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EFFECTS OF A 2,3-OXIDOSQUALENE-LANOSTEROL CYCLASE INHIBITOR, 2,3:22,23-DIOXIDOSQUALENE AND 24,25-EPOXYCHOLESTEROL ON THE REGULATION OF CHOLESTEROL BIOSYNTHESIS IN HUMAN HEPATOMA CELL LINE HepG2

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Abstract—*N*-[(1,5,9)-trimethyldecyl]-4 α, 10-dimethyl-8-aza-*trans*-decal-3 β-ol (8-azadecalin **1**), a high-energy intermediate analogue for the 2,3-oxidosqualene-lanosterol cyclase, was found to be a powerful ($IC_{50} \approx 0.1$ μM) inhibitor of cholesterol biosynthesis in human hepatoma HepG2 cells. In analogy with other mammalian cells grown in the presence of cyclase inhibitors, the decrease in C_{27}-sterol formation was accompanied by an accumulation of 2,3-oxidosqualene, 2,3:22,23-dioxidosqualene, and by the formation of a compound characterized as 24,25-epoxycholesterol, a repressor of HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase activity. In order to assess the cyclase as a potential pharmacological target for the design of hypocholesterolemic drugs, it is important to test whether inhibitors of this enzyme are able to act synergistically on the biosynthesis of cholesterol, i.e. by decreasing the amount of lanosterol formed *and* by repressing the regulatory HMG-CoA reductase via the formation of regulatory oxysterols. The accumulation of 24,25-epoxycholesterol in relationship to the decrease of C_{27}-sterol biosynthesis and of HMG-CoA reductase activity showed only a partial correlation: e.g. at [**1**] = 100 × IC_{50} only a 50% reduction in enzyme activity could be attained. In contrast, when HepG2 cells were treated with 2,3:22,23-dioxidosqualene or 24,25-epoxycholesterol, excellent correlations were found between the inhibition of C_{27}-sterol biosynthesis and the repression of HMG-CoA reductase activity, which was almost complete at the highest concentrations of these epoxides (10^{-5} M). Altogether, our results suggest that treatment of HepG2 cells with a cyclase inhibitor such as 8-azadecalin (**1**) does not lead to an intracellular accumulation of repressor molecules high enough to fully trigger a regulatory pathway resulting in a complete down-regulation of HMG-CoA reductase. At intermediary concentrations of cyclase inhibitors (IC_{50}), however, a synergistic mode of action of these inhibitors seems plausible.

Key words: cholesterol; oxysterols; 24,25-epoxycholesterol; 2,3-oxidosqualene cyclase; HMG-CoA reductase; HepG2 cells

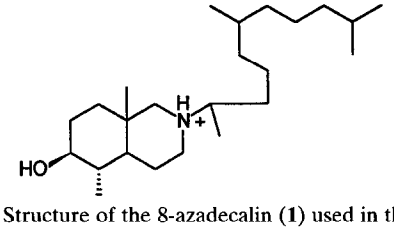

Fig. 1. Structure of the 8-azadecalin (**1**) used in this study.

8-Azadecalin (Compound **1**; Fig. 1) is an excellent inhibitor of 2,3-oxidosqualene-lanosterol cyclase (EC 5.4.99.7) [1]. In analogy with previous studies [2], this compound was designed to mimic a transient high-energy carbocationic intermediate occurring at C-8 during the transformation of 2,3-oxidosqualene into lanosterol. Recently, other high-energy intermediate analogues, based on the same concept, were similarly described as inhibitors of the cyclase [3–5]. The 8-azadecalin (**1**) was found to be a potent inhibitor of the biosynthesis of cholesterol in 3T3 fibroblasts [6]. In addition to directly reducing lanosterol production in these cells, the inhibitor also induces the accumulation of 2,3(*S*):22(*S*),23-dioxidosqualene. This metabolite is known to be cyclized into 24(*S*),25-epoxylanosterol which ultimately leads to the formation of 24(*S*),25-epoxycholesterol, a known repressor of HMG-CoA† reductase [7]. Accordingly, treatment of 3T3 fibroblasts with 8-azadecalin (**1**) also resulted in a marked reduction in HMG-CoA reductase activity [6]. This is a general observation in cultured mammalian cells treated with cyclase inhibitors [reviewed in Refs. 8 and 9]. Therefore, cyclase inhibitors might act, under certain conditions, synergistically through a combination of two mechanisms: (*i*) by decreasing the amount of lanosterol formed (*biosynthetic pathway*; Fig. 2, Pathway A) and (*ii*) by repressing the HMG-CoA reductase through the formation of oxysterols such

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† Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; RT, retention time; U18666A, 3 β-[2-(diethylamino)ethoxy]androst-5-en-17-one.

