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# **INTERSPECIES DIFFERENCES IN PHARMACOKINETICS AND METABOLISM OF S-3-(4-ACETYLAMINO-PHENOXY)-2- HYDROXY-2-METHYL-N-(4-NITRO-3-TRIFLUOROMETHYL- PHENYL)-PROPIONAMIDE: THE ROLE OF N- ACETYLTRANSFERASE**

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## **Abstract**

*N*-Acetyltransferase (NAT) is one of the major phase II enzymes involved in drug metabolism. Both species differences and polymorphism are observed in NAT expression. During the preclinical development of a novel selective androgen receptor modulator, *S*-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide (S4), we also observed species differences in S4 metabolism due to the interaction between the deacetylation metabolite M1 and NAT, which converted M1 back to S4 both in vitro and in vivo. During incubation with human liver cytosol or rat liver S9 fraction in the presence of acetyl-CoA, more than 50% of M1 (2 μM) was converted back to S4, but this conversion was not observed in the incubation with dog liver S9 fraction or human liver microsome. In vivo pharmacokinetic experiments showed that M1 could be rapidly converted back to S4 in rats, but a similar conversion was not observed in dogs. When S4 was administered, the formation of M1 was only observed in dogs due to the absence of NAT expression. Simultaneous fitting of the concentration-time profiles of both S4 and M1 showed that more than 50% of S4 was deacetylated to M1 in dogs after i.v. administration of S4, whereas more than 80% of M1 was converted to S4 in rats after i.v. administration of M1. Considering the polymorphism in NAT expression, the interaction between M1 and NAT may raise concerns for drug-drug interactions during clinical applications of S4. The observed species differences suggested that interspecies scaling might not be applicable for predicting the metabolism and disposition of S4 in humans.

*N*-Acetyltransferase (NAT) is one of the major hepatic phase II enzymes involved in drug metabolism. Humans contain two functional NAT isoforms (NAT1 and NAT2). NAT is expressed as a cytosolic protein that is expressed in a wide variety of tissues, and plays an important role in the *N*-acetylation of drugs containing aromatic amine and hydrazine groups, converting them to aromatic amides and hydrazides, respectively. The acetylation reaction requires a cofactor, acetyl-CoA, that contributes the activated acetyl group for NAT acetylation activity. As an important metabolizing enzyme in humans, the polymorphism of human NAT expression, especially NAT2, raises concerns about drug-drug interaction related to drug metabolism during clinical use. In contrast, the species differences in NAT expression (Casarett et al., 1996) could introduce species differences in drug metabolism, which also raises concerns about using certain animal species to evaluate the metabolic profile of new compounds, as we have observed with one of the leading compounds [S4, *S*-3-(4-acetylamino-phenoxy)-2-

hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide] during preclinical development of nonsteroidal selective androgen receptor modulators (SARMs).

Since the discovery of the first generation of SARMs (Dalton et al., 1998), several structural modifications have been made to improve the binding affinity, agonist activity, and in vivo metabolic profiles of this new class of compounds. The in vivo pharmacological activities of the second generation of SARMs, including compounds S1 and S4, have been well characterized in animal models (Yin et al., 2003a; Gao et al., 2004, 2005), and the metabolic profile of compound S4 was also characterized in vitro (Gao et al., 2006) and in vivo (M. A. Perera, D. Wu, K. K. Chan, D. D. Miller, and J. T. Dalton, data to be published). Similar to its structural analogs, bicalutamide (Boyle et al., 1993; Cockshott, 2004) and acetothiolutamide (Yin et al., 2003b), S4 undergoes hydrolysis and oxidation as well. However, different from other analogs of the class, in vitro metabolism study identified the deacetylated derivative (M1) as the major phase I metabolite of S4, which accounted for about half the S4 metabolized in human, rat, and dog liver S9 fraction (Fig. 1). The deacetylation of the B-ring acet-amide group introduced an aromatic amine group, which turned out to be a substrate for NAT and could convert M1 back to S4 (Fig. 1). More importantly, the species differences in NAT expression (Casarett et al., 1996) could introduce species differences in S4 metabolism, and the polymorphism of human NAT expression, especially NAT2, could raise concerns in terms of drug-drug interaction related to drug metabolism during clinical use. Furthermore, in vitro binding study and transcription activation assay suggested that M1 could be an active metabolite of S4, and might contribute to the in vivo pharmacological activity of S4. The possible species differences in S4 metabolism might also introduce species differences in in vivo pharmacological activity of S4.

The purpose of the current study was to characterize the interaction between M1 and NAT, and potential species differences in S4 metabolism in rat and dog. Because NAT is mainly located in the cytosolic fraction of the liver enzyme preparation (Casarett et al., 1996), different subcellular fractions of human liver were tested. However, because NAT is expressed in humans and rats, but not in dogs (Casarett et al., 1996), the metabolite PK profile of M1 was characterized and compared in rat and dog. Furthermore, the concentration-time profiles of both S4 and M1 were subjected to simultaneous modeling to evaluate the contribution of the deacetylation process to the overall in vivo metabolism of S4 in rat and dog. These studies provide compelling evidence of the role of *N*-acetyltransferase in disposition and preclinical development of this novel SARM.

## Materials and Methods

### Materials

Compounds S4 and M1 were synthesized by Dr. Duane Miller's research group at the University of Tennessee. Recombinant human NAT 1 and 2, pooled human, rat, and dog liver microsomes, cytosol, and S9 preparations were purchased from BD Gentest (Woburn, MA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). All analytical columns were purchased from Waters (Milford, MA).

