Altered Hemostasis in Male Rats Following Administration of the ACAT Inhibitor SKF-99085


Department of Safety Assessment, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

Received February 26, 1998; accepted July 6, 1998

SKF-99085, an acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, was evaluated in male and female Sprague-Dawley rats at oral doses of 0, 10, 100, or 400 mg/kg/day for 6 months as part of the preclinical safety assessment of this drug candidate. In male rats given 400 mg/kg/day SKF-99085, hemorrhage and death were observed in males during the first month of the study, prompting collection of blood samples at weeks 6, 17, and 24 to monitor coagulation parameters. A dose-related increase in activated partial thromboplastin time (APTT) and Thrombotest clotting time (TCT) was observed in all male drug-treated groups. Mean APTT values for male rats given 10, 100, or 400 mg/kg/day were increased maximally to 17.5, 20.8, and 34.7 s (control, 15.4-16.0 s), and mean TCT values were increased to 86, 100, and >300 s (control, 71-74 s), respectively. Mean prothrombin times (PT) for male rats given 400 mg/kg/day SKF-99085, hemorrhage and death were observed in males during the first month of the study, prompting collection of blood samples at weeks 6, 17, and 24 to monitor coagulation parameters. A dose-related increase in activated partial thromboplastin time (APTT) and Thrombotest clotting time (TCT) was observed in all male drug-treated groups. Mean APTT values for male rats given 10, 100, or 400 mg/kg/day were increased maximally to 17.5, 20.8, and 34.7 s (control, 15.4-16.0 s), and mean TCT values were increased to 86, 100, and >300 s (control, 71-74 s), respectively. Mean prothrombin times (PT) for male rats given 400 mg/kg/day SKF-99085 were increased to 16.5 s (control, 12.9-13.1 s). Activities of factors II, VII, IX, and X were decreased in males at dosages of 10, 100, or 400 mg/kg/day. Factor V and VIII activities were unaffected. In summary, the drug-related hemorrhagic disorder observed in male rats given high doses of the ACAT inhibitor SKF-99085 was attributed to a reduction in the activity of vitamin-K-dependent coagulation factors. In contrast to humans and some other species, the APTT and TCT were more sensitive than the PT in detecting this effect.

Key Words: ACAT inhibitor; hemostasis; vitamin-K-dependent coagulation factor activity; Thrombotest clotting time.

SKF-99085 [tetraisopropyl-2-(3,5-diterbutyl-4-hydroxyphenyl)ethyl-1,1-diphosphonate] is an orally active inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT), the enzyme responsible for the acylation of cholesterol to cholesteryl esters. It has been postulated that inhibition of this enzyme could prevent excess accumulation of cholesteryl esters, resulting in suppression of atheromatous plaque formation in the intima of arterial walls (Matsuda, 1994). SKF-99085 also exhibits calcium channel blocking activity and antioxidant properties that are considered useful characteristics of an antiatherosclerotic agent.

SKF-99085 was evaluated in rats in single dose and repeat dose toxicity studies of up to 6 months duration as part of the preclinical assessment of this drug candidate. In a 6-month toxicity study, 6 of 12 male rats given doses of 400 mg/kg/day SKF-99085 died or were killed in moribund condition during the first 3 months of the study. Hemorrhage was observed in various tissues at necropsy. The purpose of this study was to further characterize this drug-related hemorrhagic disorder.

MATERIALS AND METHODS

Animals. Male and female Sprague-Dawley virus-antibody-free rats (Charles River Laboratories, Raleigh, NC) were used in this study. The rats were approximately 11 weeks of age and weighed between 322 and 403 g (males) or 197 and 245 g (females) at the start of dosing. Rats were housed individually in stainless steel cages in a controlled environment (72 ± 4°F; 50 ± 10% relative humidity) with a 12-h light/dark cycle. Male and female rats were offered at least 21 or 16 g per day, respectively, of 5002 Certified Rodent Diet and 2.5 g SK 5L34 (PMI Feeds, Inc., St. Louis, MO). Food was withheld prior to blood collection at necropsy. Filtered tap water was available ad libitum. All animal care procedures were in accordance with the Guidelines for the Use and Care of Laboratory Animals, NIH Publication No. 86-23 (U.S. Department of Health and Human Services, Bethesda, MD).

