Effects of distal cholesterol biosynthesis inhibitors on cell proliferation and cell cycle progression

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Abstract Cholesterol is a major lipid component of the plasma membrane in animal cells. In addition to its structural requirement, cholesterol is essential in cell proliferation and other cell processes. The aim of the present study was to elucidate the stringency of the requirement for cholesterol as a regulator of proliferation and cell cycle progression, compared with other sterols of the cholesterol biosynthesis pathway. Human promyelocytic HL-60 cells were cultured in cholesterol-free medium and treated with different distal inhibitors of cholesterol biosynthesis (zaragozic acid, SKF 104976, SR 31747, BM 15766, and AY 9944), which allow the synthesis of isoprenoid derivatives and different sets of sterol intermediates, but not cholesterol. The results showed that only the inhibition of sterol Δ^7-reductase was compatible with cell proliferation. Blocking cholesterol biosynthesis upstream of this enzyme resulted in the inhibition of cell proliferation and cell cycle arrest selectively in G2/M phase.—Fernández, C., M. Martín, D. Gómez-Coronado, and M. A. Lasunción. **Effects of distal cholesterol biosynthesis inhibitors on cell proliferation and cell cycle progression. *J. Lipid Res.* 2005.** 46: **920–929.**

Supplementary key words sterols • 7-dehydrocholesterol • HL-60

The cholesterol biosynthesis pathway provides cells with several compounds that are essential for cell growth and division, such as mevalonic acid (1–3), farnesyl pyrophosphate (4), geranylgeranyl pyrophosphate (5), and cholesterol (6–8). Some studies aimed at determining whether sterol intermediates may substitute for cholesterol during cell proliferation have been published in the past (9, 10). Mammalian mutant cells lacking detectable lanosterol 14α-demethylase, an enzyme of the cholesterol biosynthesis pathway, have been shown not to grow unless the culture medium contains cholesterol (10). Similarly, as shown in both HL-60 and MOLT-4 human cells incubated in cholesterol-free medium, inhibition of lanosterol 14α-demethylase with SKF 104976 leads to the inhibition of cell proliferation, with the accumulation of cells selectively in G2/M phase (8, 11). These effects were fully prevented and reversed when cholesterol was added to the culture medium (3), indicating that lanosterol and dihydrolanosterol, which accumulate in these conditions, do not support cell proliferation (10, 11).

With regard to other sterol intermediates, desmosterol appears to sustain cell proliferation. Thus, cells lacking sterol Δ^{24}-reductase normally proliferate in cholesterol-deficient media (9), which is compatible with the obvious cell growth that occurs in humans with desmosterolosis (12) or in sterol Δ^{24}-reductase-deficient mice (13). To our knowledge, there is no report directly examining the effect of 7-dehydrocholesterol on the growth of cells lacking sterol Δ^7-reductase activity incubated in cholesterol-free media. Therefore, the role of that sterol in cell proliferation remains to be elucidated. In cholesterol-auxotroph cells, such as U937 human macrophages (14), S2 mutant LM mouse fibroblasts (9), and NS-1 mouse myeloma cells (15), 7-dehycrocholesterol added to the medium supported growth at the same rate as cholesterol, but the significance of these results could not be evaluated because of the extensive conversion of that sterol to cholesterol in these cells (9, 14). In addition, 7-dehydrocholesterol has been shown to replace cholesterol in raft and caveolae formation (16, 17). Congenital defects of sterol Δ^7-reductase in humans, which causes the Smith-Lemli-Opitz (SLO) syndrome, lead to antenatal growth retardation, malformations, and multiple organ anomalies (18). This syndrome, which is reproduced in sterol Δ^7-reductase-deficient mice (19, 20) and in animals treated with sterol Δ^7-reductase enzyme inhibitors (21–23), may be prevented, at least in part, by cholesterol feeding (24, 25). This indicates the requirement of cholesterol for normal embryogenesis, but 7-dehydrocholesterol, which accumulates in this condition, also appears to be responsible for teratogenesis. It has been shown that it is actually the extensive conversion of 7-dehydrocholesterol to oxidized derivatives that induces growth retardation in cultured rat embryos

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(26). Therefore, the beneficial effect of cholesterol feeding could be attributable, at least partially, to the downregulation of the synthesis of 7-dehydrocholesterol and toxic derivatives (26). Whether 7-dehydrocholesterol affects cell cycle progression has not yet been studied.