### Animals

Male Sprague-Dawley rats (approximately 250 g) were purchased from Harlan Bioproducts for Science Inc. (Indianapolis, IN). Beagle dogs (approximately 9 kg) were purchased from an approved vendor through University Laboratory Animal Resources at The Ohio State University. The animals were maintained on a 12-h light/dark cycle with food and water available ad libitum. Animal protocols were reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

### In Vitro Metabolism Reaction

In vitro enzyme reactions were conducted according to the instructions provided by BD Gentest. Reactions using Super-somes, liver microsomes, cytosol, or liver S9 preparations were conducted at 37°C in the presence of 1 mM NADPH in 100 mM phosphate buffer (pH 7.4) for various times. The reaction time, and substrate and enzyme concentrations are noted in figure legends. Reactions were stopped by adding ice-cold acetonitrile (v/v, 1:1) containing internal standard (CM-II-87, structural analog of S4) for HPLC analysis. Protein present in the reaction mixture was precipitated by centrifugation (> 16,000g, 30 min at 4°C), and the supernatant was directly used for HPLC analysis.

Recombinant human NAT1 and NAT2 reaction buffer contained 50 mM triethanolamine (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM acetylCoA, and an acetyl-CoA regenerating system (4.6 mM acetyl-D,L-carnitine and 0.06 unit of carnitine acetyl transferase). For mixed reactions that contained both pooled liver enzyme preparations and NAT, 100 mM phosphate buffer (pH 7.4) was used. NADPH and/or acetyl-CoA (including the regenerating system) were added according to experimental design.

### HPLC Method

The disappearance of S4 or the appearance of M1 was determined using HPLC analysis. S4 and M1 were separated on a reversed-phase column (Symmetry C8, 3.9 × 150 mm) with a mobile phase of 45% acetonitrile and 50 mM phosphate buffer (pH 4.8) in deionized water, at a flow rate of 1.0 ml/min, and were detected by their UV absorbance at 230 nm. The interday variability was less than 8% at the lower limit of quantitation (0.03 µg/ml).

### Pharmacokinetic Studies of S4 and M1

The pharmacokinetic profiles of S4 and M1 in rat ( $n = 5$ ) and dog ( $n = 3$ ) were also determined. In rats, a catheter was implanted in the right jugular vein 18 h before dosing. In dogs, the saphenous vein catheter was implanted immediately before dosing. Intravenous doses (10 mg/kg) were administered via the catheters. The catheter was immediately rinsed with saline at a volume 3 times that of the dosing solution. Blood samples were withdrawn from the catheters before and after each dose. For the 10 mg/kg dose of M1 in rats, blood samples (250 µl each) were collected at 5, 10, 30, 60, 120, 240, 480, and 720 min after the i.v. dose. For the 10 mg/kg dose of S4 or M1 in dogs, blood samples (3 ml each) were collected at 2, 5, 10, 15, 30, 60, 120, 240, 480, and 720 min after the i.v. dose. All dogs were treated with S4 (10 mg/kg) during the first week of the study. One week later, M1 (10 mg/kg) was administered to the same group of animals.

Plasma samples were separated by centrifugation (3000g for 20 min at 4°C). Plasma proteins were precipitated by the addition of acetonitrile (v/v, 1:1, containing internal standard). Protein pellets were separated by centrifugation (> 16,000g, 30 min at 4°C), and the supernatant was used for HPLC analysis.

### Compartmental Analysis of the Concentration versus Time Profiles of S4 and M1

A variety of compartmental models were used to identify the model that best described the pharmacokinetic data in each species. The goodness of fit between models was compared using the Akaike information criterion (Ludden et al., 1994), observed variability in the estimated pharmacokinetic parameters, and visual inspection of plots of residual differences between actual and computer-estimated concentration-time profiles. The models presented below represented those that best represented the data for each study.

### Compartmental Modeling of Rat Pharmacokinetic Data

Plasma concentration-time profiles of S4 in the rat after an i.v. dose of S4 was computer-fitted individually using a standard one-compartment model in WinNonlin (Version 4.1, Pharsight Corp., Cary, NC). The volume of distribution ( $V_{dS4}$ ) and the elimination rate constant ( $k_{S4}$ ) of S4 were estimated as model parameters using a weighting scheme of  $1/Y$ .

The S4 and M1 data from each rat after an i.v. dose of M1 were computer-fitted using a user-defined model (Fig. 2) and WinNonlin software. The plasma concentrations of S4 and M1 were best described by the following equations:

$$Cp = \frac{\text{Dose}}{V_{dM1}} \cdot e^{-(k_a + k_{M1}) \cdot t} \quad (1)$$

for M1;

$$Cp = \frac{\text{Dose} \cdot k_a}{V_{dS4} \cdot (k_a + k_{M1} - k_{S4})} \cdot \left( e^{-k_{S4} \cdot t} - e^{-(k_a + k_{M1}) \cdot t} \right) \quad (2)$$

for S4.

The mean values of  $k_{S4}$  ( $0.0044 \text{ min}^{-1}$ ) and  $V_{dS4}$  (112 ml), determined from the S4 data after an i.v. dose of S4, were used as constants in eq. 2 during nonlinear regression. M1 and S4 data were fitted simultaneously using eqs. 1 and 2 to determine model parameters ( $k_a$ ,  $k_{M1}$ , and  $V_{dM1}$ ) describing M1 disposition.

