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DNA SYNTHESIS AND 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE ACTIVITY IN PHA STIMULATED HUMAN LYMPHOCYTES : A COMPARATIVE STUDY OF THE INHIBITORY EFFECTS OF SOME OXYSTEROLS WITH SPECIAL REFERENCE TO SIDE CHAIN HYDROXYLATED DERIVATIVES.

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The effects of a series of oxygenated sterols on DNA synthesis and HMG-CoA reductase activity were tested in human lymphocytes. The cells were stimulated by PHA and cultured in cholesterol containing medium. The inhibitory effects of sterols on DNA synthesis were strictly related to the position and the configuration of the hydroxyl on the side chain, to the side chain conformation and integrity and to the structure of the sterol nucleus. The inhibition of HMG-CoA reductase activity was less dependent on these structural features since all the sterols tested were strong inhibitors. In our experimental conditions the inhibition of DNA synthesis was not related to the suppression of the HMG CoA reductase activity. The specificity of the structures required for DNA synthesis inhibition could be explained by the involvement of a specific hydroxysterol binding protein.

1. INTRODUCTION

Some oxygenated sterols are potent inhibitors of sterol biosynthesis by suppressing the 3-hydroxy-3-methylglutaryl CoA reductase activity (1). This sterol synthesis inhibition seems to lead to the inhibition of DNA synthesis and cell division in some fibroblastic cell lines (2,3) and in mouse (4) or human (5) lymphocytes stimulated to divide by lectins and cultured in delipidated or cholesterol-poor media.

In a recent paper (6), we demonstrated that the HMG CoA reductase inhibition by sterols oxygenated at C_7 or C_{20} did not obligatorily involve an inhibition of DNA synthesis of PHA stimulated human lymphocytes provided the cells were cultured in a medium containing sufficient quantities of cholesterol (1 mM) : in these conditions, the lack of endogenous cholesterol

Abbreviations : HMG-CoA : hydroxymethylglutaryl coenzyme A
PHA : phytohemagglutinin.

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synthesis is compensated by the uptake of external cholesterol ; nevertheless, 25-hydroxycholesterol, another side chain hydroxylated sterol, was able to suppress both sterol and DNA synthesis. In order to determine whether the inhibitory effect on activated lymphocytes DNA synthesis was actually specific to the position of the hydroxyl on the side chain, we compared the effects on the HMG-CoA reductase activity and DNA synthesis of a series of side chain hydroxylated derivatives of cholesterol, lanosterol, cycloartenol and cholecalciferol.

2. MATERIAL AND METHODS :

2.1. *Materials* :

2.1.1. *Oxygenated sterols origin* :

Graciously donated

J.P. RAYNAUD (Roussel-Uclaf, Romainville, France) : cholest-5-ene-3β, 25-diol (25-hydroxycholesterol).

G. OURISSON and LUU BANG (Strasbourg, France) : cholest-5, 23-diene-3β, 25-diol (23-dehydro-25-hydroxycholesterol) ; cholest-5, 20(22)diene-3β, 25-diol (20, (22)-dehydro-25-hydroxycholesterol) ; lanost-8,25-diene-3β, 24-diol (24-hydroxy-25-dehydrolanosterol) ; 9,19-cyclo-lanost-25-ene-3β, 24-diol (24-hydroxy-25-dehydrocycloartenol).

J.E. VAN LIER (Sherbroke, Quebec) : 3β-hydroxycholest-5-ene-22-one (22-oxocholesterol) ; cholest-5-ene-3β, 22(S)-diol (22(S)-hydroxycholesterol) ; cholest-5-ene-3β, 22(R)-diol (22(R)-hydroxycholesterol) ; cholest-5-ene-3β, 22, 23-triol (22, 23-dihydroxycholesterol) ; 3β, 23-dihydroxycholest-5-ene-22-one (22-oxo-23-hydroxycholesterol) ; cholest-5-ene-3β, 23(S)-diol (23(S)-hydroxycholesterol) ; cholest-5-ene-3β, 23(R)-diol (23(R)-hydroxycholesterol) ; cholest-5-ene-3β, 24(S)-diol (24(S)hydroxycholesterol) ; cholest-5-ene-3β, 24(R)-diol (24(R)-hydroxycholesterol) ; cholest-5-ene-3β, 26 (25 R)-diol (25(R)-26-hydroxycholesterol) ; 26-nor-cholest-5-ene-3β, 25-diol (26-nor-25-hydroxycholesterol).

