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Compared Effect of Immunosuppressive Drugs Cyclosporine A and Rapamycin on Cholesterol Homeostasis Key Enzymes CYP27A1 and HMG-CoA Reductase

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Abstract: Hyperlipidaemia, i.e. increase in total cholesterol and triglycerides, is a common side-effect of the immunosuppressive drugs rapamycin (RAPA) and cyclosporine A (CsA), and is probably related to inhibition of the 27-hydroxylation of cholesterol (acid pathway of bile acid biosynthesis). This might be one of the causes for the increase in plasma cholesterol, as 27-hydroxycholesterol is a potent suppressor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), a key enzyme of cholesterol synthesis. As the sterol 27-hydroxylase (CYP27A1) inhibition by CsA is well known, we evaluated the effect of another immunosuppressive drug, RAPA, on this enzyme in HepG2 mitochondria, which confirmed the dose-dependent inhibition of mitochondrial CYP27A1 by cyclosporine (10–20 μM), while the inhibition by RAPA required a higher dose (50–100 μM). Corresponding K_i was 10 μM for CsA (non-competitive inhibition) and 110 μM for RAPA (competitive inhibition). Cotreatment with both immunosuppressive drugs showed an additive inhibitory effect on CYP27A1 activity. Later, we analysed the effect of these immunosuppressants on HMGR expression in HepG2 cells, and a dose-dependent up-regulation of HMGR gene expression was observed. The results suggest that RAPA and CsA are both inhibitors of CYP27A1 activity with slightly different mechanisms and that they may accordingly increase HMGR expression.

Rapamycin (RAPA; sirolimus) is a macrolide lactone (fig. 1), originally developed as an antifungal and antitumour drug, but the focus of current interest has shifted towards its immunosuppressive activity, which has been reported to be up to 100 times greater than cyclosporine A (CsA) (fig. 1) [1]. Unfortunately, hyperlipidaemia is a common adverse effect of treatment with RAPA or CsA [2–4]. During the first year of treatment, RAPA significantly increased both the serum cholesterol and triglycerides [5,6]. Among RAPA-treated patients, 20% displayed mean plasma cholesterol values that increased by more than 25%, and 10% had an increase of more than 50% above baseline values after 2 weeks of treatment [7]. At therapeutic concentrations, CsA had comparable effects as RAPA on the above parameters [6].

Complex mechanisms regulate the cholesterol metabolic pathways and the cholesterol homeostasis. Oxysterols, intermediate metabolites of cholesterol have a greater inhibitory effect than cholesterol, itself on HMG-CoA reductase (HMGR), which is the rate-regulating enzyme of cholesterol synthesis [8–10]. Axelson and Larsson have demonstrated that low-density lipoprotein (LDL) cholesterol was converted to 27-hydroxycholesterol and that it reduced HMGR activity [11]. On the basis of these observations it has been suggested that circulating 27-hydroxycholesterol may be a potent negative feedback regulator of HMGR in peripheral cells and possibly liver cells [11,12]. It has also been suggested that the inhibition of the liver mitochondrial sterol 27-hydroxylase (CYP27A1) activity related to CsA [13–15] may cause hypercholesterolaemia. The consequence is a decrease of the negative feedback on HMGR expression by 27-hydroxycholesterol, which could finally result in increased cholesterol synthesis.

27-Hydroxycholesterol is the most abundant hydroxycholesterol in human beings [16]. It is formed from cholesterol by CYP27A1, a mitochondrial cytochrome P450 enzyme, which is expressed in the liver and in many extra-hepatic tissues [17,18]. The CYP27A1 catalyses the initial step in the alternative (or acidic) pathway of bile acid synthesis [19]. In human hepatocytes, the alternative pathway contributes at least 50% of bile acid synthesis and leads predominantly to the formation of chenodeoxycholic acid [13,19]. Furthermore, a genetic defect in the CYP27A1 gene leads to development of cerebrotendinous xanthomatosis, an inborn error of bile acid metabolism characterized by large deposits of lipids and cholestanol and pre-mature atherosclerosis [20].

