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Crilvastatin, a new 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor, inhibits cholesterol absorption in genetically hypercholesterolemic rats

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Abstract

Crilvastatin is a new drug from the pyrrolidone family, which acts as a non-competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. The long-term effects of oral crilvastatin treatment (200 mg per day per kg body weight for 4 and 10 weeks) were investigated on in vivo cholesterogenesis in male adult normocholesterolemic (SW) and genetically hypercholesterolemic (RICO) rats. In both strains of rats, the treatment had no effect on the plasma cholesterol level, but efficiently inhibited cholesterol synthesis in liver and intestine, as shown by the decreased incorporation of exogenous [^{14}C]acetate into hepatic (3.5-fold in SW, 1.7-fold in RICO rats) and intestinal (2.5-fold in SW, 3.3-fold in RICO rats) sterols. In RICO rats in which the dietary cholesterol absorption coefficient was two-fold lower in treated (38%) than in untreated (78%) rats, this drug reduced intestinal cholesterol absorption. As a result, the total plasma cholesterol input (absorption + synthesis), measured by isotope analysis in RICO rats, was markedly lower in treated (11.3 mg per day) than in untreated animals (28.8 mg per day).

Keywords: Hypocholesterolemic drug; Liver; Intestine; Cholesterol synthesis; Dual isotope blood ratio method; Occupancy principle; (RICO rat)

1. Introduction

Crilvastatin is a drug from the pyrrolidone family (Fig. 1) which acts in vitro as a non-competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), the key enzyme of cholesterol synthesis (Esnault et al., 1988). In rats with hypercholesterolemia induced by dietary cholesterol excess, it has been shown recently that this drug reduces the plasma cholesterol level and stimulates cholesterol 7α-hydroxylase, which catalyzes the limiting step of bile acid synthesis (Clerc et al., 1993).

The genetically hypercholesterolemic (RICO) rat (Ouguerram et al., 1990) is characterized by a very high cholesterogenesis activity, mainly in the liver. This animal model is considered a very useful tool for testing drugs which may interfere with cholesterol and bile acid metabolic pathways. The main objective of this study was to evaluate, in normal and RICO rats fed a cholesterol-poor diet, the effects of crilvastatin on cholesterolemia. Moreover, the mechanisms by which crilvastatin affects the processes of cholesterol turnover, and particularly cholesterol synthesis and/or intestinal absorption, were examined in RICO rats.

2. Materials and methods

2.1. Chemicals

Chemicals of the highest purity were obtained from Sigma. Radiolabeled [1,2-^{3}H] and [4-^{14}C]cholesterol were purchased from CEA (Gif sur Yvette, France). Crilvastatin was a generous gift of Pan Medica (Carros, France).

2.2. Experimental procedures

In the first experiment, the hypocholesterolemic action of chronic oral crilvastatin treatment was tested in

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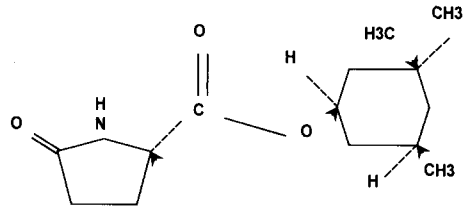

Fig. 1. Chemical structure of crilvastatin.

normal (SW) and genetically hypercholesterolemic (RICO) rats, and its effect on cholesterol synthesis was evaluated. Eight male SW and eight male RICO rats, 8 weeks old, were placed at random in stainless steel cages (one per cage). They received a control semi-purified cholesterol-poor diet (sucrose 53%, casein 23%, lard 9.2%, mineral mix 5%, skim milk 4%, yeast 2.5%, vitamin mix 2.5%, walnut oil 0.8%, cystine 0.2%, cholesterol 0.05%) for 4 weeks (Perrodin and Lutton, 1985). Four rats of each strain were maintained on this diet for another 4-week period, whereas the others received crilvastatin. The amount of drug was calculated to be such that the daily oral intake of each rat was 200 mg of crilvastatin per kg of body weight. The drug was solubilized in walnut oil before being mixed with the other food ingredients. Blood samples were drawn every week (or two weeks) by puncture from the tail vein. Plasma levels of total and free cholesterol were determined by means of commercial enzyme kits (CHOP PAP method, Boehringer, Mannheim, Germany). At the end of the study, the rats were killed at 10:00 am, 70 min after administration of [1-^{14}C]acetate in order to estimate in vivo cholesterogenesis activity on the basis of the radioactivity incorporated into hepatic and intestinal sterols (Lutton et al., 1990).

