IN VITRO AND IN VIVO EFFECTS OF THE ARYLAMINE HUMAN IMMUNODEFICIENCY VIRUS PROTEASE INHIBITOR 4R-(4α,5α,6β,7β)-1-[(3-(1-
imidazoylcarbamoyl)phenyl)methyl]-3-[(3-
aminophenyl)methyl]hexahydro-5,6-dihydroxy-4,7-bis(phenylmethyl)-2H-1,3-diazepin-2-one (SD894) ON RAT HEPATIC CYTOCHROME P450 2B AND 3A

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ABSTRACT:

The human immunodeficiency virus-1 protease inhibitor SD894 was evaluated as an inhibitor and inducer of cytochromes P450 (CYPs) in rats. After addition of 10 μM SD894 and 2 mM NADPH to liver microsomes from dexamethasone-treated rats, a type II spectrum appeared. Within 2 min, it was replaced by a type III spectrum, with absorbance maxima at 426 and 456 nm, similar to those observed with alkylamines (SKF-525A) and arylamines (p-chloroaniline). Preincubation of microsomes from dexamethasone-treated rats with SD894 and NADPH resulted in a time-dependent inhibition of testosterone 6β-hydroxylation (CYP 3A1/2 activity), which was decreased to 25% of controls after 30 min. Testosterone 6β-hydroxylation activity in microsomes from TAO-treated rats was greater than controls. Preincubation of these microsomes with potassium ferricyanide produced an additional 50% increase, consistent with disruption of a metabolite-CYP complex. Microsomes from SD894-treated rats displayed a 3-fold increase in testosterone 16β-hydroxylation. Potassium ferricyanide preincubation did not increase activity. Thus, although SD894 appears to inhibit CYP in vitro in a manner typical of other amine-containing, mechanism-based inhibitors, in vivo induction by 10 mg/kg daily doses of SD894 affects a different isozyme than does inhibition. The mechanism of induction is unknown.

SD894 [4R-(4α,5α,6β,7β)-1-[(3-(1-imidazoylcarbamoyl)phenyl)methyl]-3-[(3-aminophenyl)methyl]hexahydro-5,6-dihydroxy-4,7-bis(phenylmethyl)-2H-1,3-diazepin-2-one (fig. 1) is one of a series of potent human immunodeficiency virus protease inhibitors based on a cyclic urea core (1). SD894 features both arylamine and imidazole substituents, which may interact with the active site of CYP.1 Imidazole-containing compounds, including ketoconazole, miconazole, and clotrimazole, are potent inhibitors of CYP in vitro and in vivo (2–4). Their inhibition is short-lived and reversible and is due to binding of the free electron pair of nitrogen as the sixth heme ligand of the ferric form of CYP, as well as lipophilic binding to the apoprotein (2, 5–7). In contrast, TAO and orphenadrine are mechanism-based inhibitors of CYP, whereby inhibition is dependent on oxidation of an alkylamine moiety to an active nitrosoamine, which binds in a quasi-reversible fashion to ferrous CYP (8–11). Although less well characterized, arylamine-containing compounds such as p-chloroaniline also are activated to inhibitory species by CYP (12–14).

In general, compounds that are activated to species that bind quasi-irreversibly to CYP have dual effects on overall drug metabolism. Acutely, and in vitro, potent inhibition is the dominant effect; whereas, with chronic administration induction dominates (15). Because SD894 may be administered in combination with other therapies for treatment of acquired immunodeficiency syndrome, it is important to address the possibility of potential drug interactions. The focus of these studies was to determine the potential for SD894 or a metabolite to both inhibit and induce hepatic CYP. A secondary goal was to identify and characterize in vitro and in vivo the formation of an inhibitory complex between SD894 or a metabolite and CYP in rats.

Materials and Methods

Materials. SD894 was synthesized and characterized by The DuPont Merck Pharmaceutical Company (Wilmington, DE). Testosterone and 16β-, 6β-, and 11β-hydroxytestosterone were purchased from Steraloids (Wilton, NH). 4-MA was a gift of Merck Research Laboratories (Rahway, NJ). TAO, potassium ferricyanide, and common biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO). SKF-525A was purchased from Biomol Research Lab, Inc. (Plymouth Meeting, PA). All general solvents and chemicals were the highest grade available commercially. Antibodies to CYP 2B1 and 3A1/2 were bought from Human Biologics, Inc. (Phoenix, AZ).