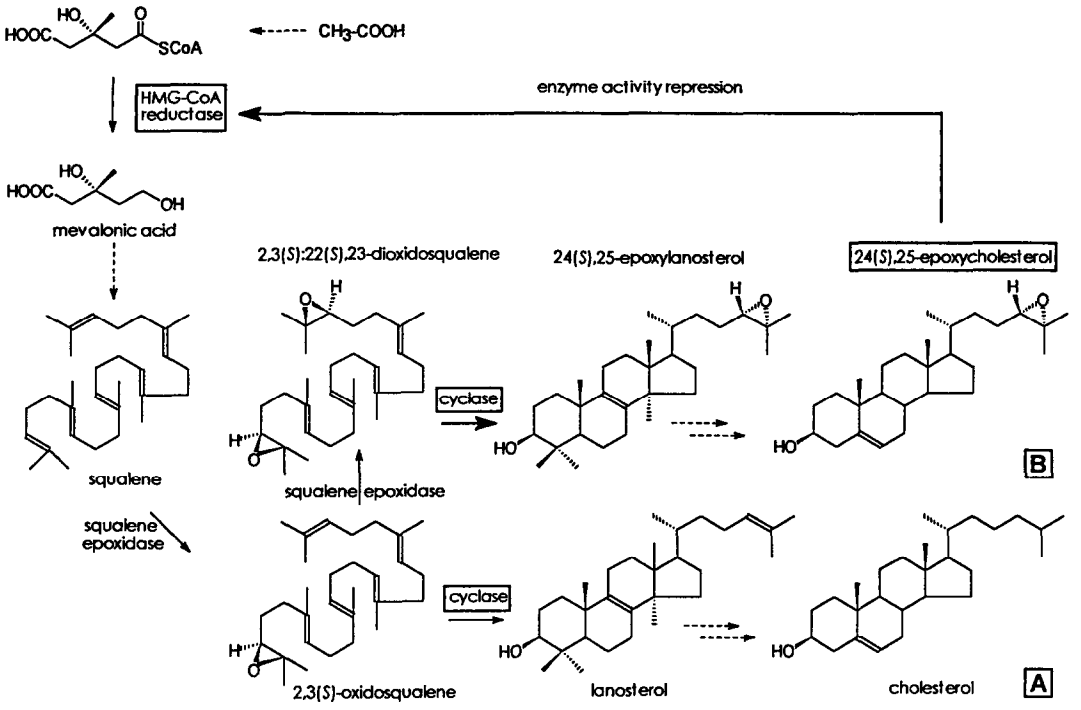

Fig. 2. Cholesterol (Pathway A) and 24(*S*),25-epoxycholesterol (regulatory Pathway B), biosynthesis.

as 24,25-epoxycholesterol (*regulatory pathway*; Fig. 2, Pathway B). This would make the 2,3-oxidosqualene-lanosterol cyclase a particularly attractive target enzyme for the design of hypocholesterolemic drugs. In agreement with such a dual mode of action, we have recently shown that compared to 2,3(*S*)-oxidosqualene, its oxidized derivative 2,3(*S*):22(*S*),23-dioxidosqualene is a better substrate for mammalian cyclases [10]. It is possible that, in the presence of a cyclase inhibitor, squalene epoxides might be preferentially channelled into the regulatory pathway. Since hepatocytes are the prime target cells for hypocholesterolemic drugs which act on cholesterol biosynthesis, we decided to study the effect of 8-azadecalin (**1**) on HepG2 cells. This human hepatoma cell line, which retains many of the functions of normal liver parenchymal cells, is considered one of the best models for the study of cholesterol biosynthesis and regulation [11]. Only limited studies with cyclase inhibitors are available with these cells [12–14]. Treatment of HepG2 with **1** resulted in a powerful inhibition of cholesterol biosynthesis and in a reduction in HMG-CoA reductase activity which was correlated, in part, with a cellular accumulation of 2,3(*S*):22(*S*),23-dioxidosqualene and 24(*S*),25-epoxycholesterol. In order to assess the importance of the two possible modes of action of **1**, i.e. through the biosynthetic and regulatory pathways, we also studied the effect of these epoxides on cholesterol biosynthesis and the activity of HMG-CoA reductase.

MATERIALS AND METHODS

Chemicals. Sodium [2-^{14}C]acetate (50 mCi/mmol), DL-[2-^{3}H]mevalonic acid lactone (35.1 Ci/mmol) and 3-hydroxy-3-methyl[^{14}C]glutaryl CoA (47.4 mCi/mmol) were purchased from NEN Dupont de Nemours (Les Ulis, France). [3-^{3}H]*R*,*S*-2,3-oxidosqualene was a kind gift from Prof. L. Cattel and Dr M. Ceruti (University of Torino, Italy). Squalene, cholesterol, desmosterol, lanosterol, mevalonolactone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$ were supplied by the Sigma Chemical Co. (L'Ile d'Abeau Chesnes, France). The 8-azadecalin (**1**) was a gift from Marion Merrell Dow (Cincinnati, OH, U.S.A.). Compactin was a gift from the Laboratoires Fournier (Dijon, France). Cell culture medium and serum were obtained from Gibco and the culture flasks were from Falcon. All other chemicals were of the highest purity available.

2,3-Oxidosqualene and 2,3:22,23-dioxidosqualene were prepared, according to the literature, from squalene via the intermediary formation of mono- and di-bromhydrins [15, 16]. The purity of the compounds was assessed by analytical TLC using various migration conditions and by GC [17]. 24,25-Epoxycholesterol was obtained chemically by a modification of a classical method [18]. Briefly, a solution of desmosterol in CH_2Cl_2 was treated with 1.4 eq *m*-chloroperbenzoic acid for 3 hr at room temperature. Analysis of the reaction mixture by TLC, using *n*-hexane/ethyl acetate (85:15) as solvent, revealed the presence of several spots corresponding to mono- and di-epoxidized reaction products. 24,25-Epoxycholesterol ($R_F = 0.2$ compared to 0.25 for desmosterol), which was identified by GC/MS (see below), was the least polar

oxidized derivative. The compound was isolated by preparative TLC and its purity assessed by GC. Its fragmentation (at 70 eV) was in agreement with published spectra [18, 19].