CL and AUC were determined as:

$$CL_{M1} = (k_a + k_{M1}) \times V_{dM1} \quad (3)$$

$$AUC_{M1} = \frac{\text{Dose}}{CL_{M1}} \text{ or } AUC_{S4} = \frac{\text{Dose} \cdot k_a}{V_{dS4} \cdot k_{S4} \cdot (k_a + k_{M1})} \quad (4)$$

The fraction of M1 that was converted to S4 ( $f_m$ ) after an i.v. dose of M1 to rats was determined by:

$$f_m = \frac{k_a}{k_a + k_{M1}} \quad (5)$$

### Compartmental Modeling of Dog Pharmacokinetic Data

Since the S4 and M1 pharmacokinetic studies were completed in a crossover design with the same group of dogs, the three sets of pharmacokinetic data were fitted simultaneously using the two-compartment model shown in Fig. 2. The plasma concentrations of S4 after an i.v. dose of S4 to dogs were best described by:

$$Cp = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} \quad (6)$$

with  $A$ ,  $B$ ,  $\alpha$ , and  $\beta$  defined as:

$$A = \frac{\text{Dose} \cdot (\alpha - k_{21} - k_h)}{V_{d1} \cdot (\alpha - \beta)} \quad \text{and} \quad B = \frac{\text{Dose} \cdot (k_{21} + k_h - \beta)}{V_{d1} \cdot (\alpha - \beta)} \quad (7)$$

$$\begin{aligned} \alpha + \beta &= k_{21} + k_h + k_{12} + k_{10} \\ \alpha \cdot \beta &= k_{12} \cdot k_h + k_{10} \cdot k_{21} + k_{10} \cdot k_h \end{aligned} \quad (8)$$

The plasma concentrations of M1 after an i.v. dose of M1 were best described by:

$$Cp = A' \cdot e^{-\alpha' \cdot t} + B' \cdot e^{-\beta' \cdot t} \quad (9)$$

with  $A'$ ,  $B'$ ,  $\alpha'$ , and  $\beta'$  defined as:

$$A' = \frac{\text{Dose} \cdot (\alpha' - k_{21}')}{V_{d1}' \cdot (\alpha' - \beta')} \quad \text{and} \quad B' = \frac{\text{Dose} \cdot (k_{21}' - \beta')}{V_{d1}' \cdot (\alpha' - \beta')} \quad (10)$$

$$\begin{aligned} \alpha' + \beta' &= k_{21}' + k_{12}' + k_m \\ \alpha' \cdot \beta' &= k_{21}' \cdot k_m \end{aligned} \quad (11)$$

Separate fitting of the S4 data after an i.v. dose of S4 and the M1 data after an i.v. dose of M1 revealed that  $\beta$  and  $\beta'$  values were very similar. Thus, the plasma concentrations of M1 after an i.v. dose of S4 were described by a simplified equation:

$$Cp = C \cdot (e^{-\beta \cdot t} - e^{-\beta' \cdot t}) + D \cdot e^{-\alpha \cdot t} - E \cdot e^{-\alpha' \cdot t} \quad (12)$$

with  $C$ ,  $D$ , and  $E$  defined as:

$$\begin{aligned} C &= \frac{\text{Dose} \cdot k_{12}' \cdot k_{21}' \cdot k_h}{V_{d1}' \cdot (\beta' - \beta) \cdot (\alpha - \beta) \cdot (\alpha' - \beta)}; \\ D &= \frac{\text{Dose} \cdot k_{12}' \cdot k_{21}' \cdot k_h}{V_{d1}' \cdot (\alpha' - \alpha) \cdot (\alpha - \beta) \cdot (\alpha' - \beta)}; \\ E &= \frac{\text{Dose} \cdot k_{12}' \cdot k_{21}' \cdot k_h}{V_{d1}' \cdot (\alpha' - \alpha) \cdot (\alpha' - \beta) \cdot (\alpha' - \beta')} \end{aligned} \quad (13)$$

$$\text{AUC}_{M1} = C \cdot \left( \frac{1}{\beta} - \frac{1}{\beta'} \right) + \frac{D}{\alpha} - \frac{E}{\alpha'} \quad (14)$$

Equations 7, 10, and 12 were used to fit the three sets of data from each dog (i.e., both S4 and M1 concentration after an i.v. dose of S4, and M1 concentration after an i.v. dose of M1) simultaneously. The fraction of S4 that was deacetylated to M1 ( $f_m$ ) was determined using eq. 15.

$$f_m = \frac{\text{AUC}_{M1} \cdot \text{Dose}}{\text{CL}_{M1}} \quad (15)$$

### Conversion of M1 Back to S4 by NAT

The conversion of M1 to S4 by NAT was first examined using liver enzyme preparations (Fig. 3). When M1 (2  $\mu$ M) was incubated with different liver enzyme preparations in the presence of acetyl-CoA (Fig. 3A), M1 to S4 conversion was observed in human liver cytosol, human liver S9 fraction, and rat liver S9 fraction, but not in HLMs or dog liver S9 fraction. Over the 2-h incubation period, the rate of M1 acetylation was the highest in rat liver S9 fraction, with the percentage of M1 converted to S4 being approximately 3-fold and 20-fold higher than that observed in human and dog liver S9 fractions, respectively.

### In Vitro Characterization of the Species Difference in NAT Expression

The opposing reactions of S4 deacetylation and M1 acetylation (Fig. 1) suggested that reversible metabolic transformation might occur in some species. To test the reversibility of

the reaction in different species, S4 was incubated with different liver enzyme preparations in the presence of NADPH, with or without acetyl-CoA, a necessary cofactor for NAT (Fig. 3B).

Without acetyl-CoA, S4 (2  $\mu$ M) was deacetylated to M1 by HLMs, human liver cytosol, human liver S9 fraction, rat liver S9 fraction, and dog liver S9 fraction in the presence of NADPH. There were no significant species differences in S4 deacetylation. M1 was formed in rat, dog, and human S9 liver preparations, although the rate of S4 deacetylation in rats seemed to be significantly slower than that observed in dogs and humans. Since a similar amount of total protein (2 mg/ml) was included in these reactions, the higher deacetylation activity observed in human and dog liver preparations suggested higher P450 enzyme activities in these species. However, other cytosolic enzymes might also catalyze the deacetylation of S4, since M1 formation was also observed in reactions with human liver cytosol preparations (Fig. 3A).

When S4 was incubated with the same enzyme preparations in the presence of both NADPH and acetyl-CoA, no significant change in the extent of S4 deacetylation was observed with HLMs, human liver cytosol and S9 fraction, and dog liver S9 fraction, which could be caused by the relatively lower level of NAT activity. In contrast, S4 deacetylation was completely abolished in reactions containing rat liver S9 fraction. The differences observed in this *in vitro* experiment suggested that species differences in S4 metabolism exist *in vivo*.