In the second experiment, the effect of crilvastatin on dietary cholesterol absorption was studied in RICO rats. After a pre-treatment period of two weeks, seven rats received oral crilvastatin (200 mg per kg body weight and per day) for ten weeks, as shown in Fig. 2.

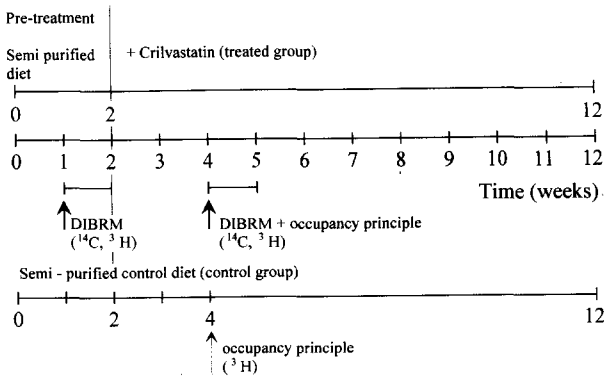

Fig. 2. Schematic protocol of the long-term experiment.

The absorption coefficient of dietary cholesterol was measured during the pre-treatment period, then after two weeks of treatment, using the dual-isotope blood ratio method (DIBRM) of Zilversmit (Zilversmit and Hughes., 1980), that had been critically evaluated and validated in our laboratory (Lutton, 1980). The radioactive doses of [1,2-^{3}H]cholesterol (intravenous administration) and [4-^{14}C]cholesterol (oral administration) used in the second experimental period were ten times higher than the doses used before the treatment. These conditions allowed us to study the cholesterol turnover in crilvastatin-treated RICO rats by measuring the total plasma cholesterol input (absorption + synthesis), using the occupancy principle (Magot et al. 1987). This method was based on the analysis of long-term decay of the specific radioactivity of [^{3}H]-cholesterol in plasma. The results were compared with data obtained by the same method in seven untreated RICO rats fed the control diet, and observed during the ten weeks following the intravenous administration of [^{3}H]cholesterol (Fig. 2).

2.3. *In vivo [^{14}C]acetate incorporation into sterols*

Sodium [1-^{14}C]acetate, 1.85 MBq (1.66–2.22 GBq/mmol) was dissolved in 0.2 ml normal saline solution and injected subcutaneously between 8:00 and 10:00 a.m. (Lutton et al., 1990). The rats were killed by intraaortic puncture under pentobarbital anesthesia, 70 min after injection. Blood, liver and small intestine were collected. After saponification in 2 N ethanolic KOH, the sterols were extracted with petroleum ether and radioactivity was measured (Lutton et al., 1990), using a liquid scintillation spectrometer (Kontron MR 300, Montigny le Bretonneux, France).

2.4. *Cholesterol absorption coefficient measurement (DIRBM)*

The dose of [4-^{14}C]cholesterol (37–74 kBq, for the first measurement, 10 times higher for the second) was mixed with 3 g of semi-purified control diet. The dose of [1,2-^{3}H]cholesterol (74–148 kBq per animal for the first measurement, ten times higher for the second) was mixed with Tween 80 and dispersed in 0.9% NaCl (Zilversmit and Hughes, 1980). The oral dose of [^{14}C]cholesterol was ingested by the animals in several minutes, after an overnight fast. Immediately after, the dose of [^{3}H]cholesterol (0.5 ml) was administered through the jugular vein under light diethylether anesthesia. Blood samples (0.2–0.3 ml) were collected from the venous orbital sinus 48, 72 and 120 h after dual isotope administration. After saponification in 2 N ethanolic potassium hydroxide for 2 h, blood cholesterol was extracted with petroleum ether, and radioactivity was determined by liquid scintillation counting.