Cell cultures. HepG2 cells, obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were routinely maintained as monolayers in DMEM supplemented with 10% (v/v) foetal calf serum in plastic culture flasks. The cells were grown in a humidified atmosphere (95% air/5% CO_2) at 37°. Lipid-depleted serum was prepared from foetal calf serum according to Ref. 20. The different compounds were added to the cells as solutions in absolute ethanol. Final concentrations of solvent in culture medium did not exceed 0.4% (v/v) and an equivalent amount was added to the control cultures. No significant effect of ethanol on the different parameters studied could be detected under these conditions.

Incorporation of [^{14}C]*acetate into non-saponifiable sterols.* Cells derived from stock cultures were seeded at about 1×10^6 cells in 60 mm diameter plastic Petri dishes (35×10^3 cells/cm^2) in 5 mL DMEM containing 10% (v/v) delipidated serum. On the second day of culture, the cells were treated at designated times (generally 4 hr) with given concentrations of cyclase inhibitor (**1**) or epoxy-compounds. Labelled acetate (20 μCi per dish) was then added to the culture medium. After 2 hr of incubation, the medium was removed and the dishes rinsed three times with phosphate-buffered saline, pH 7.4. The cells were then treated twice for 30 min with 2 mL of 0.1 N NaOH. Aliquots were removed for protein determination [21] and the remainder saponified with 1 vol. of 6% (w/v) potassium hydroxide in methanol for 1 hr at 55°. The non-saponifiable lipids were extracted with *n*-hexane and analysed by TLC essentially as described previously [6, 17].

Transformation of 2,3:22,23-*dioxidosqualene into* 24,25-*epoxycholesterol by HepG2 cells.* Ten 80 cm^2 dishes each containing 3×10^6 cells, 15 mL DMEM and 10% delipidated foetal calf serum were given 5×10^{-7} M of 2,3:22,23-dioxidosqualene and 20 μCi sodium [2-^{14}C]acetate. After 5 hr of incubation, the cells were worked-up as above. The compound (2.35 μg) migrating on TLC as 24,25-epoxy-cholesterol ($R_F = 0.2$) was isolated and identified by GC ($RT_X/RT_{cholesterol} = 1.19$) and by GC/MS (see below).

Assay of HMG-CoA reductase activity. After incubation, the cells were washed three times with phosphate-buffered saline, harvested by scraping into buffer A (5 mM dithithreitol, 10 mM EDTA and 50 mM potassium phosphate buffer, pH 7.4), frozen in liquid nitrogen and finally stored at −20° for further analysis. After thawing and disruption by sonication, aliquots of approx. 50–100 μg cell extract protein were assayed for enzyme activity, essentially as described before [6]. In most cases, the reaction mixture was preincubated for 15 min at 37°, thus allowing the dephosphorylation of HMG-CoA reductase [22], and the assay initiated by addition of 3-hydroxy-3-methyl[^{14}C]glutaryl CoA.

Analytical procedures. The non-saponifiable lipid extracts were separated by one-dimensional TLC on

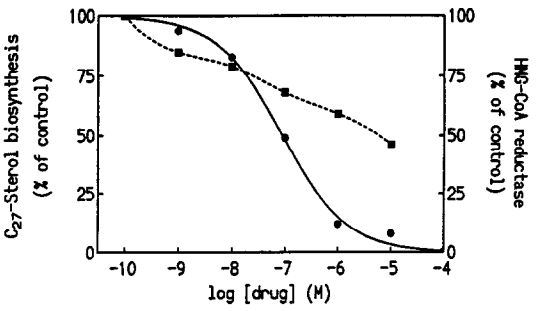

Fig. 3. Influence of varying concentrations of **1** on the biosynthesis of C_{27}-sterols and on the activity of HMG-CoA reductase in HepG2 cells. Cells were plated at 1×10^6 cells/60 mm dish and cultured for 24 hr in 5 mL delipidated growth medium. The cells were then preincubated for 4 hr in the presence of the indicated concentrations of the drug, and collected for the measurement of HMG-CoA reductase activity (--■--) or pulsed with [^{14}C]acetate (20 μCi per dish) for 2 hr and the incorporation of the label in the C_{27}-sterol fraction (—●—) determined (see Materials and Methods). The results are expressed as percentages of [^{14}C]-C_{27}-sterols and reductase activity measured in the absence of the drug: 100% corresponds, to an average of 98×10^3 dpm/mg cell protein and 75 pmol mevalonate formed/min/mg cell protein respectively.

Silica Gel F_{254} plates (Merck) as detailed elsewhere [17] using *n*-hexane/ethyl acetate (85:15, v/v) as solvent. The radioactivity associated with the different compounds was read with a Berthold automatic TLC-linear analyser (LB 284/285) and the peaks integrated by a data acquisition system. The radioactive bands were also scraped off into scintillation vials and counted. Under our work-up conditions recovery of [^{14}C]cholesterol, added to control experiments, was approx. 65%.

Gas chromatography and mass spectrometry. Compounds (e.g. 2,3-oxidosqualene, 2,3:22,23-dioxidosqualene, 24,25-epoxycholesterol) were analysed and quantified by gas chromatography on a 25 m 0.25 mm i.d. DB1 silica capillary column, using ergosterol or cholesterol as internal standards, essentially as described previously [6, 17]. Gas chromatography coupled with mass spectrometry was used to identify e.g. synthesized and cellular 24,25-epoxycholesterol. In this case a 25 m SE-30 capillary column was used and the mass spectrometer was operated at 70 eV.