Comparing the results in Fig. 3, A and B, it is apparent that both rat liver S9 fraction and human liver cytosol showed greater NAT activity (Fig. 3A), but lower deacetylation activity (Fig. 3B) compared with human liver S9 fraction. In the presence of both NADPH and acetylCoA, the rate of S4 deacetylation was decreased to a greater extent with rat liver S9 fraction and human liver cytosol compared with that observed with human liver S9 fraction, which suggested that the relative content and activity level of P450 enzymes and NAT might be directly related to the metabolism profile of S4 *in vivo*. Furthermore, the strong NAT activity observed in rat liver S9 fraction suggested that M1 could be rapidly converted back to S4 in rat and that M1 formation might not be observed *in vivo*.

Genetic polymorphism in NAT in humans is well documented, especially human NAT2 (Gross et al., 1999; Meisel, 2002). The possible interaction between M1 and human NAT was characterized using recombinant human NAT1 and NAT2 enzymes (Fig. 4). M1 could be acetylated by both NAT1 and NAT2, and NAT2 showed higher affinity for M1 than did NAT1. However, due to the limited solubility of M1 in the reaction buffer used for NAT enzyme reaction, the  $V_{\max}$  was not reached in these experiments.

### In Vivo Characterization of the Species Differences in S4 Metabolism

The pharmacokinetic profile of S4 in rats was determined in our previous study (Kearbey et al., 2004). *In vitro* studies demonstrated that deacetylation to an amine metabolite (M1) represented one of the primary metabolic conversions of S4. After the identification of the species difference in S4 metabolism, the pharmacokinetic profiles of S4 and the deacetylated metabolite M1 in rat ( $n = 5$ ) and dog ( $n = 3$ ) were determined as well. In rats, after an i.v. bolus dose (10 mg/kg) of S4, only S4, and no M1, was detected in the plasma (Fig. 5A). After an i.v. dose (10 mg/kg) of M1, both M1 and S4 were observed in the plasma (Fig. 5C). Opposite results were observed in dogs. After an i.v. dose of S4 (10 mg/kg), both S4 and M1 were detected in the plasma (Fig. 5B). However, after an i.v. dose of M1 (10 mg/kg), no S4 was detected in the plasma of dogs (Fig. 5D).

Since the pharmacokinetics of S4 and M1 in rats was studied in separate groups of animals, the data were computer-fitted separately. Plasma concentration-time profiles of S4 (Fig. 5A) after an i.v. dose of S4 were computer-fitted using a one-compartment model. Pharmacokinetic parameter estimates are summarized in Table 1. The volume of distribution for S4 in rats was



112 ml, and elimination rate constant  $k_{10}$  was  $0.0044 \text{ min}^{-1}$  (Table 1). Plasma concentration-time profiles of M1 and S4 after an i.v. dose of M1 were computer-fitted using the model shown in Fig. 2A. Since no M1 was detected in rats after an i.v. dose of S4, we assume that the acetylation rate in vivo was much higher than the deacetylation rate. Therefore, only the acetylation of M1 ( $k_a$ ) was considered in the proposed model (Fig. 2A). The M1 and S4 PK data obtained after an i.v. dose of M1 were fitted simultaneously using the proposed model. S4 and M1 data from each animal were fitted individually, and the mean and standard deviation of each estimated parameter are listed in Table 1. The elimination rate constant of M1 ( $k_{M1}$ ) and the acetylation rate constant ( $k_a$ ) were  $0.017 \text{ min}^{-1}$  and  $0.073 \text{ min}^{-1}$ , respectively. After the i.v. dose of M1, 81% of the M1 was converted to S4. The acetylation occurred so rapidly that M1 disappeared from the systemic circulation within 1 h after dosing.

The plasma concentration-time profiles of both S4 and M1 after an i.v. dose in dog showed two exponential phases (Fig. 5, B and D). When S4 and M1 data were fitted separately using the two-compartment model, the results suggested that the volume of the central compartment was approximately 400 to 600 ml (similar to the results shown in Table 2), which is close to the blood volume of dog (Davies and Morris, 1993). Since the deacetylation occurs in the hepatocytes, we consider the liver as part of the peripheral compartment, and deacetylation ( $k_h$ ) only happens in the peripheral compartment (Fig. 2B).

Plasma concentration-time profiles of S4 and M1 in dog after an i.v. dose of either S4 or M1 were computer-fitted simultaneously using the model shown in Fig. 2B. The fitting results showed that after an i.v. dose of S4 in dogs, 53% of S4 was metabolized through the deacetylation pathway. Representative fitting results are shown in Fig. 6. Although the clearance of M1 was more than 2 times higher than that of S4, both S4 and M1 had a very similar terminal half-life of about 2 h. The elimination rate constant of M1 ( $k_m$ ) was more than 20 times the deacetylation rate constant ( $k_h$ ), showing that deacetylation of S4 is the rate-limiting process.

## Discussion

The major in vitro metabolism pathway of S4 in rats, dogs, and humans was identified as the deacetylation of the acetamide group (Fig. 1). Among the cytochrome P450 enzymes tested, CYP3A4 was one of the P450 enzymes responsible for the deacetylation reaction observed in vitro. Kinetics studies showed that S4 has an affinity ( $K_m$ ) to CYP3A4 similar to that of testosterone. However, the deacetylation reaction observed with the phase I enzymes could be reversed by NAT in vitro and in vivo. Species differences in NAT expression also introduced species differences into S4 metabolism and pharmacokinetics in rats, dogs, and humans.