Table 1
Plasma levels of total and free cholesterol after one to four weeks of control diet without (untreated) or with crilvastatin added (+crilvastatin) in normo-cholesterolemic (SW) or in genetically hypercholesterolemic (RICO) rats

Group	Time (weeks)	Total cholesterol (mg per ml)	Free cholesterol (mg per ml)
SW			
untreated	4	0.84 ± 0.03	0.33 ± 0.01
+crilvastatin	2	0.85 ± 0.04	0.27 ± 0.02
	4	0.89 ± 0.03	0.28 ± 0.02
RICO			
untreated	4	2.10 ± 0.02 [a]	0.82 ± 0.02 [a]
+crilvastatin	1	2.16 ± 0.03 [a]	0.85 ± 0.03 [a]
	2	1.99 ± 0.04 [a]	0.78 ± 0.03 [a]
	3	1.91 ± 0.04 [a]	0.75 ± 0.03 [a]
	4	1.96 ± 0.04 [a]	0.77 ± 0.03 [a]

Results are expressed as means ± S.E.M. ($n = 4$ animals per group). At 1, 2 or 3 weeks, cholesterolemia was measured in blood samples obtained by puncture from the tail vein. [a] Significantly different from untreated SW rats ($P < 0.05$).

The absorption coefficient of cholesterol (AC) was given by the ratio: AC = (percent oral dose per ml plasma/percent intravenous dose per ml plasma) × 100. Measured in plasma or blood samples, this ratio is constant 48 h after isotope administration (Lutton, 1980).

2.5. Cholesterol turnover

Cholesterol turnover was studied by analyzing the long-term decay of the specific radioactivity of plasma cholesterol after the second dose of [^{3}H]cholesterol in treated RICO rats (Fig. 2), or after the single dose of [^{3}H]cholesterol in untreated RICO rats. Blood samples (0.2 ml) were drawn from the tail vein at different time intervals over ten weeks. Compartmental analysis (Perl and Samuel, 1969) of the decay curve allowed us to calculate the parameters of the cholesterol biodynamics, according to a two-pool model to which the system was approximated (Perl and Samuel, 1969). The area under the curve ($\int a_i \cdot dt$) was measured and the total plasma cholesterol input (R), which represents the total flow of synthesized and absorbed cholesterol into the plasma, was calculated according to the occupancy principle (Goodman and Noble, 1968) from the relation:

$$R = q_i \int a_i \, dt$$

where q_i is the injected radioactivity (Perl and Samuel, 1969).

2.6. Expression of results and statistical analyses

The results were expressed as mean values ± S.E.M., and the statistical significance of differences was calculated with Student's t-test.

3. Results

3.1. Effects of 4-week crilvastatin treatment on cholesterol synthesis in SW and RICO rats

The body weight of the animals and the mean food consumption were not modified by the drug during the

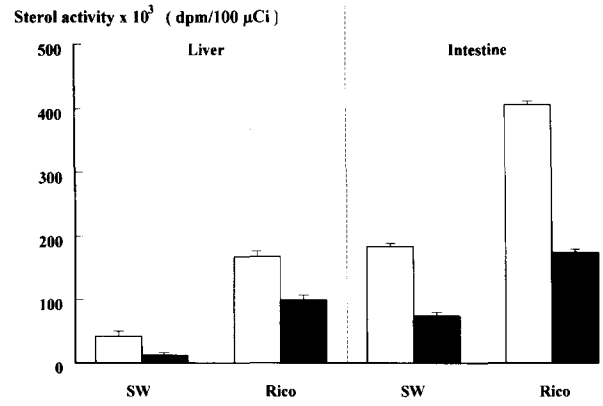

Fig. 3. Sterol radioactivity (dpm per 100 µCi[^{14}C]acetate) in liver and intestine, measured 70 min after [^{14}C]acetate administration in genetically hypercholesterolemic RICO rats, open columns: before, filled columns: after crilvastatin treatment.