Expression of results. The data in Figs 3–8 represent the means of duplicate determinations on replicate dishes not found to differ by more than 10% and are representative of at least two separately performed experiments.

RESULTS AND DISCUSSION

Effect of **1** *on sterol biosynthesis.*

The effect of 8-azadecalin (**1**) on cholesterol biosynthesis was studied on HepG2 cells which had been growing for 24 hr in the presence of 10% (v/v) delipidated foetal calf serum. Under these conditions

these cells actively incorporate [^{14}C]acetate into the C_{27}-sterol (cholesterol) fraction and exhibit an HMG-CoA reductase activity close to its maximal level. Since we had previously shown that **1** rapidly exerts its inhibitory action in cells [6], the HepG2 cells were preincubated for 4 hr with the inhibitor; this resulted in a dose-dependent decrease of cholesterol biosynthesis (Fig. 3) and an IC_{50} (half-maximal inhibition) of 0.1 μM was measured. The specificity of **1** for the sterol biosynthetic pathway is suggested by the fact that the ^{14}C radioactivity associated with total non-saponifiable lipids was essentially unchanged after the cells were treated with drug concentrations up to 1 μM. It appears that in HepG2 cells, **1** is somewhat less potent (about 5-fold) than in Swiss 3T3 fibroblasts [6]. Comparatively, compactin, a powerful inhibitor of HMG-CoA reductase [23], presented in HepG2 cells under the same experimental conditions, a similar potency than **1** in the inhibition of cholesterol synthesis (i.e. $IC_{50} = 0.1$ μM; not shown).

Analysis of the radiochromatograms (TLC) of the non-saponifiable fractions isolated from cells treated with increasing concentrations (10^{-9}–10^{-5}M) of 8-azadecalin (**1**) revealed, as would be expected from the mode of action of this compound, an accumulation of radiolabelled 2,3-oxidosqualene ($R_F = 0.86$) and of 2,3:22,23-dioxidosqualene ($R_F = 0.65$) at the expense of the C_{27}-sterols. Both squalene oxides were characterized as described previously [6, 17]. A compound with $R_F = 0.20$), i.e. more polar than cholesterol ($R_F = 0.30$), was also found to accumulate; it co-migrated with authentic 24,25-epoxycholesterol indicating that in HepG2 cells inhibition of the 2,3-oxidosqualene-lanosterol cyclase leads to the observation of a regulatory pathway. HPLC analysis of this polar metabolite, using an on-line radiochromatography detector, also revealed an elution profile identical to that of 24,25-epoxycholesterol (not shown; D. Levasseur, unpublished). The relative quantities of the polar metabolite accumulated in the treated cells were too small to be amenable to GC/MS analysis. In order to formally identify cellular 24,25-epoxycholesterol we studied the formation of this oxysterol from exogenous unlabelled 2,3:22,23-dioxidosqualene by HepG2 cells pulsed with [^{14}C]acetate (see Materials and Methods). GC analysis of the band co-migrating on TLC with authentic 24,25-epoxycholesterol showed the presence of this oxysterol (about 50 ng/mg cellular protein). Moreover the identity of this compound was unambiguously confirmed by its fragmentation (at 70 eV) in GC/MS; *m/z* (relative intensity): 400 (43), 382 (41), 367 (29), 271 (83), 255 (30). This fragmentation pattern, which gives the typical ions at 271 and 255 (i.e. M-H_2-side chain and M-H_2O-side chain, respectively), is similar to that of an authentic sample and to published data [18, 19]. These results, which reveal the presence of 24,25-epoxycholesterol in HepG2 cells, indicate that this cell line is able to convert 2,3:22,23-dioxidosqualene into this regulatory oxysterol and that it can be formed endogenously by a partial inhibition of the cyclase. A similar transformation was previously described, *in vitro*, with rat liver homogenates [24]; moreover endogenous 24,25-epoxycholesterol was also found within concentration ranges required for repressing HMG-CoA reductase in human liver [19] and cultured fibroblasts [25].

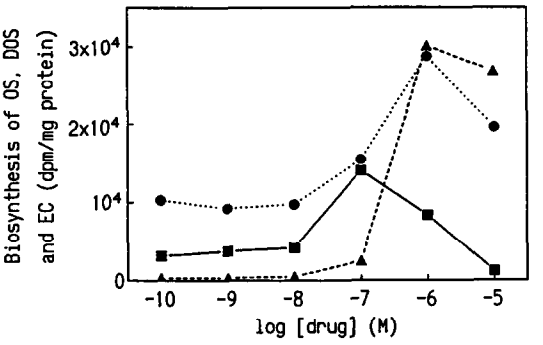

Fig. 4. Formation of 2,3-oxidosqualene, 2,3:22,23-dioxidosqualene and 24,25-epoxycholesterol in HepG2 cells treated with **1**. The non-saponifiable fractions obtained from cells grown as indicated in the legend of Fig. 3 were analysed by TLC (see Materials and Methods). The bands corresponding to 2,3-oxidosqualene, (OS; ···●···), 2,3:22,23-dioxidosqualene (DOS; --▲--) and 24,25-epoxycholesterol (EC, —■—) were isolated and counted. The values given are the recovered radioactivity expressed per mg of cellular protein. The [^{14}C]-C_{27}-sterols measured in the absence of the drug was 98×10^3 dpm/mg cell protein.