The deacetylated product, M1, was converted back to S4 in vitro by rat and human liver enzyme preparations that contained NAT activity. However, such conversion was not observed in the dog liver enzyme preparation, due to the absence of NAT expression. Similar results were observed during in vivo experiments. In rats, the acetylation reaction was so rapid and efficient that M1 was not detected in plasma samples after an i.v. dose of S4. Furthermore, 81% of M1 was converted back to S4 after an i.v. dose of M1. In comparison, due to the lack of NAT activity in dog, approximately 53% of S4 was metabolized by deacetylation, but S4 was not observed after an i.v. dose of M1. Therefore, the pharmacokinetic profiles of S4 and M1 in dogs were qualitatively and quantitatively different from those in rats.

Furthermore, although both rats and humans express NAT, the relative expression levels and enzyme activities of P450 enzymes and NAT could also affect the in vivo metabolism of S4 and result in interspecies differences. As observed during in vitro experiments (Fig. 3B), the strong NAT activity in rat liver S9 fraction completely reversed the deacetylation reaction of

S4, which might explain the fact that no M1 was detected in rat plasma samples after i.v. doses of S4. In human liver enzyme preparations, the NAT activity was not strong enough to completely reverse the deacetylation process. Thus, M1 is very likely to be observed in humans after administration of S4. Detailed analysis of clinical data may determine whether the NAT polymorphisms (Gross et al., 1999; Meisel, 2002) will have any effects on S4 pharmacokinetics and metabolism in humans.

Our previous in vitro experiment showed that M1 binds to androgen receptor and can initiate transcription activation, suggesting that it might be an active metabolite in vivo. However, M1 was rapidly converted back to S4 in rats (Fig. 5C), with an extremely short half-life of 8 min (Table 1), which excluded the possibility that M1 might contribute to the pharmacologic effects of S4 observed in male rats (Yin et al., 2003a). In comparison, the in vivo activity of M1 in humans will be more difficult to predict, considering the large variations observed in the expression level and the genetic polymorphism of NAT in humans.

Conversely, since NAT can reverse the deacetylation process in rats and humans, other metabolic pathways of S4, such as hydrolysis, reduction, and oxidation, might actually contribute more to S4 metabolism in vivo. For instance, since the deacetylation process was reversed by NAT in rats, S4 is mainly metabolized through hydrolysis, reduction, and oxidation (Gao et al., 2006), as predicted from the in vitro data reported previously.

Interspecies scaling is often used to estimate the appropriate dosage for humans based on the pharmacokinetic profile of the drug in animals. For S4, however, the species differences in drug metabolism and disposition of S4 could greatly affect the accuracy of the approach. The relatively higher P450 enzyme activity in human liver preparation, the absence of NAT in dog, and known genetic polymorphisms in human NAT suggest that interspecies scaling of S4 might be confounded by numerous issues. Therefore, in vitro-in vivo correlation might be a better approach to predict the in vivo pharmacokinetics and metabolism of S4 in humans.

In summary, both in vitro and in vivo data showed that S4 was metabolized by the deacetylation of the acetamide group and that species differences exist for the metabolism of S4. However, the deacetylated metabolite, M1, does not seem to contribute to the in vivo pharmacologic activity of S4 in rats, which excluded the possibility that previously observed tissue selectivity of S4 in rat was mediated by active metabolites. Also, similar species differences in metabolism will not be observed with other structural derivatives that do not contain the acetamide group.

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#### ABBREVIATIONS

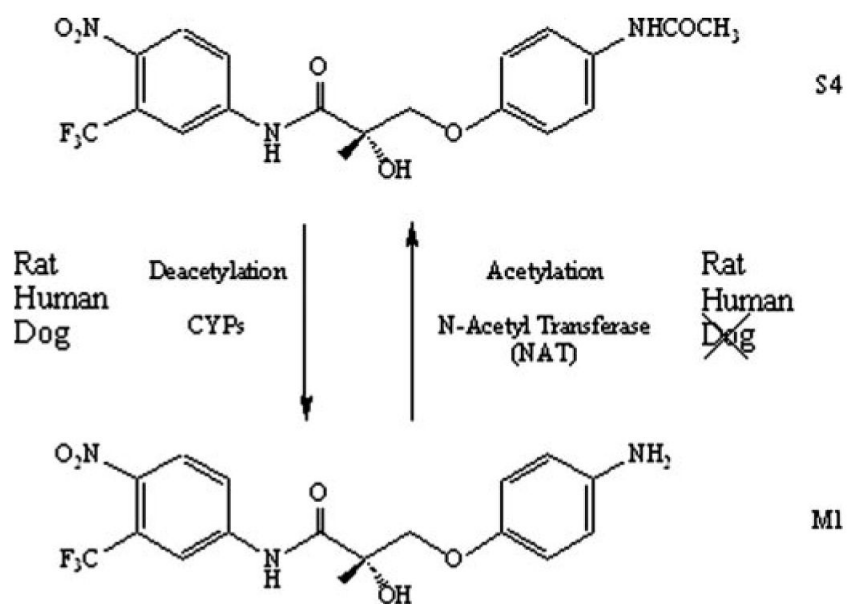
NAT, *N*-acetyltransferase; S4, *S*-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide; S1, *S*-3-(4-fluoro-phenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-propionamide; CM-II-87, 2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethyl-phenyl)-butyramide; SARM, selective androgen receptor modulator; PK, pharmacokinetic; HPLC, high performance liquid chromatography; HLM, human liver microsome; P450, cytochrome P450.