Animal Treatment and Microsome Preparation. To selectively induce CYP 3A1, male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) weighing between 250 and 500 g were given ip doses of dexamethasone (150 mg/kg/day), po doses of PCN (150 mg/kg/day) for 4 days, or ip doses of TAO (50 mg/kg/day) for 7 days. For induction of CYP 2B1, animals were administered four daily ip doses of phenobarbital (100 mg/kg/day). Additionally, another group of rats were administered seven daily iv doses of 10 mg/kg

1 Abbreviations used are: CYP, cytochrome P450; TAO, troleandomycin; 4-MA, 17β-N,N-diethylcarbamoyl-4-methylaza-5α-androstan-3-one; PCN, pregnenolone 16α-carbonitrile; MI, metabolite intermediate.

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SD894 in 80% polyethylene glycol 400 in 0.01 N HCl; control animals received vehicle alone. Starting after the first dose, food and water were given ad libitum. The animals were killed by decapitation and the livers were removed and weighed 24 hr after the final dose; livers were also harvested from animals given a single dose of either SD894 or TAO. Microsomes were prepared by standard methods from individual livers immediately after removal (16). Microsomal protein was measured according to the method of Lowry et al. (17) and CYP content by the method of Omura and Sato (18).

**Regioselective Testosterone Oxidation.** Testosterone oxidation to 6β- and 16β-hydroxy metabolites was measured using a modification of a standard method (20). Briefly, microsomes (0.3–0.5 mg/ml) were mixed with 0.1 M phosphate buffer (pH 7.4) containing 200 μM testosterone, 1 mM EDTA, 3 mM MgCl₂, and 1 μM 4-MA. NADPH (1 mM) was added to start the incubation. The reaction was terminated 10 min later by the addition of 6 volumes of methylene chloride. At this time, the internal standard 11β-hydroxytestosterone (20 μM) was added to each sample. The samples were vortex-mixed for 5 min and centrifuged at 2000g for 5 min to separate the phases; the top phase was discarded. The remaining organic phase was dried under nitrogen, and the residue was reconstituted in 0.2─0.3 ml of mobile phase. Aliquots (50–100 μl) of the samples were analyzed using a HPLC system composed of a Supelcosil C₁₈ column (4 × 250 mm, 5 μm), a C₁₈ guard column (Supelco, Bellefonte, PA), and a UV detector (Waters, Milford, MA) monitoring at 254 nm. Mobile phase was pumped at 1.5 ml/min. The guard column (Supelco, Bellefonte, PA), and a UV detector (Waters, Milford, MA) monitoring at 254 nm. Mobile phase was pumped at 1.5 ml/min. The initial conditions of 44% methanol/54% water were maintained isocratically for 1 min; a concave gradient increased the methanol composition to a maximum of 74% at 38 min. For 1 min; a concave gradient increased the methanol composition to a maximum of 74% at 38 min. 6β-hydroxytestosterone at 25 min, and 11β-hydroxytestosterone at 32 min. Metabolite concentrations were determined by comparison of peak area ratios with a standard curve. All samples were assayed in triplicate.

**Spectral Binding.** UV difference spectra were obtained by mixing microsomes from dexamethasone- or phenobarbital-treated rats (1 nmol/ml CYP) with 10 μM SD894 in Tris-HCl buffer (50 mM, pH 7.4), containing 150 mM KCl and 10 mM MgCl₂, at 25°C. Complex formation was initiated by addition of NADPH (2 mM) to the sample cuvette. The absorbance between 400 and 500 nm was recorded every other minute for 4 min, using a Perkin Elmer model Lambda 18 dual-wavelength/double-beam UV spectrophotometer (Norwalk, CT). Potassium ferricyanide (50 μM) was added to both reference and sample cuvettes and, after a 20-min incubation at 37°C, the spectrum was again recorded. Approximately 3 mg of sodium dithionite was added to the cuvettes before the last spectrum was recorded.

**Time-Dependent CYP Inhibition.** Microsomes (0.3–0.5 mg/ml) from dexamethasone-treated rats were incubated in 0.1 M potassium phosphate buffer, containing 1 mM EDTA, 3 mM MgCl₂, and 1 μM 4-MA, with 10 μM SD894 in dimethylsulfoxide, or with dimethylsulfoxide alone, and with or without 1 mM NADPH for up to 30 min at 37°C. Testosterone (200 μM) was then added in 20 μl of ethanol (this volume of ethanol was not inhibitory) to initiate the reaction; NADPH (1 mM) was added to all samples at this time. After a 10-min incubation, samples were extracted and analyzed as described above.