Figure 4 summarizes the quantitative data of the changes in the different metabolites in HepG2 cells treated with 8-azadecalin (**1**). Several observations can be made. (i) In the 10^{-8}–10^{-6} inhibitor concentration range, the decrease of cholesterol biosynthesis corresponds to an accumulation of squalene epoxides. (ii) Between 10^{-8} and 10^{-7} M of **1**, a decrease of cholesterol synthesis is paralleled by an increase in the formation of 24,25-epoxycholesterol. This result is in favour of a preferential cyclization, at a cellular level, of 2,3:22,23-dioxidosqualene (regulatory pathway) over 2,3-oxidosqualene (biosynthetic pathway) and extends our previous *in vitro* studies with the cyclase (see Introduction). At 10^{-6} M of 8-azadecalin (**1**) (i.e. $10 \times IC_{50}$) the ratio of labelled 24,25-epoxycholesterol/cholesterol was highest, with a value of about 0.8; this indicates that at this inhibitor concentration, the incorporation of [^{14}C]acetate into both sterols is nearly equivalent. (iii) At high inhibitor concentrations ($\geq 10^{-5}$ M) the formation of 24,25-epoxycholesterol is abolished as would be expected from its mode of formation.

Taken together these data indicate that in HepG2 cells 8-azadecalin (**1**) is a powerful inhibitor of the 2,3-oxidosqualene-lanosterol cyclase which, through the accumulation of 2,3:22,23-dioxidosqualene allows the formation of 24,25-epoxycholesterol, a known repressor of HMG-CoA reductase activity (Fig. 2, Pathway B). We shall now examine the effect of **1** on the cellular activity of this regulatory enzyme.

Effect of **1** *on cellular HMG-CoA reductase activity.*

To test whether the inhibitory potency of **1** on the

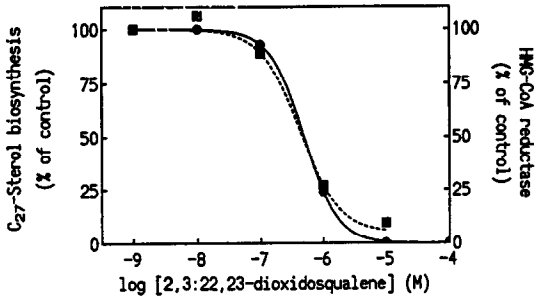

Fig. 5. Influence of the concentration of 2,3:22,23-dioxidosqualene on the biosynthesis of C_{27}-sterols and HMG-CoA reductase activity in HepG2 cells. Cells which had been grown for 24 hr in a delipidated medium were incubated for 4 hr in the presence of the indicated concentration of the diepoxide. They were further pulsed for 2 hr with [^{14}C]acetate to determine the biosynthesis of the C_{27}-sterols (—●—) or collected to assay HMG-CoA reductase activity (- -■- -). The 100% values (control) correspond to 116×10^3 dpm/mg protein and to 82 pmol mevalonate formed/min/mg protein, respectively.

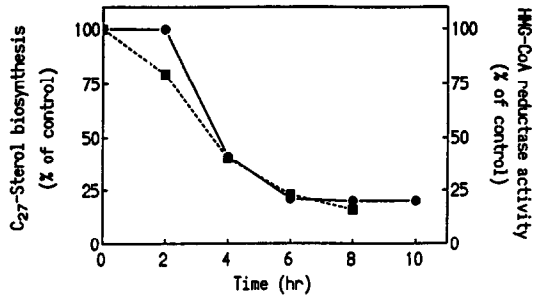

Fig. 6. Time-course of HMG-CoA reductase activity repression and C_{27}-sterols biosynthesis inhibition upon treatment of HepG2 cells with 2,3:22,23-dioxidosqualene. Cells which had been grown for 24 hr in a delipidated medium were incubated for the given times with 1 μM 2,3:22,23-dioxidosqualene. The cells were then collected for the determination of HMG-CoA reductase activity (—■—). To determine the biosynthesis of the C_{27}-sterols (- -●- -), the cells were pulsed with [^{14}C]acetate for the last 2 hr of incubation. The results are expressed as percentages of reductase activities and [^{14}C]-C_{27}-sterols measured in controls which were run for each time point in the absence of the diepoxide; at t_0, 100% corresponds to an average of 98 pmol mevalonate formed/min/mg protein and 119×10^3 dpm/mg protein, respectively.

biosynthesis of cholesterol is also due to the regulatory pathway, i.e. repression of HMG-CoA reductase activity, HepG2 cells were treated with the 8-azadecalin (**1**) under similar experimental conditions as those above. As shown in Fig. 3, a gradual decrease in reductase activity was observed, suggesting that accumulated 24,25-epoxycholesterol indeed plays a role in the mode of action of **1**. Measured HMG-CoA reductase activity, however, was not suppressed, reaching a minimum of 45% of control cells when concentration of 8-azadecalin (**1**) was 10^{-5} M (i.e. $100 \times IC_{50}$). A concentration-dependent decrease in HMG-CoA reductase activity was found with this compound in 3T3 fibroblasts [6]. This contrasts with the "paradoxical" effects observed with other 2,3-oxidosqualene-lanosterol cyclase inhibitors [26, 27]; e.g. in HepG2 cells exposed to the non-specific U16888A, after an initial decrease to 50% of the control value, higher concentrations of the compound cause a gradual increase in reductase activity above the control value [12]. This was ascribed to a complete blockage of the cyclase, which thereby prevented the transformation of accumulated 2,3:22,23-dioxidosqualene into the repressor 24,25-epoxycholesterol. We confirmed this observation with HepG2 cells treated with 1 μM U16888A (D. Levasseur, unpublished). It is possible that the concentration of **1** used in this study was not high enough to reach such a rebound effect. Nevertheless, in studies where the effect of cyclase inhibitors were tested on cellular HMG-CoA reductase, a complete repression of its activity was never observed. Comparing the data in Figs 3 and 4 thus indicates *a priori* a lack of a stringent correlation between residual HMG-CoA reductase activity, inhibition of C_{27}-sterol biosynthesis and cellular levels of [^{14}C]-24,25-epoxycholesterol formed according to the regulatory pathway. It is important to compare the effects of **1** and those of the metabolites of the regulatory pathway in HepG2 cells, i.e. 2,3:22,23-dioxidosqualene and 24,25-epoxycholesterol, on the C_{27}-sterol biosynthesis and HMG-CoA reductase activity.