Commercial origin

From Hoffman-La Roche (Bâle, Switzerland) : 9,10,seco-5,7,10(19)-cholestatriene-3β, 25-diol (25-hydroxycholecalciferol) ; 9,10-seco-5,7,10(19)-cholestatriene-1α, 3β, 25-triol (1α, 25-dihydroxycholecalciferol) ; 9,10-seco-5,7,10(19)-cholestatriene-3β, 24(R), 25-triol (24(R), 25-dihydroxycholecalciferol).

2.1.2. *Other materials* :

[Methyl-^{3}H] thymidine (25 Ci/mmole), C.E.A. ; DL-3-hydroxy-3-methyl-3 [^{14}C]-glutaryl CoA (49,5 mCi/mmole), New England Nuclear Corp. ; DL-mevalonic acid-2-[^{3}H] lactone (382 mCi/mmole), Amersham ; 3-hydroxy-3-methylglutaryl CoA sodium salt, PL Biochemicals ; DL-mevalonic acid lactone, dithioerythritol, Sigma ; NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, Boehringer ; EDTA, Merck ; Kyro EOB, Procter and Gamble ; sodium metrizoate solution, Flobio ; Ficoll 400, Pharmacia ; Trypan Blue, Flow Laboratories ; gentian violet and bactophytohemagglutinin-P, Difco ; butyl-PBD, naphtalene and phenyl ethylamine, Koch-Light Laboratories ; scintillation fluid Ready Solv HP Beckman ; organic solvents, Prolabo (RP grade) or Merck ; microtest II plates, Falcon ; Kieselgel 60F254 plates for thin-layer chromatography, Merck.

Multiple automatic sample harvester : Titertek skatron ; oxidizer : Oxymat Intertechnique ; radioactivity counting, Packard Tri-Carb liquid scintillation spectrometer Model 3320 or 460C.

2.2 *Methods* :

Collection and separation of human peripheral lymphocytes were achieved as previously described (7,8). Cells were cultured in sterile RPMI-1640 (Gibco) buffered with Hepes (40 mM) (Sigma), pH = 7.4 containing 142 mg/l gentamicin sulfate (Unilabo) supplemented with 20 % human AB serum (gift from voluntary donors).

Blastic transformation was evaluated by [^{3}H]-thymidine incorporation of PHA stimulated human lymphocytes according to (9). The effect of oxygenated sterols on blastic transformation was determined following the method previously described (6) ; in the results, the inhibition of blastic transformation (%) was expressed as $(1-\frac{I}{C}) \times 100$ (I = dpm of [^{3}H]-thymidine incorporated by the cells cultured with oxygenated sterol, C = dpm incorporated by control cells) at the concentration of PHA giving the optimal stimulation.

The determination of HMG-CoA reductase activity is described in (8) ; the oxygenated sterol was added in the culture medium simultaneously with PHA and the assays of HMG-CoA reductase activity were made after 18 hr of culture. Results are expressed as a percentage of inhibition.

Oxygenated sterols were added to the culture medium in ethanolic solution (final concentration of ethanol : 1 % v/v).

3. RESULTS :

None of the oxygenated sterols tested were cytotoxic as demonstrated by the Trypan Blue exclusion test and the cell count performed after 3 days of culture in absence of PHA (in presence of PHA, lymphocyte agglutination rendered erroneous the cell count).

3.1. *Effects on DNA synthesis* :

3.1.1. *Derivatives of cholesterol* :

Table I shows the effect of 14 compounds derived from cholesterol on DNA synthesis of stimulated lymphocytes.

