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THE IMPORTANCE OF SERUM LIPOPROTEINS IN THE CYTOLYTIC ACTION OF 7β-HYDROXYCHOLESTEROL ON CULTURED HEPATOMA CELLS

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SUMMARY: The toxicity of 7β-hydroxycholesterol for cultured HTC cells is 10 times greater if serum lipids and lipoproteins are absent from the culture medium. A water-soluble derivative of 7β-hydroxycholesterol, sodium 3,7-bishemisuccinate, showed the same toxicity as the original molecule and was also 8 times more toxic when serum lipids and lipoproteins were absent. But the rapid inhibition of DNA synthesis was similar in cells treated with both compounds, whether lipids and lipoproteins are present or not. Thus the absence of serum lipids and lipoproteins enhances the lytic effect of both substances but does not increase their intracellular action on DNA synthesis. This first parallel study on lipophilic 7β-hydroxycholesterol and its water-soluble homologue shows the importance of the serum lipids and lipoproteins in the cytotoxicity of such sterols.

Many authors have reported that hydroxysterols inhibit cholesterol synthesis in mammalian cells (1-6). The most powerful inhibitors were 25- and 20-hydroxycholesterol which affect sterol biosynthesis at concentrations of 10^{-6} to 10^{-7}M (7). Their action mechanism is still hazy, though they are held to interfere with 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.3.4.), which catalyses mevalonic acid formation from HMG-CoA. We found earlier that 25- and also 7β-hydroxycholesterol (10^{-5}M) inhibited HMG-CoA reductase activity in cultured HTC cells (8). Kandutsch et al. reported that cells cultured with 25-hydroxycholesterol show abnormal features if extracellular cholesterol carried by serum lipoproteins is absent. They can no longer synthesise DNA and remain blocked in a reversible growth stage, at the G_1 cell cycle phase (5,9). Their transport of cations, e.g., Rb^+ and K^+, is abnormal (10) and the cholesterol/phospholipid ratio in the plasma membranes is lower than in untreated cells (1). Since all these functional and biochemical anomalies are reversible with cholesterol or mevalonate in culture medium containing 25-hydroxy

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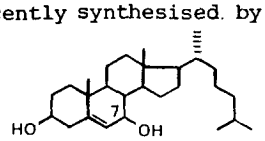
cholesterol, they have been attributed to lack of cholesterol. However, Yachnin et al. showed that the ability of some hydroxysterols to inhibit sterol synthesis has little or no relation to their ability to modify cells, e.g., by provoking E-rosettes in the lymphocytes (11), by annulling chemotaxis in the leucocytes (12), or by changing red globules into echinocytes (13). They envisaged the insertion of the hydroxysterols into cell plasma membranes, and showed that serum lipoproteins modulate this insertion into human red globules (14). They attributed this to the ability of lipoproteins to bind 50 to 80% of hydroxysterols which cannot then penetrate the cells. In contrast, we found that 7β-hydroxycholesterol was highly toxic for cultured HTC cells, with rapid cell lysis, even with lipoproteins in the culture medium (15-17).

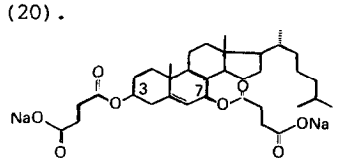
In this paper we show that the toxic effect of 7β-hydroxycholesterol (7-HC) is 10 times greater when serum lipids and lipoproteins are absent from the culture medium, but the inhibition of DNA synthesis is not enhanced in cells treated with this sterol and protein synthesis is not affected. The sodium 3,7-bishemisuccinate (7-HC BHS), water-soluble derivative of 7-HC, shares the same toxicity, and is also 8 times more cytotoxic when lipids and lipoproteins are absent. Its effect on the synthesis of DNA or proteins remains similar whether lipoproteins are present or not. Finally, in lipid- and lipoprotein-deprived medium, both products are more toxic for cultured HTC cells and at concentrations which do not affect the synthesis of DNA or proteins.