In the present work, we focused on the mechanism of hypercholesterolaemia caused by CsA or RAPA. In order to assess if the proposed mechanism for CsA could be extended to RAPA, we compared the effects on mitochondrial CYP27A1 activity from HepG2 cells. The activity of CYP27A1 was

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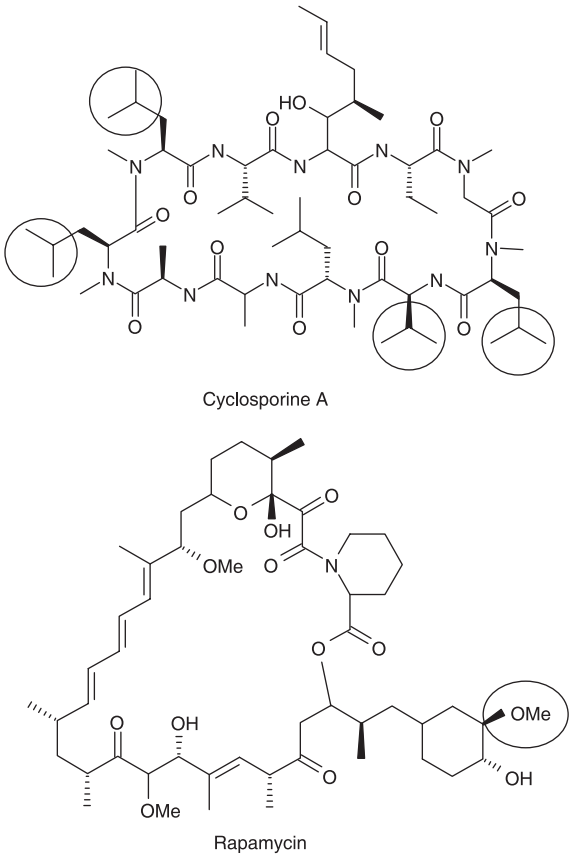

Fig. 1. Chemical structure of cyclosporine A (CsA) and rapamycin (RAPA; sirolimus). The four isopropyl chains out of the monocyclic ring of the CsA molecule and the 39-O-demethylation-metabolic group of RAPA are marked by circles.

determined in the presence or absence of the compounds. The HMGR gene expression was evaluated in HepG2 cells treated with these immunosuppressors.

Materials and Methods

Materials. Human hepatoblastoma HepG2 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cell culture medium RPMI 1640, L-glutamine, antibiotics and cholesterol were from Sigma-Aldrich (St. Louis, MO, USA). Foetal calf serum (FCS) was from Boehringer Mannheim (Mannheim, Germany). Plasticware used for cell culture was from Costar (Cambridge, MA, USA). [4-^{14}C] cholesterol was purchased from NEN Life Sciences (Boston, MA, USA). Sodium isocitrate, isocitrate deshydrogenase, nicotinamide adenine dinucleotide phosphate (NADPH) and thin-layer chromatography (TLC) plates on silica gel G were from Sigma-Aldrich. All solvents were from Merck Eurolab (Strasbourg, France).

Cell culture. The HepG2 cells were grown to confluence in T-75 flasks at 37°C in a 5% carbon dioxide humidified atmosphere. The cell culture medium was supplemented with 10 % FCS, glutamine 4 mM and antibiotic 1% (penicillin, streptomycin, and amphotericin). After development in flasks, cells were harvested by scraping in 3 ml of cold phosphate-buffered saline (PBS) and then were pelleted at 1000 ×g for mitochondrial preparation. For RNA preparation, HepG2 cells were seeded onto 6-well plates at a density of 6.10^5 cells per well. At confluence, cells were washed with RPMI without FCS during 24 hr to remove traces of FCS. Cells were then incubated in fresh RPMI with or without immunosuppressive drugs (CsA or RAPA) with or without cholesterol water soluble (Sigma, Saint-Quentin Fallavier, France) for up to 72 hr. Cells were recovered for RNA isolation.

RNA isolation. Isolation of total RNA was performed by guanidinium thiocyanate (GTC)-phenol mixture based on the technique described by Chomczynski [21] according to the manufacturer's instructions for RNA InstaPure (Eurogentec, Angers, France). Quantification of RNA was obtained by absorbance at 260 nm. The integrity of the total RNA was checked by performing denaturating electrophoresis in 1% agarose gel.

Reverse transcription. Three micrograms of total RNA isolated from HepG2 cells were mixed, denatured for 5 min. at 65°C, then reversely transcribed in a final volume of 50 µl containing 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiotreitol, 1 mM each deoxynucleotide, 40 units RNasine, 0.5 µl BSA 100×, 5 ng oligodt and 200 units Moloney murine leukaemia virus (MMLV) reverse transcriptase. The mixture was incubated for 2 hr at 37°C and the enzyme was then inactivated by heating for 2 min. at 95°C.