Table 2
Absorption coefficient of dietary cholesterol (AC) measured by the DIBRM, and values of cholesterol turnover calculated according to a two-pool model, in genetically hypercholesterolemic RICO rats fed the diet without (untreated) or with crilvatatin added (+crilvastatin).

Group	Parameters of the two-pool model					
	AC (%)	Q1 (mg)	K_{12} (per day)	K_{21} (per day)	K_{01} (per day)	R_{10} (mg per day)
RICO						
untreated	78 ± 3	210 ± 5	0.11 ± 0.02	0.18 ± 0.02	0.14 ± 0.01	28.9 ± 1.7
+crilvastatin	38 ± 2 [a]	129 ± 5 [a]	0.08 ± 0.01	0.12 ± 0.01	0.07 ± 0.01 [a]	11.2 ± 0.3 [a]

Results are expressed as means ± S.E.M. (n = 7 animals per group). AC was measured in the same animals, fed first the control diet (two weeks), then the diet with crilvastatin added. Parameters of the two-pool model were measured on other groups of RICO fed the control diet without (untreated) or with crilvastatin added (see Fig. 2). The abbreviations are the same as in Fig. 5. [a] Significantly different from untreated RICO rats ($P < 0.05$).

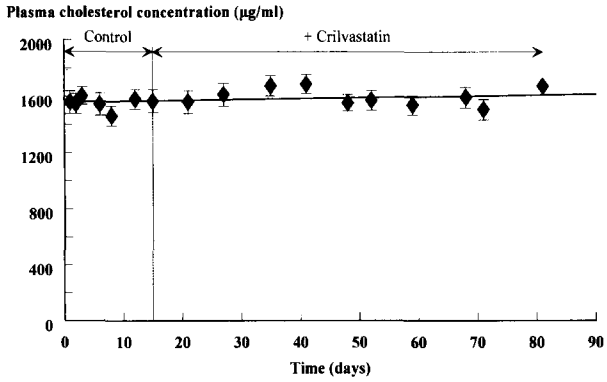

Fig. 4. Plasma cholesterol level in RICO rats before (two weeks) and during long-term chronic crilvastatin treatment (ten weeks).

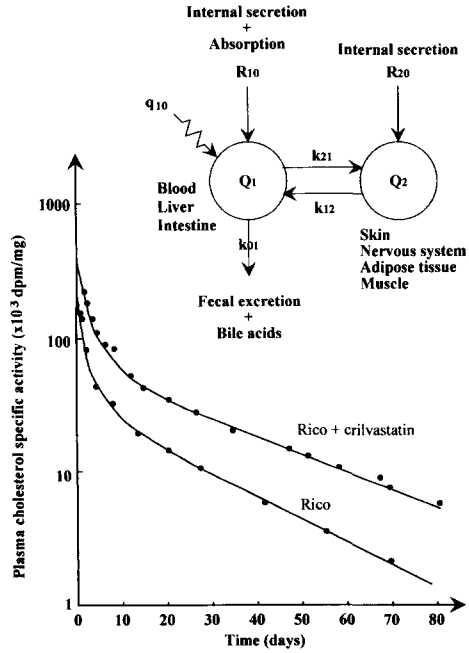

Fig. 5. Time course of the specific radioactivity of plasma cholesterol (dpm per mg) following intravenous injection of [^{3}H]cholesterol, in control or in crilvastin-treated RICO rats (n = 7). The lines represent the best fit generated by the two-pool model. *Right top*: representation of the two-pool model of cholesterol turnover. Compartment 1: rapidly exchangeable cholesterol pool; Compartment 2: slowly exchangeable cholesterol pool; k: rate constant (per day), Q: mass (mg), R: flux (mg per day).