MATERIALS AND METHODS

Chemistry: 7β-hydroxycholesterol was first extracted in our laboratory from the Chinese drug "Bombyx cum Botryte" long reputed in traditional medicine (18, 19) and then synthesised from cholesterol by Cheng et al. (15). The water-soluble sodium salt of 7β-hydroxycholesterol bishemisuccinate was more recently synthesised by one of us, Sh. Rong (20).



7β-hydroxycholesterol (7-HC)



sodium 3,7-bishemisuccinate of 7β-hydroxycholesterol (7-HC BHS)

Cytotoxicity tests: The tests were performed using the HTC cell-line (21) derived from a rat Morris hepatoma. Cells were cultured in suspension in Swim's S77 medium containing 10 % newborn calf serum (Gibco), either whole or minus lipids and lipoproteins. The latter was prepared according to Watson et al. (22). The

serum, enriched with 3.2 g of anhydrous KBr per 100 ml of serum, was centrifuged for 48 h in 30 ml polycarbonate tubes at 30 000 rpm in a Beckman S 30 rotor. The upper fractions from the tubes (5 ml) were discarded and the lower lipoprotein-deprived fractions (25 ml) were dialysed for 72 h against a 0.15M NaCl solution enriched with 0.05 % of L-serine. The dialysis solution was renewed every 24 h , at 4 l per 100 ml of serum. Serum was then sterilised by ultrafiltration and stored at -20°C. Cells were grown in 75 ml culture flasks with magnetic stirring, at 37°C, in normal atmosphere. The initial concentration was adjusted to 10^5 cells/ml. Under these conditions, cell growth was exponential for about 3 days, with a generation time of 28 h., before stabilising at a density of 6 to 7.10^5 cells/ml. The sterols, dissolved either in ethanol (7-HC) or in water (7-HC BHS) were added at various concentrations. Control tests showed that ethanol (100 µl/75 ml of culture) had no effect on culture development. Cell growth was evaluated from cell-counts with a Neubauer microcytometer, and the number of living cells was determined by the Trypan blue exclusion test (23). Cell viability is given for different times as the ratio of living cells to initial cells x 100.

DNA and protein synthesis: The incorporation of ^{3}H-thymidine or ^{3}H-leucine in HTC cells cultured in suspension, at a density of 4.10^5 cells/ml, was measured by pulse labelling. After various periods of exposure to the sterols, 2 ml samples of the cell suspensions were incubated in test tubes for 30 min with either 25 µl of ^{3}H-leucine (Amersham, spec. act. 40 Ci/mM, total activity 625 nCi/ml), or with 25 µl of methyl-^{3}H-thymidine (spec. act. 24 Ci/mM, total activity 156 nCi/ml). The samples were then washed by centrifugation at 1000 rpm with a buffer solution (10 mM TRIS, 0.15 M NaCl, pH 7.4) to eliminate free radioactivity. The final cell pellet was suspended in NaOH 1 N and hydrolysed for 30 min at 50°C. Protein content was measured on 100 µl aliquots according to Lowry and 300 µl aliquots were precipitated with 1 ml of 15 % cold trichloroacetic acid . The precipitates were collected on Millipore HAWP filters (pore Ø: 0.45 µm), washed with 5 % trichloroacetic acid and with 95 % ethanol. The dried filters were dissolved in scintillation liquid (Filter-Solv ^{TM}HP, Beckman) and radioactivity was measured with an Intertechnique SL 32 liquid scintillation counter. Results are given either in cpm/mg of protein or as % of incorporation in control culture. At each stage, cell viability was tested on a cell suspension aliquot as already described, and given as a % of that in the control.

RESULTS

1 - The cytolytic effect of 7β-hydroxycholesterol.