SKF-99085. SKF-99085 was administered orally by gavage (10 ml/kg) as a suspension in 1% aqueous carboxymethylcellulose to male and female rats (12/sex/group) at dosages of 0 (vehicle), 10, 100, or 400 mg/kg/day for 26 weeks.

Plasma samples. Blood was collected for coagulation testing from control and drug-treated rats prior to the daily dose at weeks 6, 17, and 24 of the study and at necropsy (week 26). Rats were restrained in a heating apparatus for approximately 5 min to dilate the tail vein for blood collection. A 21-gauge, 3/4-in. butterfly needle was inserted into the lateral tail vein, and approximately 0.9 ml of blood was allowed to flow into tubes (Teklab Medical Laboratories Ltd., Durham, UK) containing trisodium citrate (0.13 M, 1 part anticoagulant to 9 parts blood). At necropsy, blood samples were collected from the vena cava into 10-ml plastic syringes, and 4.5 ml of blood was transferred immediately to citrated Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) and inverted gently. Blood samples were centrifuged at 4°C at 900# for approximately 15 min, and citrated plasma was removed and refrigerated for immediate testing or recentrifuged as indicated above and frozen at approximately −70°C until assayed.

Measurements. Prothrombin time (PT) and activated partial thromboplastin time (APTT) and coagulation factors II, V, VII, VIII, IX, and X were measured using the Electra 1000C Automatic Coagulation Timer (Medical
Effect of Oral Administration of SKF-99085 for 24 Weeks on Hemostasis in Rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>APTT (seconds) Males</th>
<th>APTT (seconds) Females</th>
<th>PT (seconds) Males</th>
<th>PT (seconds) Females</th>
<th>TCT (seconds) Males</th>
<th>TCT (seconds) Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.0 ± 0.1</td>
<td>14.9 ± 0.3</td>
<td>13.1 ± 0.1</td>
<td>12.3 ± 0.1</td>
<td>71 ± 2.5</td>
<td>57 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>17.5 ± 0.1b</td>
<td>15.8 ± 0.9</td>
<td>12.8 ± 0.1</td>
<td>12.3 ± 0.1</td>
<td>84 ± 3.0*</td>
<td>56 ± 1.3</td>
</tr>
<tr>
<td>100</td>
<td>20.8 ± 0.6b</td>
<td>14.9 ± 0.7</td>
<td>12.5 ± 0.1</td>
<td>12.2 ± 0.1</td>
<td>100 ± 5.7*</td>
<td>56 ± 1.6</td>
</tr>
<tr>
<td>400</td>
<td>34.7 ± 1.4b</td>
<td>14.7 ± 0.9</td>
<td>15.9 ± 0.5b</td>
<td>12.1 ± 0.1</td>
<td>&gt;300 ± 0.0*</td>
<td>56 ± 1.5</td>
</tr>
</tbody>
</table>

* Values for activated partial thromboplastin time (APTT), prothrombin time (PT), and Thrombotest clotting time (TCT) expressed as means ± SE, n = 10 to 12, except n = 6 for males given 400 mg/kg.

** Difference from control is statistically significant at P < 0.05.

*** Difference from control is statistically significant at P < 0.0001.

DISCUSSION

Increased prothrombin and activated partial thromboplastin times (weeks 6, 17, and 24), Thrombotest clotting times (weeks 17 and 24), and decreased plasma coagulation factor II, VII, IX, and X activities (week 26) were observed in male drug-treated groups. The increase in APTT values was explained by a decrease in the activities of factors IX and X, and the increase in TCT was consistent with decreases in activities of factors II, VII, IX, and X; decreases in the activity of these coagulation factors have been associated with vitamin K deficiency in the rat (Godsafe et al., 1992).