Effect of HepG2 treatment with 2,3:22,23-dioxidosqualene on sterol biosynthesis and HMG-CoA reductase activity.

Since HepG2 cells, treated with **1**, accumulate 2,3:22,23-dioxidosqualene, which in turn is efficiently converted into the regulatory metabolite 24,25-epoxycholesterol (see above), it was worth investigating whether one could trigger, and to what extent, the regulatory pathway by giving exogenous squalene diepoxide. Figure 5 shows that 2,3:22,23-dioxidosqualene is an excellent inhibitor of cholesterol biosynthesis ($IC_{50} \approx 0.5$ μM) in HepG2 cells pulsed with [^{14}C]acetate. Such an inhibition could be due to two factors: (i) since squalene diepoxide is a preferential substrate of the cyclase [10], it could compete with the cyclization of endogenously formed [^{14}C]-2,3-oxidosqualene and thus inhibit the formation of [^{14}C]cholesterol via the metabolic pathway, (ii) the diepoxide is converted into the regulatory 24,25-epoxycholesterol which decreases HMG-CoA reductase activity. TLC analysis of the non-saponifiable fractions revealed that an accumulation of labelled 2,3:22,23-dioxidosqualene and 24,25-epoxycholesterol is much less pronounced than in cells treated with 8-azadecalin (**1**); e.g. [^{14}C]-24,25-epoxycholesterol was detectable only in cells incubated with 1 μM of the diepoxide (not shown). As evidenced in Fig. 5, treatment of HepG2 cells with 2,3:22,23-dioxidosqualene resulted, under similar experimental conditions, in an almost complete suppression of HMG-CoA reductase activity. This is a remarkable contrast to the more limited extent (about 50%) reached with cyclase

inhibitors such as **1** (see above). In this case an IC_{50} of about 0.5 μM was also found; in fact this value must be even smaller considering that racemic squalene diepoxide was used and only the 2,3(*S*)-enantiomer is transformed by 2,3-oxidosqualene-lanosterol cyclase. To understand further the mode of action of 2,3:22,23-dioxidosqualene we studied the rates by which this metabolite is able to affect cholesterol biosynthesis and the HMG-CoA reductase activity. To this end, HepG2 cells were treated with 1 μM of the diepoxide for different periods. Figure 6 shows that 2,3:22,23-dioxidosqualene influences fairly rapidly the two parameters which plateau at about 20–25% of control. It should be noted that, for experimental reasons, the time courses in Fig. 6 cannot be strictly compared, at least in the early parts: in contrast to HMG-CoA reductase, where activity can be measured immediately after treatment with the diepoxide for the different time periods, each time point for the C_{27}-sterol biosynthesis determinations includes a final 2 hr [^{14}C]acetate-labelling period (the fact that the 2 hr data point is so close to the control might indicate that simultaneous addition to the cells of the diepoxide and the labelled acetate does not result in a noticeable decrease of [^{14}C]cholesterol synthesis). Nevertheless, approximately 3.5 hr of treatment with the diepoxide are required to reduce the apparent cholesterol biosynthesis and the activity of the reductase in HepG2 cells by half; in comparison, a half-life of about 2 hr for HMG-CoA reductase was estimated in cultured rat hepatocytes [27]. If the diepoxide acts only by channelling the cyclase towards the regulatory pathway (see Fig. 2), the relative match of the two curves in Fig. 6 would imply a virtual absence of a lag period between the formation of a repressor molecule from 2,3:22,23-dioxidosqualene which had penetrated the cells and the inhibition of cholesterol biosynthesis. This seems improbable (see below). Several conclusions may be drawn from these data. (i) Exogenous 2,3:22,23-dioxidosqualene, which must be readily taken up by HepG2 cells, is able to rapidly inhibit cholesterol biosynthesis both by inhibiting endogenous 2,3-oxidosqualene- cyclization and by establishing the regulatory pathway resulting in a repression of HMG-CoA reductase activity. (ii) The ultimate metabolite responsible for the decrease in reductase activity is probably 24,25-epoxycholesterol, a known repressor of this enzyme (see Introduction). It was found by Panini *et al.* [26] that 2,3:22,23-dioxidosqualene does not inhibit the isolated reductase and moreover has no effect on HMG-CoA reductase activity in cultured rat intestinal epithelial cells treated with the cyclase inhibitor U18666A. An intermediary metabolite such as 24,25-epoxylanosterol [27] (see Fig. 2) could, however, also be responsible for suppressing the reductase, but it does not seem to accumulate, to a measurable extent, in HepG2 cells treated with **1**. (iii) In HepG2 cells, HMG-CoA reductase activity can be almost completely suppressed via the regulatory pathway, triggered by 2,3:22,23-dioxidosqualene. The fact that treatment of the cells with **1** resulted only in a partial diminution (about 50%) in reductase activity, indicates that, at high concentrations ($\geq 10^{-6}$ M), the