experiment. In normocholesterolemic (SW) as well as in hypercholesterolemic (RICO) rats, no significant hypocholesterolemic effect of crilvastatin at the daily dose of 200 mg per kg of body weight for four weeks was observed (Table 1). At the daily dose of 400 mg per kg (results not shown), no significant modification in food consumption, body weight or cholesterol plasma level was noted. In both strains of rats, however, crilvastatin (200 mg per day per kg) inhibited very efficiently the in vivo incorporation of radioactive acetate into hepatic and intestinal sterols (Fig. 3). Sterol radioactivity (expressed in dpm per 100 μCi or 3.7 MBq [^{14}C]acetate injected) in liver and intestine was decreased 3.5- and 2.5-fold in SW rats, and 1.7- and 3.3-fold in RICO rats, respectively.

3.2. Effects of ten-week crilvastatin treatment on dietary cholesterol absorption and cholesterol turnover in RICO rats

As seen above for the four-week treatment experiment, crilvastatin at the daily dose of 200 mg per kg of body weight was always without significant effect on cholesterolemia through a ten-week treatment in RICO rats (Fig. 4). The absorption coefficients (AC) of dietary cholesterol, measured by the DIBRM in the same rats before and after two weeks of crilvastatin treatment, are shown in Table 2 (first column). In rats observed in the pre-treatment period (control RICO), AC reached 78 ± 3%. Although at a low concentration in the diet (0.5%), crilvastatin strongly reduced the absorption of dietary cholesterol in RICO rats, since AC reached only 38% after two weeks of treatment.

The inhibition of both cholesterol absorption and synthesis by crilvastatin was confirmed by comparing the decay curves of specific radioactivity of plasma [^{3}H]cholesterol in treated and untreated RICO rats (Fig. 5). Bicompartmental analysis (9), which involves a rapidly exchangeable cholesterol pool (blood, liver, intestine, adrenals....) and a slowly exchangeable pool (nervous system, adipose tissue....), indicated that crilvastatin slowed the plasma cholesterol turnover. Parameters of the two-pool model are presented in Table 2. The mass (Q_i) of the rapidly exchangeable cholesterol pool was significantly lower in treated than in untreated rats. Parameter k_{12} (which accounts for the cholesterol movements from the slowly exchangeable pool towards the plasma) was approximately the same in the both groups, but k_{21} (which accounts for the cholesterol movements from the plasma towards the slowly exchangeable pool) and above all k_{01} (fractional excretion rate) were significantly lowered by crilvastatin treatment. The total plasma cholesterol input, calculated from the area under the decay curve of the specific radioactivity of plasma cholesterol, reached only 11.3 mg per day in treated rats, versus 28.9 mg per day in untreated rats. For a mean food ingestion of 15 g daily, the mean daily amount of ingested cholesterol approximated 7.5 mg in both groups of RICO rats. However, the mean daily absorption rate of dietary cholesterol was 2.95 mg in crilvastatin-treated rats, versus 5.85 mg in untreated rats. Thus, calculated from the difference, cholesterol synthesis reached only 8.3 mg per day in crilvastatin-treated rats, versus 23 mg per day in untreated rats.

4. Discussion

Crilvastatin has been shown to act in vitro as a new non-competitive inhibitor of HMG-CoA reductase in rat liver microsomes, with an effect even more potent than that of fenofibrate (Esnault et al., 1988). In the present in vivo study, a short (four weeks) or long-term (ten weeks) crilvastatin treatment had no effect on the plasma cholesterol level, in either normolipidemic (SW) or genetically hypercholesterolemic (RICO) rats receiving a cholesterol-poor diet. It has been shown that rodents are resistant to the hypocholesterolemic action of statins, which act as competitive inhibitors of HMG-CoA reductase (Endo, 1988). However, when hypercholesterolemia was induced in male Wistar rats by a high-fat and cholesterol-rich diet (29.2% lipids, 1.2% cholesterol), crilvastatin decreased plasma cholesterol by 20–30%, and low density lipoprotein (LDL) cholesterol even further (Clerc et al., 1993). Thus, crilvastatin has been considered as an hypocholesterolemic drug in rats when given with a cholesterol-enriched diet.