Semiquantitative polymerase chain reaction amplification. The cDNA mixture (2 µl) was directly used for polymerase chain reaction (PCR) (i-Cycler, Bio-Rad, Hercules, CA, USA). The PCR mixture contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.3 µM [in the case of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], 0.5 µM (in the case of HMGR) and 2.5 units of Taq DNA polymerase in a final volume of 50 µl. The reaction profile for amplification consisted of an initial denaturation of 94°C for 5 min., followed by 32 cycles at 94°C for 1 min., 55°C for 1 min., 72°C for 1 min., with a final elongation step at 72°C for 10 min. These PCR conditions were determined empirically in two series of experiments, which were conducted to confirm that all amplifications increased exponentially and that the plateau phase of the reaction had not been reached. The PCR (10 µl) mixture was analysed after electrophoretic separation in a 3% agarose gel, visualized under ultraviolet light, digitalized and was quantified using the KDs1 Kodak software. HMGR mRNA was normalized to the GAPDH mRNA and was expressed as a percentage of the control (cholesterol-treated HepG2 cells). The sequences of the specific primers used were from Shoda et al. [22] – (HMGR) – and Andreou and Prokipcak [23].

Mitochondrial preparation. Cell pellets were homogenized in a Dounce homogenizer (Pyrex; Fisher Scientific, Springfield, NJ, USA) with 25 gentle strokes in homogenization buffer (0.1 M Tris/1 mM EDTA/ 0.1 M KCl, pH 7.4). The homogenate was centrifuged for 20 min. at 20,000 ×g and the pellet was gently resuspended in the buffer and homogenized by 15 gentle strokes with a Dounce. The homogenate was centrifuged at 2000 ×g for 10 min. The supernatant was then centrifuged at 9000 ×g for 10 min. and the mitochondrial pellet finally resuspended in Tris 10 mM, EDTA 0.1 mM and glycerol 23%, was sampled and stored at –80°C until used. The protein concentration was measured according to Lowry et al. [24].

Enzyme assays. Sterol 27-hydroxylase was assayed (according to Souidi et al. 1999 [15]) in the mitochondrial fraction by a radioisotopic method performed with 0.5 µCi [4-^{14}C] cholesterol (1.1×10^6 d.p.m., 26 nmol, 52 µM) solubilized in 4.5 mg of hydroxypropyl-β-cyclodextrin (HP-β-CD) in a reaction mixture. Briefly, 250 µg of mitochondrial protein were pre-incubated during 30 min. at 37°C with appropriate concentration of CsA or RAPA. The reaction was started by the addition of NADPH 1 mM to the incubation assay containing DL-sodium isocitrate 5 mM, isocitrate dehydrogenase 0.2 U, mitochondrial fraction and [4-^{14}C] cholesterol solubilized in HP-β-CD for a total volume of 500 µl. The reaction was stopped after 6 min. by adding 40 µl of 5 N NaOH. After neutralisation

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with HCl, the sterols were extracted with 4.3 ml dichloromethane/ethanol (5/1, v/v) plus 1.2 ml H_2O. Unlabelled 27-hydroxycholesterol (75 µg) was added and separated from cholesterol by TLC on silica gel G using single migration technique with ethyl acetate/hexane (1/1, v/v).

Cyclosporine A (Coger, Paris, France) and RAPA (generous gift from Wyeth-Ayerst, NY, USA) were added to the assay mixture dissolved in dimethyl sulfoxide (DMSO), giving a final concentration of 0.1% (v/v) DMSO in the incubation medium. The same amounts of DMSO were added to the controls.

Data analyses. Data shown are the mean ± S.E. CYP27A1 activity after treatment with CsA or RAPA (n = 4 for each group). Due to the limited number of samples simultaneously analysed, we chose the Lineweaver–Burk plotting method for the kinetic analysis.

For HMGR mRNA analysis, each experiment was performed in triplicate and repeated at least once in an independent setting. Data shown are the mean ± S.E. after treatment with CsA or RAPA at various concentrations. Statistical analyses were performed with Student's t-test. Differences were considered significant when $P < 0.05$.