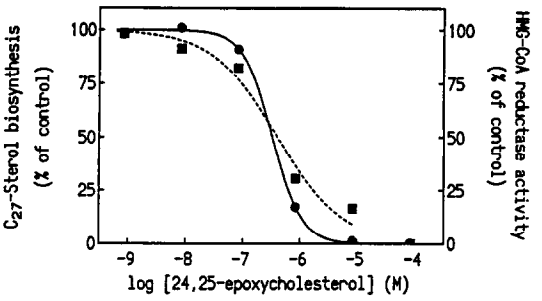

Fig. 7. Influence of the concentration of 24,25-epoxycholesterol on the biosynthesis of C_{27}-sterols and HMG-CoA reductase activity in HepG2 cells. Cells which had been grown for 24 hr in a delipidated medium were incubated for 4 hr in the presence of the indicated concentration of the epoxide. They were further pulsed for 2 hr with [^{14}C]acetate to determine the biosynthesis of the C_{27}-sterols (—●—) or collected to assay the HMG-CoA reductase activity (- -■- -). The 100% values (control) corresponds to 98.7 × 10^3 dpm/mg protein and to 81 pmol mevalonate formed/min/mg protein, respectively.

8-azadecalin (**1**) must inhibit cholesterol biosynthesis principally by blocking the cyclase.

We wanted to verify if one could also manipulate the regulatory pathway by giving 2,3-oxidosqualene to HepG2 cells. In striking contrast to the data obtained with 2,3:22,23-dioxidosqualene, when these cells were treated with unlabelled 2,3-oxidosqualene (up to 10^{-4} M) no inhibition of [^{14}C]-cholesterol formation and no accumulation of [^{14}C]-2,3-oxidosqualene could be observed. When the cells were given [3-^{3}H]-*R*,*S*-2,3-oxidosqualene (1.65 μM) for periods up to 24 hr, a limited cellular uptake of the mono-epoxide cells (less than 25%) was found; moreover the majority of the recovered counts remained associated with unmetabolized mono-epoxide whereas only a small fraction ended up in cholesterol (e.g. 5% at 8 hr) and a more sizeable one in 2,3:22,23-dioxidosqualene (e.g. 20% at 8 hr). It therefore seems that cellular uptake and the fate of squalene mono- and diepoxides are somewhat different. In this respect it would be interesting to investigate, for both epoxides, the role of the "protein supernatant factors" which were found by the Bloch group to influence their translocation and metabolization [28].

Effect of HepG2 treatment with 24,25-epoxycholesterol on sterol biosynthesis and HMG-CoA reductase activity.

The efficient inhibition of cholesterol biosynthesis and suppression of HMG-CoA reductase activity by 2,3:22,23-dioxidosqualene could in principle be ascribed to its transformation into 24,25-epoxycholesterol. We wanted to verify the effect of this oxysterol on the two parameters when given exogenously to HepG2 cells. As shown in Fig. 7, this compound behaves similarly to squalene diepoxide and affects both parameters in a dose-dependent fashion; an IC_{50} approximately 0.5 μM could be estimated from these curves. Similar data

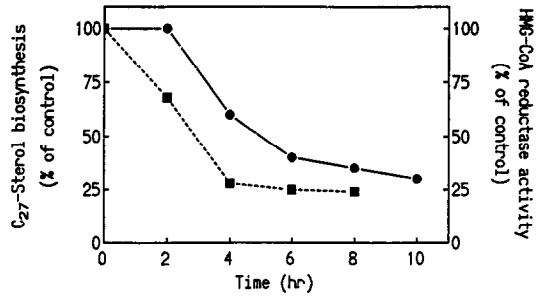

Fig. 8. Time-course of HMG-CoA reductase activity repression and C_{27}-sterols biosynthesis inhibition upon treatment of HepG2 cells with 24,25-epoxycholesterol. Cells which had been grown for 24 hr in a delipidated medium were incubated for the given times with 0.7 μM 24,25-epoxycholesterol. The cells were then collected for the determination of HMG-CoA reductase activity (--■--). To determine the biosynthesis of the C_{27}-sterols (—●—), the cells were pulsed with [^{14}C]acetate for the last 2 hr of incubation. The results are expressed as percentages of [^{14}C]-C_{27}-sterols and reductase activities measured in controls which were run for each time point in the absence of the epoxide; at t_0, 100% corresponds to an average of 89 pmol mevalonate formed/min/mg protein and 127 × 10^3 dpm/mg protein, respectively.