The APTT and TCT were more sensitive than the PT in monitoring for reductions in vitamin-K-dependent coagulation factor activities in this study. Dose-related increases in APTT and TCT were observed at doses ≥10 mg/kg/day to maximums of 2- and 4-fold, respectively, in male rats given 400 mg/kg/day. The PT for male rats given 400 mg/kg/day SKF 99085 was minimally increased (1.2-fold), despite a marked reduction in activity (10–20% control) of coagulation factors II, VII, IX, and X.

The relative insensitivity of the prothrombin time for the detection of the reduced vitamin-K-dependent coagulation fac-
HEMOSTASIS IN RATS GIVEN ACAT INHIBITOR

Because the PT is sensitive to reductions in three of the four vitamin-K-dependent coagulation factors (II, VII, and X), it is used widely in human medicine to monitor for effects on these factors in patients undergoing oral anticoagulant (coumarin) therapy (Hirsh et al., 1994) and in veterinary medicine in the detection of warfarin toxicity (Hall, 1972).

It is possible that the rabbit brain thromboplastin reagent used in this PT assay is less sensitive to alterations in rat coagulation factor activity. Animal plasmas frequently clot very rapidly in assays utilizing commercial rabbit brain reagents, resulting in an inability to detect minor deficiencies in coagulation factor activity. Some laboratories have enhanced the sensitivity of this assay by diluting the commercial reagent to produce a longer "normal" clotting time (Dodds, 1989). Human brain thromboplastin has been found to be more sensitive to some animal factor deficiencies than other thromboplastins (Mifsud, 1979). The Thrombotest has been shown to be more sensitive than the PT in the detection of reductions in vitamin-K-dependent coagulation factor activities in rats (Godsafe et al., 1992). In this laboratory, a modification of the Thrombotest assay (1:76.5 rather than a 1:51 plasma dilution) may have also contributed to the enhanced sensitivity of the assay.

The hemorrhagic bleeding disorder observed in male rats given 400 mg/kg/day SKF 99085 was attributed to a deficiency in the activity of vitamin-K-dependent coagulation factors II, VII, IX, and X. The plasma activity of factors II, VII, IX, and X decrease dramatically, particularly in male rats, in the absence of dietary vitamin K (Godsafe et al., 1992). The mechanism of the effect on vitamin-K-dependent factors in this study may be related to alterations in absorption or metabolism of vitamin K following treatment with the ACAT inhibitor. Vitamin K is lipid soluble, its absorption is facilitated by the presence of dietary fat and bile acids, and it is incorporated into and transported by plasma lipids (Newberne and Conner, 1989). The ACAT enzyme is responsible for the acylation of cholesterol to cholesteryl esters, and it has been suggested that ACAT inhibitors decrease cholesterol absorption and increase cholesterol excretion by the liver (Matsuda, 1994).

Male rats were more susceptible than female rats to the deficiency in vitamin K-dependent coagulation factor activities in this study. This finding is consistent with the known protective effect of estrogens and the accentuating effects of testosterone on the development of vitamin K deficiency in rats (Matschiner and Willingham, 1974; Uchida et al., 1985; Jolly et al., 1977; Hara et al., 1994). No evidence of a coagulation abnormality was observed in male or female dogs given SKF-99085 at doses up to 100 mg/kg/day for 6 months. The known sensitivity of male rats to vitamin K deficiency, the observation of significant coagulation effects in male rats at only the highest dose administered (400 mg/kg), and the lack of coagulation effects in female rats and male and female dogs suggest that coagulation abnormalities are unlikely to occur in humans treated with SKF-99085.

In summary, the drug-related hemorrhagic disorder observed in male rats given high doses of the ACAT inhibitor SKF 99085 was attributed to a reduction in the activity of vitamin-K-dependent coagulation factors. In contrast to humans and some other species, the APTT and TCT were more sensitive screening tests than the PT in detecting deficiencies of vitamin-K-dependent coagulation factors.
REFERENCES