At the concentration of 25 µg/ml (62.5 µM), the derivatives of cholesterol hydroxylated on the carbon 26, 25, 24(S) and 23(S) were potent inhibitors of the [^{3}H] thymidine incorporation into DNA ($\simeq$ 80 to 96 %). The relative inhibitory potencies were related to the configuration of the hydroxyl function : 23(S)-and 24(S)-hydroxycholesterol were more effective than 23(R) and 24(R)-hydroxycholesterol. This effect was particularly evident for the 23-hydroxylated derivatives : 80 % inhibition for the 23(S) versus 17 % for the 23(R) epimer. The compounds bearing an oxygenated function on the carbon 22 (22(S)-hydroxy-, 22(R)-hydroxy-or 22-oxocholesterol) did not appreciably affect the [^{3}H] thymidine incorporation ($<$ 25 %) ; the addition of a hydroxyl function in 23 enhanced the inhibitory effect (30 to 44 %). The 26-nor-25-hydroxycholesterol was 2-fold less inhibitory than the 25-hydroxycholesterol.

TABLE I : Effects of side chain hydroxylated derivatives of cholesterol on DNA synthesis and HMG-CoA reductase activity.

Sterol added. \ Sterol concentration (μg/ml)	Inhibition of DNA synthesis (%)		Inhibition of HMG-CoA reductase activity (%)	
	25	5	25	5
25-OH	90 (13)* (82-97)**	14(2) (10-18)	97(3) (96-97)	95(3) (92-96)
23-dehydro-25-OH	97(3) (95-98)	28	96	95
20(22)-dehydro-25-OH	8(4) (0-20)	0	84	68
26-nor-25-OH	56(2) (45-66)	0	nd	89
26-OH	85(3) (74-93)	9	nd	96
24(S)-OH	93(3) (88-96)	10	97	87
24(R)-OH	49(3) (16-76)	8	96	85
23(S)-OH	79(2) (74-83)	12	nd	89
23(R)-OH	17(3) (0-39)	0	nd	30(2) (26-34)
22,23-diOH	30 (3) (28-31)	nd	nd	nd
22-oxo-23-OH	44 (3) (25-64)	nd	nd	nd
22(S)-OH	7(3) (3-12)	nd	95	67
22(R)-OH	24	nd	nd	nd
22-oxo-	14(2) (0-27)	nd	96	51

* *mean of x experiments ; x = number in brackets*
** *minimal and maximal values*
nd = not determinated.

The introduction of a double bond on the side chain modified the inhibitory capacity of the 25-hydroxycholesterol : the 23-dehydro-25-hydroxycholesterol suppressed the DNA synthesis more than the 25-hydroxycholesterol, whereas the 20,(22)-dehydrocompound was ineffective on [^{3}H] thymidine incorporation.

The most inhibitory derivatives were still good inhibitors at the concentration of 12.5 μg/ml, but none of these had an effect at 5 or 2.5 μg/ml.

TABLE II : Effects of side chain hydroxylated derivatives of cholecalciferol, lanosterol and cycloartenol on DNA synthesis and HMG-CoA reductase activity.

Sterol added. / Sterol concentration (µg/ml)	Inhibition of DNA synthesis (%)		Inhibition of HMG-CoA reductase activity (%)	
	25	5	25	5
25-OH-cholecalciferol	65 (3)* (61-73)**	14(2) (0-28)	93(3) (78-100)	63(3) (45-77)
24(R),25-diOH cholecal.	73(3) (62-85)	14(2) (0-28)	93(2) (92-95)	66(2) (60-72)
1α,25-diOH-cholecal.	52(3) (36-66)	19(3) (0-33)	20(3) (0-56)	20(3) (0-45)
24-OH-25-dehydrolanosterol.	6(4) (3-11)	0	100	93
24-OH-25-dehydrocycloartenol.	25(4) (21-29)	0	100	99

* *Mean of experiments. In brackets : number of experiments.*

** *Minimal and maximal values.*

3.1.2. Derivatives of cholecalciferol and other side chain hydroxylated sterols :

Table II shows that the side chain hydroxylated derivatives of cholecalciferol were quite good inhibitors of DNA synthesis at the concentration of 25 µg/ml. The 1,25-dihydroxylated compound was less active than the 25-hydroxylated or 24,25-dihydroxylated ones : respectively 52, 65 and 73 % inhibition. At the concentration of 5 µg/ml, all three were ineffective ($\simeq$ 15 %).