have been found on the regulation of the HMG-CoA reductase by 24,25-epoxycholesterol in other cultured mammalian cells [7, 19, 25]. As above, real IC_{50} values must be smaller since in the racemic mixture, 24(*S*),25-epoxycholesterol is the naturally occurring enantiomer. According to Taylor *et al.* [7], the 24(*S*),25-epimer is approximately twice as active as the *R* form in repressing the reductase. In contrast to the squalene diepoxide, the only possibility for 24,25-epoxycholesterol to down-regulate the formation of [^{14}C]-C_{27}-sterols is through the repression of the reductase. Kinetic studies were also performed with cells treated at a fixed (0.7 μM) concentration of 24,25-epoxycholesterol (Fig. 8). Under these conditions HMG-CoA reductase activity and [^{14}C]-C_{27}-sterol biosynthesis plateaued at 24 and 30% of control, respectively. As shown in Fig. 8, the decrease in reductase activity is rapid, $t_{1/2}$ is about 2.2 hr; in contrast the inhibition of cholesterol biosynthesis is somewhat slower (apparent $t_{1/2}$ about 3.8 hr). Although the restrictions mentioned above with 2,3:22,23-dioxidosqualene for the comparison of the such kinetics apply here as well, the curves in Figs 6 and 8 are nevertheless somewhat different. The difference in apparent $t_{1/2}$ observed in Fig. 8 could account for the fact that the inhibition of cholesterol biosynthesis by 24,25-epoxycholesterol is mediated by a decrease in reductase activity; i.e. the oxysterol must first suppress the reductase before an effect on the [^{14}C]acetate incorporation becomes measurable. There are no data in the literature which allow a direct comparison with the rates observed here. Other oxysterols such as 25-hydroxycholesterol, known to induce a very rapid loss of HMG-CoA reductase activity by suppressing enzyme synthesis and enhancing enzyme degradation, have a $t_{1/2}$ of e.g. 30 min in rat intestinal epithelial IEC-6 cells [29]; the reductase in rat cultured hepatocytes treated with 24,25-epoxylanosterol has a $t_{1/2}$ of 46 min [27]. In conclusion, 24,25-epoxycholesterol presents, in HepG2 cells, an important bioavailability and its potent inhibition of cholesterol biosynthesis is well correlated with the suppression of HMG-CoA reductase activity. This is again different from the mode of action of the 8-azadecalin (**1**). Related to the interesting possibility of using 24,25-epoxycholesterol as a hypocholesterolemic agent, it is well known that, at cellular level, the repression of HMG-CoA reductase is due to the compound itself and not to a metabolite [19]; moreover some bioavailability studies have recently been performed [30].

The mechanism by which 24,25-epoxycholesterol represses HMG-CoA reductase activity is not well known. One mode of action has been ascribed, in analogy with other oxysterols which bind to oxysterol-binding protein, to the regulation of the reductase transcription [7]. Interestingly, 24,25-epoxylanosterol, which also represses HMG-CoA reductase, but which does not bind to the oxysterol-binding protein, was recently shown to down-regulate the reductase at a post-transcriptional level [27]. Preliminary results (Schuber F and Roth M; to be published) indicate that treatment of HepG2 cells with 24,25-epoxycholesterol resulted in a decrease in HMG-CoA reductase mRNA levels. In 2,3:22,23-dioxidosqualene treated cells an increase of the messenger level was observed, more in line with the absence of effect by 24,25-epoxylanosterol or with the increase of mRNA observed in HepG2 cells treated with U18666A [31].

Conclusion

We wanted to test if an inhibitor of 2,3-oxidosqualene-lanosterol cyclase such as 8-azadecalin (**1**), could act synergistically on cholesterol biosynthesis. Such a mode of action would be attractive since this compound could act by a combination of a classical enzyme inhibition, and thus slow down the biosynthetic pathway at a non-rate limiting step (Fig. 2 Pathway A), and by the generation of a regulatory pathway, acting up-stream by repressing the main rate-limiting enzyme of the cholesterol biosynthetic pathway (Fig. 2, Pathway B). Such a possibility exists since 2,3:22,23-dioxidosqualene, which initiates the regulatory pathway, is a preferential substrate of the cyclase. From the present studies, it seems that the situation is not as clear-cut. It is striking that the metabolites of the regulatory pathway, i.e. 2,3:22,23-dioxidosqualene and 24,25-epoxycholesterol, are able to almost completely suppress HMG-CoA reductase activity in HepG2 cells, this event being correlated with an inhibition of cholesterol biosynthesis. In contrast, 8-azadecalin (**1**) at 10 μM, the highest concentration used (i.e. 100 × IC_{50} observed for the C_{27}-sterol biosynthesis inhibition), leads to only a 50% reduction in reductase activity. This could mean that in cells treated with a cyclase inhibitor, intracellular concentrations of 24,25-epoxycholesterol are not high enough, through the regulatory pathway, to be able to completely down-regulate HMG-CoA reductase activity. In

intermediary inhibitor concentrations, however, the possibility for a synergistic effect exists. Thus in the 10^{-8}–10^{-6} M inhibitor range, an accumulation of 24,25-epoxycholesterol is observed which is paralleled by a decrease of cholesterol biosynthesis, i.e. we observe an inhibition of the cyclization of 2,3-oxidosqualene and a decrease in the metabolic flux due to reduced HMG-CoA reductase activity. One can therefore conclude that a synergistic effect of cyclase inhibitors exists in a limited window of concentration; it seems, however, that the direct inhibition of the cyclase represents the main contribution of these series of drugs to cholesterol inhibition. Since 2,3-oxidosqualene-lanosterol cyclase is not limiting in cholesterol biosynthesis, our results suggest that very powerful inhibitors of this enzyme will be needed to make useful hypocholesterolemic drugs; it would be of interest to know the effect of such molecules in *in vivo* systems. Ultimately, the significance for an organism of such a regulatory pathway which is uncovered and amplified by cyclase inhibitors has yet to be determined.

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