 42, the F1 generation was selected from each group. Twenty-two (22) males and 22 females were selected from each treatment group from as many litters as possible. The non-selected pups were euthanized. The selected pups were allowed to grow until they were at least 10 weeks old, at which time they were mated to other members from the same treatment group but not to siblings. Body weight changes and food consumption were comparable between groups. The mean pre-coital time was similar among groups.

Male fertility rates were decreased in pups from the 150 mg/kg/day group. This correlated with necropsy findings of small testes. For the females of the F1 generation, there were no differences between groups in body weight change, food consumption, gestation weight gain and food consumption, mean number of corpora lutea, mean number of implantations or mean number of live fetuses per litter. There was an increased pre-implantation loss and a decreased post-implantation loss in the 150 and 45 mg/kg groups compared to the control and the 15 mg/kg groups.

The NOAEL was reported to be 15 mg/kg/day for maternal F0 toxicity due to maternal behavior after giving birth and 15 mg/kg/day was reported as an acceptable NOAEL for the F1 generation males and females.

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Summary of reproductive toxicology studies

Ambrisentan affected female fertility in rats as evidenced by increases in pre-implantation losses in females at the higher doses tested. There were no test-item related effects on embryos when oral ambrisentan was administered directly to pregnant female rats (up to gestation day 6), or indirectly via treatment of males. The effect on male fertility in rats is less consistent. In one study, males demonstrated lower fertility indices and developed diffuse testicular tubular atrophy that was not consistently associated with infertility. In a second study, there was no treatment effect on male fertility, although testicular findings were present.

Ambrisentan is teratogenic in rats and rabbits when administered at any dose (7-150 mg/kg/day) between gestation days 6 and 15 and it is toxic to pregnant rabbits as evidenced by the maternal toxicities observed at doses greater than 21 mg/kg/day. Pregnant rats tolerate ambrisentan up to doses of 150 mg/kg/day. The fetal abnormalities consistently observed involved the lower jaw and/or palate. Additional findings present in rats included abnormalities of the major vessels and thymus. There was no dose in these studies at which fetal abnormalities were not observed.

When administered by daily oral gavage to pregnant rats from gestation day 15 through postpartum day 21, ambrisentan did not have any adverse effects on the specific pre- or post-natal developmental milestones of the offspring. A decrease in pup survival was present at 0-4 days post-partum at the higher dose that may represent toxicity affecting maternal behavior. An effect on pup nursing behavior cannot be ruled out, however. Male offspring at this high dose exhibited small testicles and decreased fertility rates at the highest doses employed.

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APPENDIX/ATTACHMENTS

1) BSF 208075: A 39-week oral toxicity study in beagle dogs followed by a 20-week recovery period. **(Page 77)**

2) BSF 208075: Study for effects on fertility and early embryonic development to implantation after oral administration (gavage) in the Wistar rat. **(Page 85)**

3) BSF 208075: Study for effects on pre- and postnatal development including maternal function in the Han Wistar rat. **(Page 101)**

4) Review of IND Amendment 64915 Serial No. 010: 26 week toxicology studies in rat and dog. **(Page 111)**

5) Rat carcinogenicity protocol review (IND Amendment 64915 Serial No. 034) including 13 week toxicology studies. **(Page 136)**

6) Mouse carcinogenicity protocol review (IND Amendment 64915 Serial No. 036) including 13 week toxicology studies. **(Page172)**

7) LU 208075: Effect on the cardiovascular and respiratory systems (i.v.) in the dog. **(Page 193)**

8) Mouse carcinogenicity study review. **(Page 197)**

9) Rat carcinogenicity study review. **(Page 224)**

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