The 24-hydroxy-25-dehydrolanosterol and 24-hydroxy-25-dehydrocycloartenol were poor inhibitors at 25 µg/ml and no inhibitors at 5 µg/ml.

3.2. *Effect on the HMG-CoA reductase activity* :

3.2.1. Derivatives of cholesterol :

Owing the small amounts of the sterols available, all the 14 compounds studied on DNA synthesis were not tested for their activity on HMG-CoA reductase. For the same reason, some were only studied at a concentration of 5 µg/ml.

At 25 µg/ml (table I) : 25,24(S), 24(R), 22(S)-hydroxycholesterol and 22-oxocholesterol were potent inhibitors (> 95 %). The 23-dehydro-25-hydroxycholesterol had the same effect as the 25-hydroxycholesterol (> 95 %), but the 20(22)-dehydro-25-hydroxycholesterol was slightly less inhibitory (84 %).

At the concentration of 5 µg/ml, the differences between the inhibitory capacities of these compounds appeared more clearly : 30 to 68 % inhibition was observed with the 20(22)-dehydro-25-, 23(R)-and 22(S)-hydroxycholesterol and 22-oxocholesterol whereas more than 85 % was observed for the other hydroxycholesterols.

3.2.2. Derivatives of cholecalciferol and other side chain hydroxylated sterols :

At 25 µg/ml (table II), the 25-hydroxy and the 24,25-dihydroxycholecalciferol almost completely inhibited the HMG-CoA reductase activity (≃ 90 %), contrary to the 1,25-dihydroxylated compound (20 %). The inhibitory capacity of the first two sterols was still appreciable at 5 µg/ml.

At 5 or 25 µg/ml, the 24-hydroxy-25-dehydrolanosterol and the 24-hydroxy-25-dehydrocycloartenol were strong inhibitors (≃ 100 %).

4. DISCUSSION :

25-hydroxycholesterol is known as potent inhibitor of HMG-CoA reductase activity and cellular growth in several fibroblastic cell lines (2, 3) and lymphocytes (4, 5). The present work is a comparative study of the effects of a series of other side chain oxygenated derivatives of cholesterol on DNA synthesis and HMG-CoA reductase activity of human lymphocytes stimulated by PHA. This model is a human experimental model easy to obtain ; the physiological cellular environment of lymphocytes can be maintained by adding human serum in the culture medium ; since circulating lymphocytes are resting cells (Go state), the induction of lymphoblastic transformation by lectin results in a pseudo-synchronized model.

Our results are difficult to compare with the literature since to our knowledge, most of the sterols of this series have not been studied in any animal or human cellular system.

Three general remarks can be made :

a) In our experimental conditions, none of these sterols are cytotoxic.

b) The present results as those of the preceding paper (6) show that the inhibitory effects of the oxygenated sterols on HMG-CoA reductase activity and on DNA synthesis can be dissociated since normal DNA synthesis

can be observed in spite of complete inhibition of HMG-CoA reductase activity. But when an oxygenated sterol is simultaneously inhibitor of these two processes, different concentrations are required to inhibit DNA synthesis (25 or 12.5 μg/ml) and HMG-CoA reductase activity (5 μg/ml). This difference between the effective concentrations remains unexplained until now.

c) Our results are expressed for the dose of PHA giving the optimal lymphocyte stimulation. But the inhibitory power of the hydroxylated sterols is constantly more elevated when non-optimal doses of PHA are used (data not shown). The same unexplained phenomenon was reported by others not only with 25-hydroxycholesterol (Yachnin, 10) but also with glucocorticoïds (Segel, 11) and prostaglandin E_2 (Goodwin, 12).