Fig. 1 shows viability curves for HTC cells cultured for 3 days with various 7-HC doses. In cell cultures containing 10 % whole calf serum (fig. 1a), 80 µM of 7-HC caused the lysis of all cells within 3 days, while 160 µM had the same effect within 24 h. If lipid- and lipoprotein-deprived calf serum was used (fig. 2b), cell mortality occurred at far lower doses : with 8 µM of 7-HC, all cells were lysed in 3 days, and with 16 µM, in 24 h. Thus, 7-HC is 10 times more toxic for HTC cells cultured in a lipoprotein-deprived medium. The effect is proportional to the dose and exposure time.

Water-soluble 7-HC BHS had the same toxic features (data not shown), i.e., rapid cell lysis depending on dose and exposure time. At concentrations of 160 µM in whole medium and 20 µM in lipoprotein-deprived medium, it was fatal

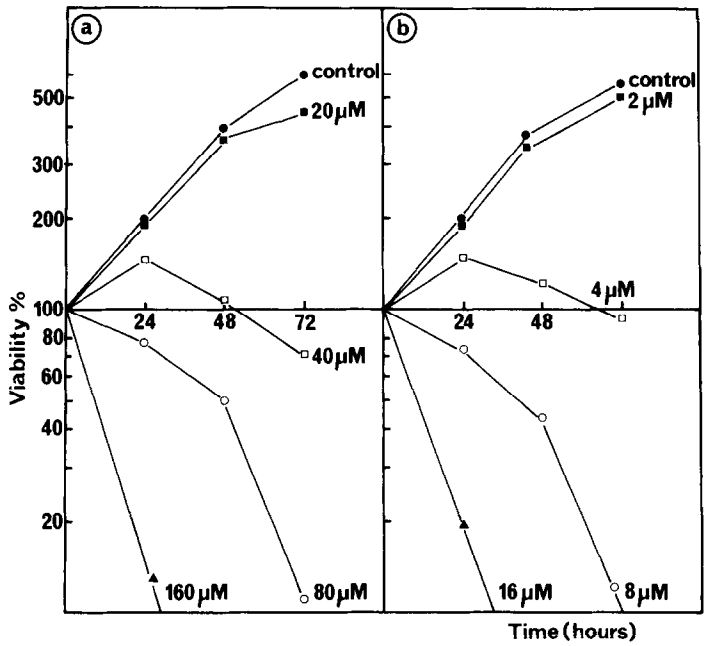


Figure 1 : Viability curves of HTC cells treated with various 7-HC doses in function of time. Cells were cultured in a medium containing 10 % calf serum either whole (a) or deprived of lipids and lipoproteins (b). Results show living cells as a % of initial cells.

for all cells after 3 days. Despite being water-soluble, 7-HC BHS is slightly less toxic than 7-HC, but its toxicity is likewise enhanced, by a factor of 8, in a medium containing lipoprotein-deprived calf serum.

2 - The effect of 7β-hydroxycholesterol on DNA and protein synthesis.

Experiments covered the first 7 h after hydroxysterol addition, as this was the maximum overall survival time for cells treated with an 80 μM concentration (fig. 2). Over this time, incorporation of ^{3}H-thymidine into DNA of control cells was constant. The curves for ^{3}H-thymidine incorporation in the DNA of cells cultured in whole medium and treated with 80 μM of either 7-HC or 7-HC BHS (fig. 2a) show that the former inhibited DNA synthesis after 3 h , reaching 80 % after 7 h., while the latter was more rapid, inhibition occurring after 1 h and reaching 90 % after 7h. DNA synthesis was almost completely inhibited when cell lysis occurred, which was 30% after 12 h contact with 7-HC, and 15% with 7-HC BHS.