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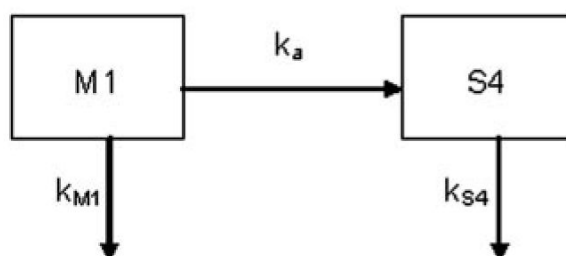


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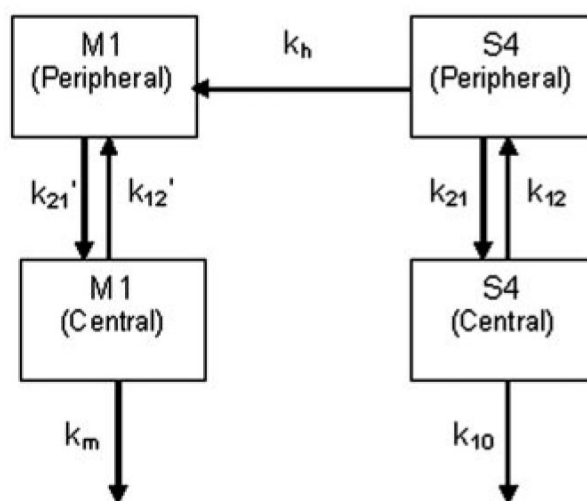
**Fig 1.**

Species differences in S4 metabolism are related to the species differences in NAT expression. Deacetylation product M1 can be converted back to S4 by NAT, but similar conversion does not happen in dog because NAT is not expressed in dog.