The effect on DNA synthesis of the sterols derivatives depends on five parameters :

a) hydroxyl position on the side chain : the inhibitory power increases as the distance of the hydroxyl function from the sterol nucleus increases.

b) hydroxyl configuration : the 23(S) and 24(S) compounds are more efficient than the corresponding (R) epimers. The inverse is observed with 22-hydroxylated derivatives : the 22(R) is a slightly better inhibitor of $[^3H]$ thymidine incorporation than 22(S) (likewise, Zander et al.(13) reported that the 22(R)-hydroxydesmosterol was more efficient than the 22(S) for inhibiting hepatoma cells growth with a cytolytic effect). The change in the optimal configuration of the hydroxyl on C22(R) in comparison with C23 and C24(S) is not surprising : the study of Dreiding's model shows that the most probable conformation of the side chain (maximal stretching) results in the orientation of the 22(R), 23(S) and 24(S) hydroxyl groups on the same side of the plane of the chain.

c) Side chain configuration and integrity : a Δ 20 desaturation suppresses the inhibitory power of the 25-hydroxylated derivative of cholesterol whereas a Δ 23 desaturation increases it ; moreover, the lack of a carbon atom decreases the inhibitory effect of the 25-hydroxycholesterol.

d) Structure of the polycyclic nucleus : this seems to be of some importance since the hydroxylated derivatives of vitamin D_3 are good inhibitors of lymphoblastic transformation whereas the hydroxylated derivatives of cycloartenol and lanosterol are not.

In conclusion, the position and the configuration of the hydroxyl on the side chain and the structure of the polycyclic nucleus seem to be

determinant for the effects of the cholesterol derivatives on the lymphoblastic transformation. This relationship appears less evident considering their effects on the HMG-CoA reductase activity.

At the concentration of 25 μg/ml, all the hydroxylated sterols tested are strong inhibitors of the HMG-CoA reductase ; but contrary to what is observed for the DNA synthesis, these hydroxysterols are potent inhibitors of the enzymatic activity even at 5 μg/ml. At this low concentration, the small differences observed in their inhibitory capacity are less dependent on the position of the hydroxyl on the side chain and on the (R) or (S) hydroxyl configuration. Similarly, the modification of the side chain configuration by the introduction of a desaturation or the loss of a carbon atom is less critical for the inhibition of the HMG-CoA reductase activity than for that of DNA synthesis (however, in an other cell system (L cells, 1), the 26-nor-25-hydroxycholesterol was reported as much less active inhibitor of HMG-CoA reductase activity than 25-hydroxycholesterol).

The structure of the polycyclic nucleus also seems to be not important since lanosterol, cycloartenol and vitamin D_3 hydroxylated derivatives are strong inhibitors, 1α,25-dihydroxycholecalciferol excepted. The hydroxylated compounds derived from the cholecalciferol have been shown to be inhibitors of HMG-CoA reductase activity in L_2C cells (14) : in this model, the 1,25-dihydroxylated compound was also less active than the 25-hydroxylated one. As for the hydroxylated derivatives of lanosterol, only the 32-hydroxylated ones have been tested : the Δ7-compound in activated human lymphocytes (15) and the Δ7 and Δ8 in L cells (16) ; in these two models, they appear less inhibitory than 25-hydroxycholesterol.

The mechanism of inhibition of HMG-CoA reductase by oxygenated sterols has been recently discussed : there is no direct inactivation either by 25-hydroxycholesterol alone or by 25-hydroxycholesterol-cytosolic protein complexes (16) ; a phosphorylation-dephosphorylation mechanism is not involved (17). Cavenee et al.(18) give evidence that the cell nucleus is necessary for HMG-CoA reductase inhibition by hydroxysterols and they suggest as Sinensky et al.(19) that 25-hydroxycholesterol could decrease the synthesis of the enzyme.