Results

First, we examined the inhibition potential of RAPA compared with CsA on CYP27A1 activity in the mitochondria of HepG2 cells by an elevated dose of each immunosuppressor, 120 µM (data not shown). At this concentration, the activity of CYP27A1 was completely inhibited by CsA (undetectable activity) while RAPA caused an inhibition of less than 70%. Cyclosporine A dose dependently (5–20 µM) inhibited CYP27A1 activity (fig. 2A). Various concentrations of RAPA were added to the mitochondria, and CYP27A1 inhibition started at 50 µM when 0.1 µM (plasma concentration in the treated patients) had no effect (fig. 2A). The addition of both immunosuppressive drugs on HepG2 mitochondria increased the inhibitory effect on CYP27A1 activity (fig. 2C). This additional effect was obvious only for higher concentrations of RAPA (100 µM) (fig. 2C).

Then the relative affinities of CYP27A1 for its substrate (cholesterol) or its inhibitors (CsA or RAPA) were determined to analyse the mechanism of CYP27A1 inhibition. By means of the Lineweaver–Burk analysis we calculate, for CYP27A1 activity, an apparent K_m of 85 µM of cholesterol and a V_{max} of 0.91 nmol/min./mg of protein without immunosuppressive drugs (control, fig. 3). In the presence of CsA, we reached a K_i of 10 µM of CsA. The K_m was similar to the control, indicating that the mechanism of inhibition would be of a non-competitive type (fig. 3A). Rapamycin was responsible for a competitive inhibition (V_{max} = 0.89 nmol/min./mg) and required a higher concentration to inhibit the CYP27A1 activity (K_i = 110 µM) (fig. 3B).

Finally, the HMGR gene expression by HepG2 cells was analysed by semiquantitative PCR to confirm the hypothesis of an up-regulation of HMGR in the presence of CsA or RAPA. Preliminary experiments with cholesterol-treated HepG2 cells were done to determine the high expression of HMGR in serum-free condition. Without addition of cholesterol, no modification of HMGR gene expression by immunosuppressive drug treatment was observed in the HepG2 cells (data not shown). In the cholesterol-treated

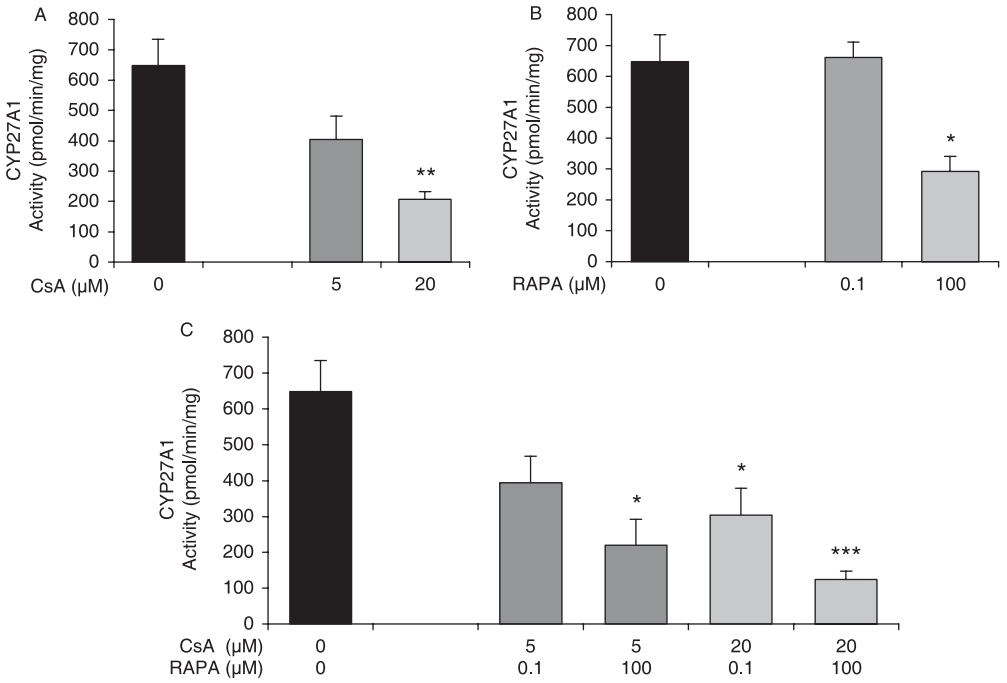

Fig. 2. Comparison of the effects of treatment with (A) cyclosporine A (CsA, 5 and 20 µM) and (B) rapamycin (RAPA, 0.1 and 100 µM) alone or (C) together (co-treatment) on sterol 27-hydroxylase (CYP27A1) activity in HepG2 mitochondria. Immunosuppressive drugs were added DMSO giving a final concentration of 0.1% during a pre-incubation time of 30 min. at 37°C. The same amount of DMSO was added to the controls. Data shown are the mean ± S.E. (n = 4 for each group) of CYP27A1 activity after treatment with CsA or RAPA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significantly different from the control group (Student's t-test).