**A.** Compartment model for M1 pharmacokinetics in rat after an i.v. dose of M1.

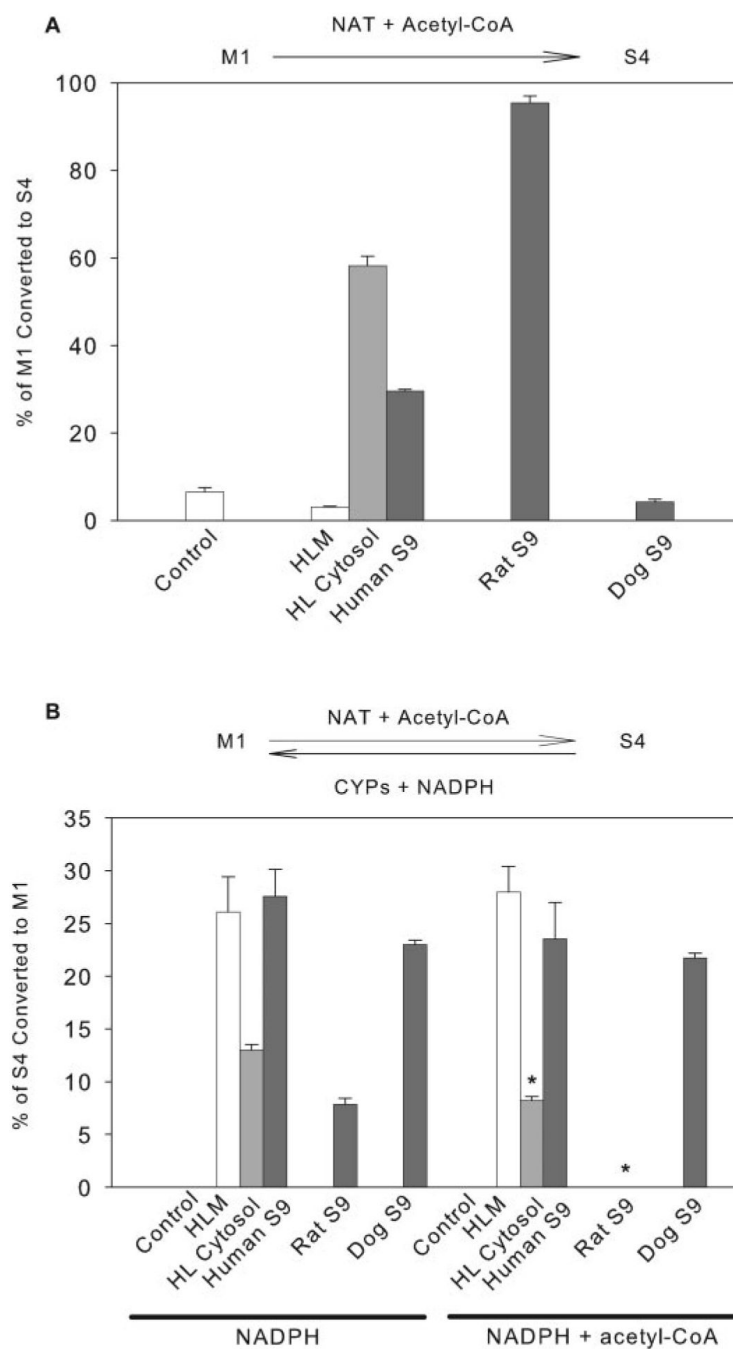


**B.** Compartment model for S4 pharmacokinetics in dog

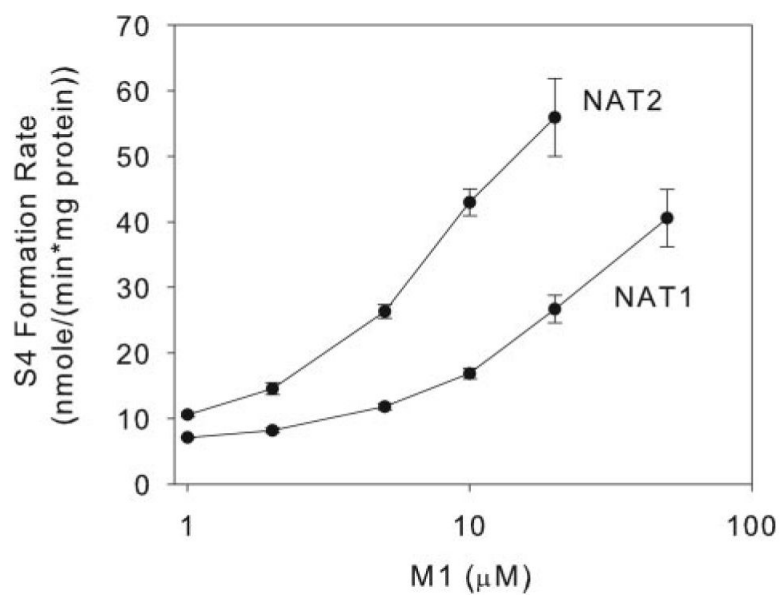


**Fig 2.**

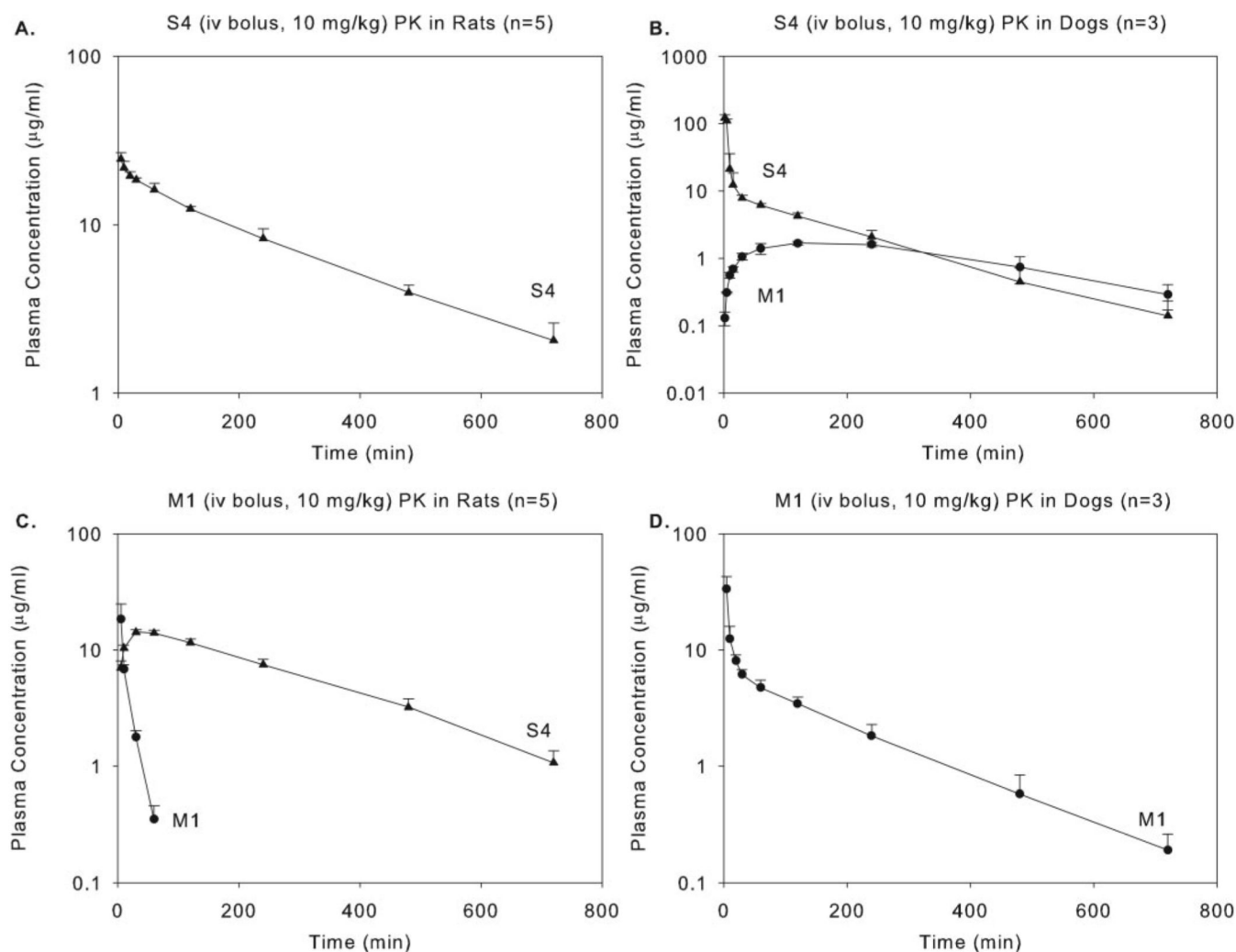
Compartment models proposed for simultaneous fitting of S4 and M1 PK profile. A, M1 pharmacokinetics in rat.  $k_a$  is the rate constant for M1 acetylation in rat. B, S4 pharmacokinetics in dog.  $k_h$  is the rate constant for S4 deacetylation in dog.

**Fig 3.**

In vitro conversion of M1 (2  $\mu$ M) to S4 in the presence of acetyl-CoA (A) and NADPH (B) with different enzyme preparations ( $n = 3$ ). The appearance of S4 was measured after 2 h of incubation, and the result is presented as mean  $\pm$  S.D. \*,  $p < 0.05$ , compared with corresponding reaction performed without acetyl-CoA.