**Reisolation of Microsomes after Incubation with SD894.** SD894 (10 μM) was added to pooled rat hepatic microsomes (10 mg/5 ml) from PCN-treated rats, in 0.1 M potassium phosphate buffer (pH 7.4). The reaction was started by the addition of NADPH (2 mM) to one set of samples while an equal volume of buffer was added to the other. All samples were then incubated for 30 min at 37°C and transferred to centrifuge bottles containing ice-cold homogenization buffer (20 ml). Identical incubations were conducted with either SKF 525A (100 μM) or vehicle. Microsomes were then reisolated by ultracentrifugation according to standard procedures (16). Total protein concentrations were determined according to the method of Lowry et al. (17).

**Evaluation of In Vivo Effects of SD894.** To determine the effect of in vivo CYP complexation on isozyme activity, microsomes (2.5 nmol CYP/ml) from SD894-, TAO-, or vehicle-treated rats were incubated in triplicate with 100 μM potassium ferricyanide in 0.1 M potassium phosphate buffer (pH 7.4) at room temperature for 10 min. Control samples were preincubated with buffer alone. Aliquots containing 0.25 nmol of CYP were then assayed in triplicate for testosterone oxidation activity using the method outlined above.

**Statistics.** All values are means ± SD for experiments where replicates are mentioned. Significance was evaluated by two-tailed Students’ t tests, with p < 0.05.

**Results**

**Spectral Binding.** Incubation of SD894 with microsomes from dexamethasone-treated rats in the presence of NADPH resulted in the formation of a classic type III binding spectrum, with maxima at 426 and 456 nm (fig. 2). Oxidation with potassium ferricyanide eliminated both peaks; re-reduction with sodium dithionite did not restore the characteristic type III peaks. Microsomes from phenobarbital-treated rats incubated with SD894 and NADPH also formed a type III binding spectrum, albeit to a lesser extent.

**Time-Dependent Inhibition.** Preincubation of microsomes with SD894 under oxidative conditions (i.e. with NADPH) for up to 30 min resulted in a time-dependent reduction in the 6β-hydroxytestosterone formation rate, demonstrating inhibition by an oxidative metabolite that may be bound to CYP (fig. 3A). The rate of 16β-hydroxytestosterone formation, catalyzed by CYP 2B1, was not significantly affected by preincubation with SD894 (fig. 3B).

**Persistence of Microsomal Inhibition by SD894 In Vitro.** Microsomes from PCN-treated rats, preincubated with SD894 and NADPH and then washed and reisolated by ultracentrifugation, formed 6β-hydroxytestosterone at 25% the rate of microsomes preincubated with vehicle and NADPH (fig. 4). This inhibition was due to a metabolite-CYP complex and not residual SD894, because microsomes preincubated with SD894 but without NADPH formed 6β-hydroxytestosterone at the same rate as microsomes preincubated with vehicle alone. Also, SD894 was not detected by HPLC in extracts of microsomes.
reisolated after incubation with SD894 in the presence or absence of NADPH. The limit of quantitation for this analysis was 1 μM. The quasi-irreversible inhibitor SKF525A decreased CYP 3A activity to 42% of control (fig. 4). These reisolation experiments were also conducted with microsomes from phenobarbital-treated rats, to confirm the isozyme selectivity of inhibition resulting from formation of the SD894 metabolite-CYP complex. 16β-Hydroxytestosterone formation, reflecting CYP 2B activity, was not affected, whereas 6β-hydroxytestosterone formation was significantly decreased, similar to the microsomes from PCN-treated rats (fig. 5).

CYP Induction In Vivo. Immunoblots of liver microsomes harvested from rats dosed with 10 mg/kg SD894 iv, developed with antibodies to CYP 2B1 and 3A1/2, demonstrated increased levels of CYP 2B, compared with microsomes from vehicle-treated animals (fig. 6). Microsomes from rats treated with 10 mg/kg SD894 daily for 7 days formed 16β-hydroxytestosterone at approximately twice the rate of microsomes from vehicle-treated animals (fig. 7). Microsomes from SD894- and vehicle-treated rats formed 6β-hydroxytestosterone at identical rates. The increased activity of CYP 3A in TAO microsomes, measured as a 1.5-fold
increase in the rate of formation of 6β-hydroxytestosterone was further augmented by a 10-min preincubation with potassium ferricyanide. No additional CYP 2B activity was produced in SD894 microsomes by preincubation with potassium ferricyanide.