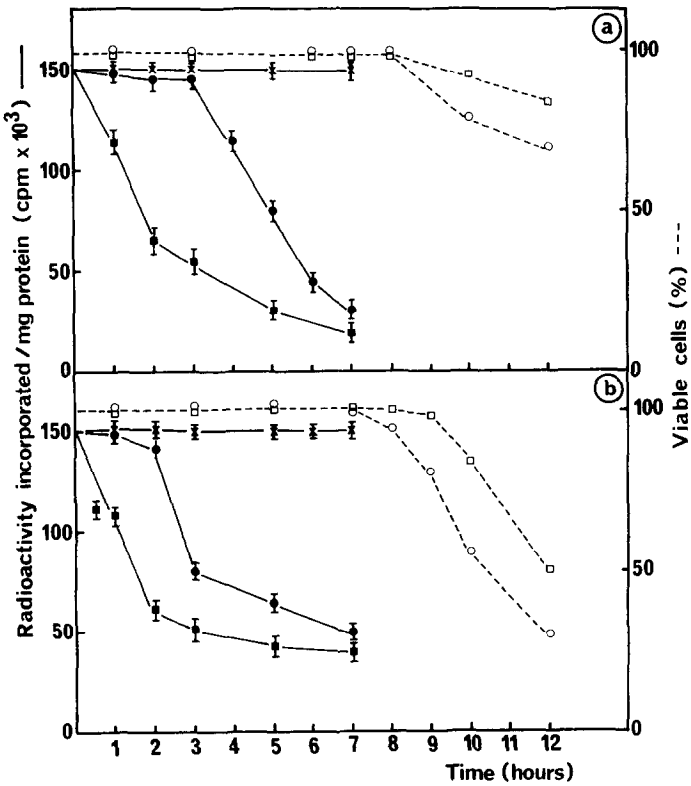


Figure 2 : Inhibition of DNA synthesis as a function of time after treatment with 7-HC 80 uM (●——●) or 7-HC BHS 80 uM (■——■) (^{3}H-thymidine pulse labelling). Fig. 2a shows results obtained with cells cultured in the whole medium and Fig. 2b with cells cultured in the lipid- and lipoprotein-deprived medium. DNA synthesis in control cultures (✱——✱). Cell viability after treatment with 7-HC (○——○) or 7-HC BHS (□——□). The plots represent the mean values from 3 different experiments.

In cells cultured without lipids and lipoproteins (fig. 2b) 80 µM of 7-HC inhibited DNA synthesis after a 2 h contact, and 7-HC BHS did so within one hour, giving respectively 65 and 70 % inhibition after 7 h. The intracellular effect of both products on DNA synthesis was thus slightly lower, although cell viability was more affected: cell lysis was 70 % and 50 % respectively for 7-HC and 7-HC BHS after 12 h contact.

A similar study was made with different concentrations of each compound to clarify this phenomenon. With HTC cells cultured in whole medium (fig. 3a), 7-HC inhibited DNA synthesis from a concentration of 20 µM, while it took 40 µM of 7-HC BHS. The former did not affect protein synthesis until the 80 µM concentration, whereas it took only 40 µM of 7-HC BHS for partial inhibition, which rose to 40% for 80 µM.