Several hypotheses on the mechanism of the inhibition of lymphoblastic transformation by hydroxylated sterols can be considered :

a) The effect of the hydroxysterols on the cell division is generally thought to be related to their inhibitory effect on endogenous cholesterol biosynthesis (2,3,4). Our work (as our previous report (6)) shows that there is a dissociation between the inhibition of the HMG-CoA reductase activity and the inhibition of the DNA synthesis : some sterols strongly inhibit this en-

zyme without affecting DNA synthesis because in our experimental conditions the cells are independent on their endogenous sterol synthesis (the culture medium is supplemented with lipoproteins (20 % human AB serum) and the external cholesterol can be taken up by the lymphocytes). Nevertheless, in the same culture conditions, other hydroxylated derivatives of cholesterol inhibit the DNA synthesis : this specific inhibitory effect is dependent on the position and the configuration of the hydroxyl on the side chain.

Chen (20) and Cuthbert et al.(21) have demonstrated that the inhibition of the lymphoblastic transformation by the 25-hydroxycholesterol or 7-oxocholesterol (1 µg/ml in delipidated or cholesterol-poor medium) was reversed by the addition of mevalonate or cholesterol to the culture medium. These results are not contradictory to ours since in our experimental conditions we observed no effect on DNA synthesis at the concentration of 5 µg/ml. Moreover, Cuthbert et al.(21) reported no reversion by the addition of mevalonate when the concentration of hydroxylated sterol was increased and concluded an action of hydroxysterols on DNA synthesis independent on their action on sterol synthesis.

b) Recently it has been demonstrated that fibroblastic cell growth requires certain mevalonate derived isoprenoid compounds : dolichol (22,23), ubiquinone (24) or isopentenyl t RNA (25). A low residual HMG-CoA reductase activity could be sufficient to ensure their production (26). But when HMG-CoA reductase activity is totally abolished (as is the case for the lanosterol or cycloartenol 24-hydroxy-derivatives in our study), the production of these isoprenoid compounds should be totally suppressed ; nevertheless, no DNA synthesis inhibition occurs in the lymphocytes. Thus, it does not follow from our study that the biosynthesis of the isoprenoid compounds could be a key process controlling cell division.

c) Havel et al.(27) have demonstrated an increased synthesis of methyl sterols by primary rat hepatocyte cells cultured in the presence of 25-hydroxycholesterol. Likewise an impaired demethylation of lanosterol is described by Ortiz de Montellano et al.(28) in HTC cells cultured in the presence of 7α , 7β -hydroxycholesterol or 22(R)-hydroxydesmosterol. The methyl sterols are considered as cytotoxic (29) and their accumulation could be involved in the inhibition of DNA synthesis when activated lymphocytes are cultured in presence of hydroxysterols. Such an accumulation cannot occur since we have not observed any cytotoxicity after three days of culture ; moreover, Ortiz de Montellano et al.(28) showed that there was no parallelism between the cytotoxicity of the sterols and the cellular accumulation of the methyl sterols.

d) The "membrane insertion hypothesis" (Yachnin, 30) of the hydroxylated sterols does not seem tenable : indeed, in spite of the high external

concentration necessary to obtain an inhibition of lymphoblastic transformation, we have determined by capillary gas chromatography that the quantity of the 25-hydroxycholesterol in the lymphocytes represents only 1 % of total cell cholesterol. This very low level could not be responsible for variations of membrane fluidity of permeability interfering with the primary phenomena of activation by lectins.

e) Our hypothesis is that the effect of the hydroxysterols is mediated by a specific cytosolic binding protein on account of the strict specificity of the hydroxysterols acting on DNA synthesis. We have shown evidence for such a binding protein specific for the side chain hydroxylated sterols in the cytosol of the resting lymphocytes (31). Such a mechanism has been put forward by Kandutsch et al.(16) to explain the inhibitory effect of the hydroxysterols on the HMG-CoA reductase.

The physiological role of these hydroxylated sterols still remains speculative.

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