Discussion

This study has demonstrated that arylamine SD894 both inhibits and induces CYP in rats. This dual response, acute inhibition and chronic induction, is not unusual for compounds containing primary amines. Although orphenadrine maintains significant inhibition of CYP even after 1 week of dosing (8), SKF-525A and TAO, given as multiple doses, produce induction of CYP in rats after an initial inhibitory period of 24 hr or less (21–30). Both effects of SKF-525A and TAO are related to the formation of inactive CYP-MI complexes that produce a quasi-reversible inhibition of drug metabolism that may stimulate the production of new CYP (31). These complexes can be observed as type III difference spectra, with Soret peaks of 445–460 nm (10, 32). MI complexes exhibiting these spectral characteristics develop in microsomes incubated with aliphatic or aromatic amines under oxidative conditions (in the presence of NADPH) and result from coordinate binding between the oxidized amine species, probably a nitrosoamine (28, 29), and ferrous CYP. Additional stability of the complex is due to hydrophobic binding of the drug molecule in the active site pocket.

Inhibitory complexes of arylamines and CYP have been observed and characterized (12–14). SD894 contains a primary aniline substituent, linked to the rest of the molecule at the meta-position. Primary amines with meta-substituents undergo N-oxidation catalyzed preferably by CYP, rather than flavin-containing monooxygenase, and thus there is a greater likelihood of forming a MI-CYP complex (30). Unlike complexes involving aliphatic nitrosoamines, arylnitrosoamine complexes are unstable to sodium dithionite; addition of the reducing agent to a microsomal/arylamine incubation mixture results in complete elimination of the 455-nm absorbance (12, 32). This instability was observed with the SD894 MI complex formed in vitro.

Complex formation of SD894 with phenobarbitral-induced microsomes was not observed. It therefore seems that the putative SD894 nitroso metabolite suspected to quasi-reversibly coordinate with heme is formed by CYP 3A. According to Bensoussan et al. (33), the CYP 3A family has a greater propensity to form complexes with amines.

Formation of an inhibitory complex with SD894 was rapid, achieving a maximum at 10 min. A 30-min preincubation with SD894 decreased CYP 3A activity to 25% of control levels. Moreover, microsomes that had been preincubated for 30 min with SD894 and NADPH and then washed and resolubilized exhibited a rate of testosterone 6β-oxidation that was 30% of the vehicle control. If the inhibition observed in the preincubation experiments had been due solely to a reversible metabolite-CYP complex, resuspension of microsomes incubated with SD894 (and NADPH) might be expected to dilute and thereby diminish this inhibition. However, only a slight and insignificant difference existed in the extent of inhibition with or without a resuspension step, suggesting very tight binding.

Direct binding to the CYP heme by the unoxidized aniline nitrogen atoms is also possible (12, 30, 34). This interaction generally results in a type II spectrum and reversible inhibition of CYP activity. A type II spectrum was observed on addition of SD894 to microsomes from dexamethasone-treated rats. However, CYP 3A was inhibited only by the metabolically activated species. Although imidazoles represent a class of agents that form type II ligands with and inhibit CYP in a reversible manner, the imidazole ring of SD894 is linked to the rest of the molecule at the 2-position, a substitution that sterically hinders the nitrogen free pair of electrons and thus decreases the potential for CYP binding (2, 3).

SD894 induction of microsomal CYP 2B was determined by immunoblotting and measurement of testosterone oxidase activity. No spectral complex was observed with these microsomes or with microsomes obtained 24 hr after a single 10 mg/kg dose. In contrast, TAO administration results in a detectable type III spectrum in microsomes prepared from livers harvested 24 hr after the last of four daily doses, although not 1 hr after one dose (25). Thus, detection of microsomal CYP-MI complexes may be time and compound dependent. Furthermore, microsomes from SD894-treated rats incubated with 100 μM potassium ferricyanide immediately before incubation with testosterone were not more active than microsomes preincubated with buffer. Potassium ferricyanide oxidizes complexed CYP to the ferric state, destabilizing nitroso-CYP complexes and thus freeing CYP (35). Microsomes from TAO-treated rats gained an additional 30% in testosterone 6β-hydroxylation activity after potassium ferricyanide treatment. Thus, it is likely that the slight induction of CYP 2B by SD894 is not directly due to complex formation. Although TAO is one of the more potent CYP 3A inducers, clotrimazole induces CYP 3A to the same extent and yet does not form a detectable complex (36). Many other inducers do not form detectable CYP complexes (27, 37, 38).

In conclusion, SD894 is similar to other arylamine-containing compounds in that it is transformed, via metabolic oxidation, to a species that combines in a quasi-reversible manner with CYP. This metabolic activation results in inhibition of CYP 3A-mediated metabolism in vitro. Induction of rat CYP 2B by multiple doses of SD894 appears to be independent of metabolite-CYP complexation.
References