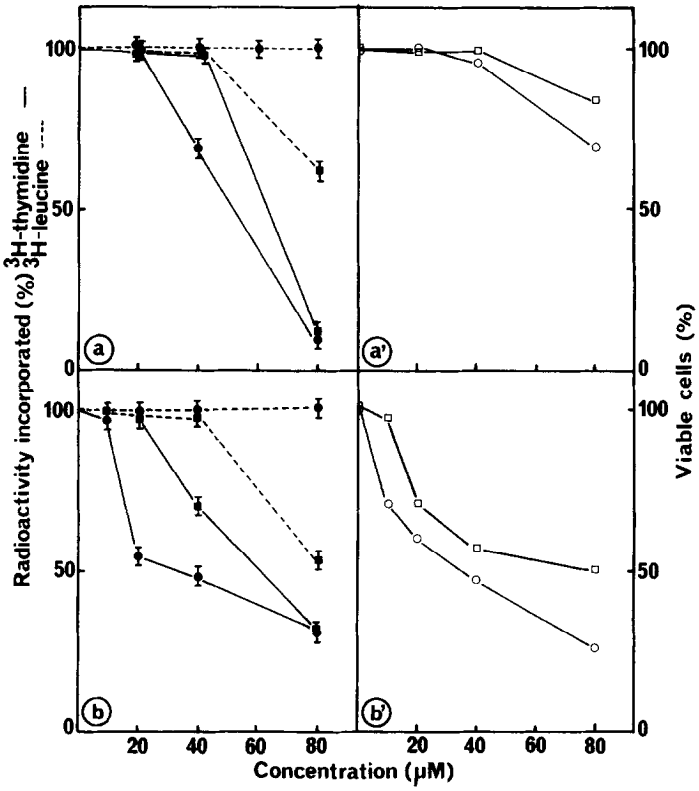


Figure 3 : Inhibition of DNA synthesis (30 min. ^{3}H-thymidine pulse labelling) in function of various concentrations of 7-HC (●——●) or 7-HC BHS (■——■) after 7 h treatment, and likewise of protein synthesis (30 min. ^{3}H-leucine pulse labelling) in presence of 7-HC (●- - -●) or 7-HC BHS (■- - -■). The results are expressed as % of controls. Cell viability after 12 h treatment with 7-HC (○——○) or 7-HC BHS (□——□). The plots represent the mean values from 3 different experiments. Fig. 3a, a' represent results obtained with cells cultured in the whole medium and fig. 3b, b' in lipoprotein-deprived medium.

In lipid- and lipoprotein-deprived culture medium (fig. 3b), both compounds inhibited DNA synthesis at lower concentrations which had no or little effect in whole medium : at 20 μM, 7-HC gave 50% inhibition, and 7-HC BHS at 40 μM gave 30% inhibition of DNA synthesis. The action threshold for both products was thus quite lower. On protein synthesis, no effect was observed with 7-HC till 80 μM, whereas with 7-HC BHS, similar inhibition was obtained at 80 μM, as in whole medium.

It should be noted that for cells cultured in lipoprotein-deprived medium, the lower concentrations of 7-HC and 7-HC BHS, which did not inhibit DNA synthesis, were nevertheless cytolytic (fig. 3b'), e.g., 8 μM of 7-HC and 20 μM of 7-HC BHS gave rise to 30% cell lysis after 12 h contact. This feature was not

observed for cells cultured in whole medium (fig. 3a'): only doses high enough to inhibit DNA synthesis showed, after a 12 h contact, a slight cytolytic action.

DISCUSSION AND CONCLUSIONS

The lipophilic 7-HC proved highly cytotoxic for cultured HTC hepatoma cells, depending on dose and exposure time. The hydrophilic derivative 7-HC BHS showed similar toxicity. Both compounds were far more toxic in lipid- and lipoprotein-deprived culture medium. 7-HC and 7-HC BHS inhibit DNA synthesis before cell lysis occurs, and to the same degree whether the cells are cultured in the whole or in lipid-and lipoprotein-deprived medium. It is noteworthy that, for cells cultured in lipoprotein-deprived medium, the cytolytic effect of both compounds occurred at concentrations which had no effect on DNA synthesis.

Yachnin et al. (7) reported that lipoproteins can bind hydroxysterols and reduce their free concentration in the culture medium, which might explain the lower action threshold of 7-HC and 7-HC BHS on DNA synthesis in lipoprotein-deprived medium, but not the far higher cytolytic effect at concentrations not affecting synthesis of DNA or proteins.

Although the hydrophilic derivative 7-HC BHS has the toxic features of the lipophilic 7-HC, it does not seem to be rapidly hydrolysed into the latter, since it has an effect of its own, inhibiting protein synthesis. Furthermore, this water-soluble compound is also far more active when lipids and lipoproteins are absent.

Finally, our results evidence the important role of serum lipids and lipoproteins in the cytolytic action of these sterols. We are now fractionating the serum lipids and lipoproteins to investigate which specific fractions or molecules antagonise predominantly the lytic effect of 7-HC and 7-HC BHS on HTC cells.

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