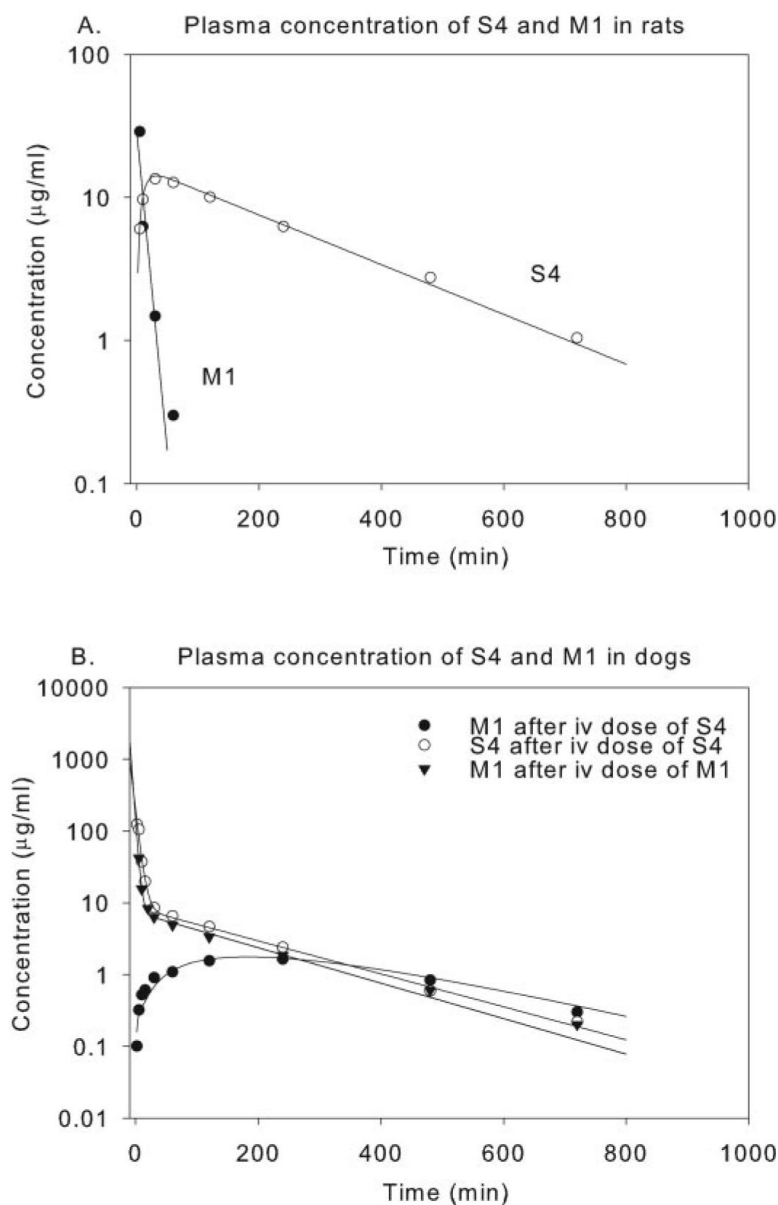
**Fig 4.**

In vitro conversion of M1 to S4 by recombinant human NAT1 and NAT2 ( $n = 3$ ). Data are presented as mean  $\pm$  S.D. Various concentrations of M1 were incubated with recombinant human NAT1 and NAT2 (0.25 mg/ml total protein) at 37°C for 5 min. The appearance of S4 was measured by HPLC analysis.

**Fig 5.**

Pharmacokinetic profiles of S4 and M1 in rat and dog. Data are presented as mean  $\pm$  S.D. A and C, S4 and M1 pharmacokinetic profiles in rats. M1 was not observed in rats after an i.v. dose of S4, but S4 was observed in rats after an i.v. dose of M1. B and D, S4 and M1 pharmacokinetic profiles in dogs. M1 was observed in dogs after an i.v. dose of S4, but S4 was not observed in dogs after an i.v. dose of M1.





**Fig 6.**  
Representative fitting results of S4 and M1 concentration-time profiles in rat (A) and dog (B).

**TABLE 1**  
Pharmacokinetic parameters of S4 and M1 in rats (n = 5)

| Parameter      | Unit                                     | Mean $\pm$ S.D.     |
|----------------|--|---------------------|
| S4 i.v.        |  |                     |
| $V_{dS4}$      | ml                                       | 112 $\pm$ 6         |
| $k_{S4}$       | min <sup>-1</sup>                        | 0.0044 $\pm$ 0.0008 |
| $t_{1/2}$      | min                                      | 160 $\pm$ 30        |
| $Cl_{S4}$      | ml/min                                   | 0.49 $\pm$ 0.07     |
| $AUC_{S4}$     | $\mu\text{g} \cdot \text{min}/\text{ml}$ | 5144 $\pm$ 738      |
| M1 iv          |  |                     |
| $V_{dM1}$      | ml                                       | 115 $\pm$ 20        |
| $k_a$          | min <sup>-1</sup>                        | 0.0734 $\pm$ 0.0071 |
| $k_{M1}$       | min <sup>-1</sup>                        | 0.0172 $\pm$ 0.0069 |
| $t_{1/2}$ (M1) | min                                      | 8 $\pm$ 1           |
| $Cl_{M1}$      | ml/min                                   | 10.32 $\pm$ 1.82    |
| $AUC_{M1}$     | $\mu\text{g} \cdot \text{min}/\text{ml}$ | 248 $\pm$ 38        |
| $AUC_{S4}$     | $\mu\text{g} \cdot \text{min}/\text{ml}$ | 4099 $\pm$ 275      |
| $f_m$          |  | 0.81 $\pm$ 0.05     |

**TABLE 2**  
Pharmacokinetic parameters of S4 and M1 in dogs (n = 3)

| Parameter  | Unit                                     | Mean $\pm$ S.D.     |
|------------|--|---------------------|
| S4         |  |                     |
| $V_{d1}$   | ml                                       | 423 $\pm$ 49        |
| $k_{10}$   | min <sup>-1</sup>                        | 0.046 $\pm$ 0.017   |
| $t_{1/2}$  | min                                      | 125 $\pm$ 25        |
| $Cl_{S4}$  | ml/min                                   | 41.3 $\pm$ 8.0      |
| $AUC_{S4}$ | $\mu\text{g} \cdot \text{min}/\text{ml}$ | 2227 $\pm$ 377      |
| $k_h$      | min <sup>-1</sup>                        | 0.0039 $\pm$ 0.0009 |
| M1         |  |                     |
| $V_{d1}'$  | ml                                       | 644 $\pm$ 171       |
| $k_m$      | min <sup>-1</sup>                        | 0.090 $\pm$ 0.035   |
| $t_{1/2}$  | min                                      | 118 $\pm$ 25        |
| $Cl_{M1}$  | ml/min                                   | 54.5 $\pm$ 8.3      |
| $AUC_{M1}$ | $\mu\text{g} \cdot \text{min}/\text{ml}$ | 894 $\pm$ 205       |
| $f_m$      |  | 0.53 $\pm$ 0.08     |