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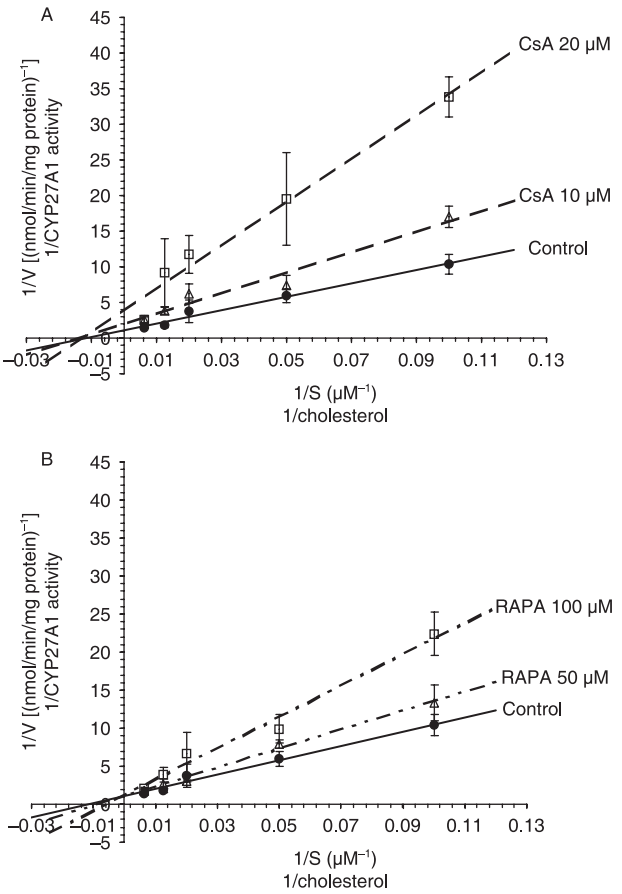

Fig. 3. Inhibition of the sterol 27-hydroxylase (CYP27A1) activity in HepG2 mitochondria by 10 and 20 μM of cyclosporine A (CsA) (A) or 50 and 100 μM of rapamycin (RAPA) (B). The sterol 27-hydroxylase activity was measured in mitochondria isolated from HepG2 confluent cells as described in the Materials and Methods. The final concentration of cholesterol varies from 10 to 160 μM. Theses graphs show Lineweaver–Burk's curves allowing the determination of the enzyme kinetics in the absence (control) or presence of cyclosporine or RAPA. Data shown are the mean ± S.E. of sterol 27-hydroxylase activity (n = 4 for each group).