The aim of the present study was to investigate the in vivo action of crilvastatin on cholesterol synthesis in normocholesterolemic (SW) and in genetically hypercholesterolemic (RICO) rats. The possible effect of crilvastatin on other steps of cholesterol metabolism, in particular on the dietary cholesterol absorption, was also studied. The RICO strain is characterized by a very high cholesterol synthesis (25 ± 2 mg per day versus 16 ± 1 mg in normal rats) (Cardona et al., 1987). In RICO rats compared with SW rats, cholesterol production studied by in vivo incorporation of [^{14}C]acetate into sterols has been shown to increase two-fold in the intestine and three-fold in the liver (Cardona et al., 1987). The results obtained in the untreated RICO animals of the present study are in agreement with these previous data. Particularly, the AC of dietary cholesterol measured by isotope analysis (78 ± 3%) in young adults (10–12 weeks old), is similar to that previously measured by the isotope equilibrium method in older male RICO (20 weeks old) fed the same diet. Moreover, the whole cholesterol synthesis estimated from the occupancy principle in this experi-

ment (23.0 ± 2 mg per day) is also similar to that previously measured by the isotope equilibrium method (25.3 ± 2 mg per day) (Cardona et al., 1987).

When crilvastatin was administered at the daily dose of 200 mg per kg body weight, the radioactivity of hepatic and intestinal sterols after administration of [^{14}C]acetate decreased 3.5- and 2.5-fold in SW rats, and 1.7- and 2.3-fold in RICO rats, respectively. Thus, crilvastatin appeared an efficient inhibitor of hepatic and intestinal cholesterol synthesis in both strains of rats. In terms of relative efficacy in the liver, the drug seemed to be slightly more efficient in the normal heterozygous (SW) than in the genetically hypercholesterolemic (RICO) rat. Although the effect of crilvastatin on cholesterol synthesis in other tissues was not investigated, the total cholesterol synthesis reached only 7–8 mg per day in treated RICO rats, versus 25 mg per day in untreated rats.

The present results demonstrated that crilvastatin also acts as a strong inhibitor of cholesterol absorption. Measured in RICO rats by the dual isotope blood ratio method, the absorption coefficient of dietary cholesterol was similar to that obtained by the isotope equilibrium method (70–78%) in RICO, as well as in normocholesterolemic SW rats (Cardona et al., 1987). Crilvastatin, at a concentration as low as 0.5% in the diet, was able to decrease two-fold the dietary cholesterol absorption in RICO rats. Such an effect may be expected in normocholesterolemic rats. However, because a dietary cholesterol excess depresses cholesterol synthesis (Mathé and Chevallier, 1979), the inhibition of cholesterol synthesis by crilvastatin could be less efficient in cholesterol-fed rats than in rats fed a diet without cholesterol added. Thus, the inhibition of dietary cholesterol absorption could play a major role in the hypocholesterolemic action of crilvastatin observed in rats fed a cholesterol-rich diet (Clerc et al., 1993). Although the drug could interfere with micellar processing in the intestinal lumen, another mode of action may be proposed, according to results of a previous study which showed that crilvastatin inhibited intestinal acylCoA-cholesterol acyltransferase (ACAT) and the intramucosal esterification step of cholesterol absorption in hamsters (unpublished results from the authors). Finally, as could be expected from the strong inhibition of both cholesterol absorption and synthesis, crilvastatin treatment produced a net reduction of cholesterol turnover (absorption + synthesis: 11 mg per day in treated rats versus 29 mg per day in untreated rats), as proved by the analysis of the decay curve of the plasma cholesterol labeling, following a [^{3}H]cholesterol intravenous pulse.

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