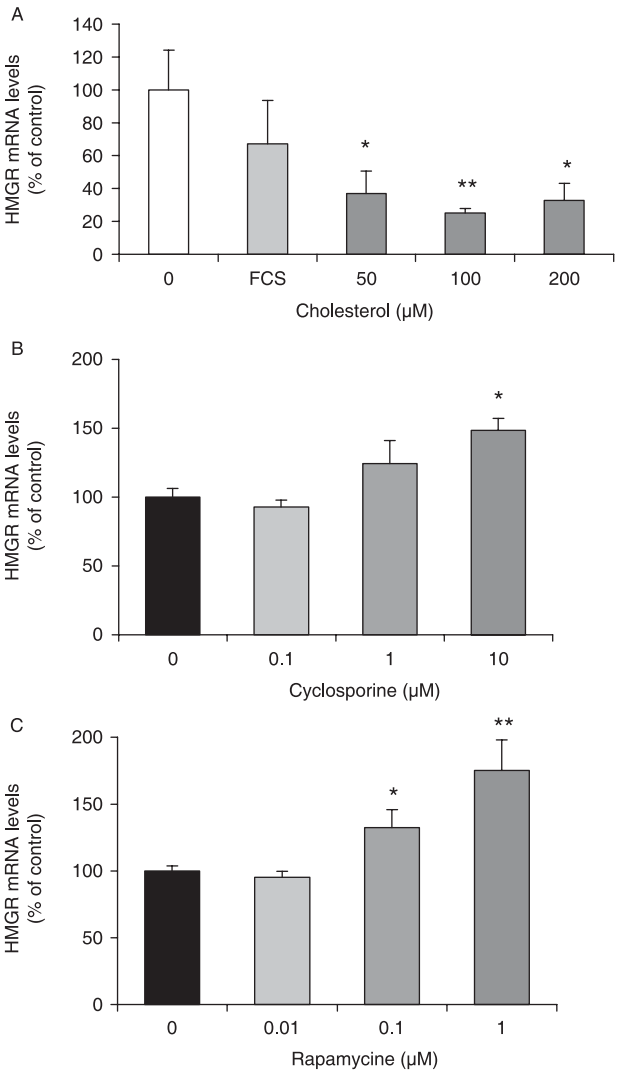

Fig. 4. Effect of (A) cholesterol alone (50 to 200 μM) and dose-dependent effect of (B) cyclosporine A (CsA) (0.1 to 10 μM) and (C) rapamycin (RAPA) (0.01 to 1 μM) on HMG-CoA reductase (HMGR) mRNA levels in HepG2 cells in the presence of cholesterol (100 μM) after 72 hr. Data shown are mean ± S.E. of three different experiments (n = 3 for each group) of HMGR mRNA levels after treatment with CsA or RAPA. HMGR mRNA relative expression was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) as described in Materials and Methods. *P < 0.05, **P < 0.01, significantly different from the cholesterol group (Student's t-test; FCS, foetal calf serum).

cells, a significant decrease was observed starting at 50 μM ($P < 0.05$, fig. 4A), probably due to the feedback down-regulation of cholesterol or oxysterols on HMGR transcription. In this concentration range (50–200 μM) no dose-effect was observed; 50 μM was probably sufficient to decrease HMGR mRNA levels at a maximum rate. The mRNA levels for the rate-limiting enzyme HMGR was increased in a dose-dependent manner in CsA and RAPA-treated cells (fig. 4B and C). Addition of 1 μM of CsA to the culture medium led to a non-significant increase of HMGR mRNA, while 10 μM significantly increased HMGR gene expression compared to control cells treated with cholesterol alone (100 μM) (fig. 4B). Smaller amounts of RAPA than CsA were needed to increase HMGR gene expression: 0.01 μM had no effect while 0.1 μM and 1 μM of RAPA significantly increased HMGR by 33% ($P < 0.05$) and 75% ($P < 0.01$), respectively (fig. 4C).

With the same conditions, CYP27A1 mRNA levels were not affected by cholesterol nor by addition of CsA or RAPA in the culture medium (data not shown). These immunosuppressive drugs would specifically affect CYP27A1 activity.

Discussion

Cholesterol homeostasis is the result of equilibrium between dietary cholesterol absorption, cellular *de novo* synthesis and hepatic catabolism in bile acids. A dysregulation of

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these input and output pathways produces metabolic disorders leading to risk of development of lipid disease. In primary cultures of rat or human hepatocytes, the addition of CsA (10 μM) decreased the conversion of cholesterol into bile acids by approximately 50%, suggesting that the acid pathway accounts for 50% of the total bile acid biosynthesis [13]. The aim of this study was to compare first the *in vitro* effects of CsA and RAPA on the CYP27A1 activity. While both immunosuppressive drugs were found to inhibit this activity, RAPA appeared to be a weaker inhibitor of CYP27A1 activity than CsA. This inhibition was studied in HepG2 mitochondria as a model system for human liver cells. In this model, high concentrations of immunosuppressive drugs were used to study the pharmacokinetics of inhibition of CYP27A1 activity. It was reported to be a suitable model for studying the regulation of bile acid biosynthetic enzymes [25] primarily because of the high mitochondrial CYP27A1 enzyme content in HepG2 and because chenodeoxycholic acid is the primary bile acid mainly secreted by these cells [26].

In this work, we have shown that CsA is a non-competitive inhibitor of CYP27A1 activity. Experiments with radio-labelled CsA indicated binding to the purified enzyme [27] and probably to a site other from cholesterol. These authors proposed the same type of inhibition as us while for Winegar et al. (1996) [14] it seems competitive. In addition to this inhibitory effect on its activity, transcriptional effects were observed for CsA [28], but in our study no modification of CYP27A1 gene expression was observed after CsA or RAPA treatment (data not shown). We have shown for the first time that RAPA causes a competitive inhibition of CYP27A1 activity, contrary to what was observed with CsA. This inhibition of a key enzyme in bile acid synthesis is in accordance with the reduced bile flow observed in rat [29,30]. In the present study co-treatment with both drugs caused a cumulative inhibitory effect on CYP27A1 activity (fig. 2). Moreover, RAPA seems to enhance the hyperlipidaemic effects of CsA in therapeutic treatment [5], which can be partly explained by the inhibition of CYP27A1 activity.

The inhibition of CYP27A1 activity by both CsA and RAPA could be explained by a roughly monocyclic chemical structure (cyclopeptide and macrolide respectively) of these immunosuppressants (fig. 1). The two compounds have a lipophilic pole: a carboxylic chain for CsA and a conjugated double bound for RAPA. The (non-competitive) inhibition due to CsA would result from interaction with the active site of the enzyme of one isopropyl chain among the four branched out of the macrocyclic ring of the CsA molecule. Indeed, the CYP27A1 activity is also inhibited by various oxysterols like 7β-hydroxycholesterol, cholestanol or epicoprostanol, all bearing such a chain [15]. The (competitive) inhibition caused by RAPA might involve the conformational position of an O-methyl substituent on the cyclohexyl ring, the major metabolic group of the molecule (encircled, fig. 1), which undergoes a 39-O-demethylation by CYP3A [31]. In addition, regions of the amino acid sequence containing the haem-binding core region are highly conserved in all cytochrome P450s [32]. Kuhn et al. [31] suggest that hydrogen donor functionalities close to the metabolic site and in appropriate relative position to it are important for anchoring the substrate at the catalytic haem centre. So, the cyclohexyl-ring of RAPA might stock in the catalytic haem centre of CYP27A1, thus becoming a competitive inhibitor of cholesterol. This difference in the intimate structure of the molecules might account for the different types of inhibition observed in this work.

Besides inhibiting a key enzyme of cholesterol degradation, CsA and RAPA could mediate their hyperlipidaemic effect by up-regulation of a key cholesterol synthesis enzyme, indeed an up-regulation of HMGR gene expression was observed after treatment with cyclosporine or RAPA. Several oxygenated cholesterol derivatives (oxysterols), such as 27-hydroxycholesterol, are highly active suppressors of HMGR and may inhibit cellular cholesterol synthesis [8,11]. Circulating 27-hydroxycholesterol may be a potent negative feedback regulator of HMGR activity both in liver and in peripheral cells [33]. We hypothesized that increased HMGR expression observed in hepatoma cells, human fibroblast cells [11] and in a mouse model [34] was a consequence of the CYP27A1 inhibition. People with deficient CYP27A1 show accumulation of cholesterol and its metabolite, cholestanol, in multiple tissues. Moreover, over-expression of CYP27A1 is coupled with a decreased cholesterol biosynthesis (50% HMGR activity) in Chinese hamster ovary (CHO) cells [35]. 27-Hydroxycholesterol is an oxysterol present in atherosclerotic plaques [36], as the formation of 27-hydroxycholesterol by CYP27A1 normally prevents the accumulation of cholesterol excess in tissues [37]. In addition, when alveolar macrophages expressing CYP27A1 were exposed to an inhibition by CsA, there was an accumulation of cholesterol in these cells [38]. These reports suggest that CYP27A1 may act as an anti-atherosclerotic enzyme in the wall of vessels by favouring the elimination of cholesterol.

In conclusion, we report that RAPA (sirolimus) is a competitive inhibitor of CYP27A1 activity. This immunosuppressive drug is a less powerful inhibitor (K_i = 111.2 μM) of the CYP27A1 activity than cyclosporine (K_i = 10.5 μM). Furthermore, the two immunosuppressive drugs act by different mechanisms, RAPA as a competitive and CsA as a non-competitive inhibitor. These inhibitions are concomitant with an increase in the HMGR expression. Our results partly explain the hypercholesterolaemia caused by RAPA or CsA, however chemical structure modifications of these two immunosuppressive drugs might diminish this side-effect.

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