Certain cholesterol analogs may substitute for cholesterol during cell proliferation with some restrictions. Ergosterol, a fungal sterol, may sustain animal cell growth, provided that suboptimal amounts of cholesterol are also available (27, 28). Similar results were obtained with the combination of other sterols in different organisms (29–31). The existing sterol synergism indicates that low concentrations of the natural sterol, which are insufficient to substantially sustain cell growth per se, elicit synergistic effects when added in combination with another sterol (27, 32, 33). These studies led to the postulation of a regulatory role for sterols during cell growth, which appears to be highly specific and fulfilled by very low levels of the natural sterol. In contrast, the bulk membrane function, which accounts for the major amount of the sterol in the cell, is potentially accomplished by different sterols with broad specificity (27, 29–31).

The aim of the present work was to define the stringency of the regulatory requirement for cholesterol during proliferation and cell cycle progression by comparison with other sterols from the cholesterol biosynthesis pathway. For this, HL-60 cells were cultured in a cholesterol-free medium in the presence of different distal inhibitors of cholesterol biosynthesis. The results show that 7-dehydrocholesterol can substitute for cholesterol, at least partially, during cell proliferation, whereas sterols that are synthesized before 7-dehydrocholesterol in the pathway are ineffective in supporting cell proliferation in the absence of cholesterol.

MATERIALS AND METHODS

Materials

HL-60 cells (human promyelocytic cells) were obtained from the American Type Culture Collection (CCL 240). Cholesterol-free medium (DCCM-1) was purchased from Biological Industries. Antibiotics were from Gibco BRL. Cholesterol and 7-dehydrocholesterol were from Steraloids. Trolox was purchased from Aldrich, mevalonic acid from ICN, and 5-fluordeoxyuridine from Sigma Chemical Co. Cholesterol biosynthesis inhibitors were gifts from the following laboratories: zaragozic acid from Merck Research Laboratories, SKF 104976 from SmithKline Beecham Pharmaceuticals, SR 31747 from Sanofi, AY 9944 from Wyeth-Ayerst Research, and BM 15766 from Boehringer Mannheim. [^{14}C]acetate (53 mCi/mmol) and [*methyl*-^{3}H]thymidine (5 Ci/mmol) were provided by Amersham Biosciences. All other chemical products were of analytical grade.

Cell culture

HL-60 cells were maintained in DCCM-1 supplemented with antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml, and 10 μg gentamicin/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

Proliferation assay

To measure cell proliferation, HL-60 cells were plated on sterile 96-well plates (Multiscreen-HV; Millipore, Molsheim, France), each well containing 1.5×10^4 cells in 225 μl of DCCM-1 supplemented or not with cholesterol (dissolved in ethanol) in the absence (control) or presence of the drugs under study (dissolved in DMSO). In other experiments, cells were also supplemented with increasing concentrations of 7-dehydrocholesterol dissolved in ethanol. Final concentrations of DMSO and ethanol in the medium were 0.044% and 0.44%, respectively. At the indicated times, the wells were supplemented with 10 mM 5-fluordeoxyuridine, and 1 h later with 0.5 μCi of [*methyl*-^{3}H]thymidine. The cells were processed for radioactivity incorporation into DNA 18 h later, as described elsewhere (34).

Analysis of sterol biosynthesis and cholesterol cell content by HPLC

To study cholesterol biosynthesis, HL-60 cells (7.5×10^6) were preincubated for 2 h in 10 ml of DCCM-1 in the absence (control) or presence of the different drugs. The medium was then supplemented with 40 μCi of [^{14}C]acetate and incubated for another 8 h. In other experiments, to determine the total cholesterol cell content, HL-60 cells (7.5×10^6) were incubated in the presence of the drugs for 24, 36, 48, 60, and 72 h in 10 ml of DCCM-1. At the end of the incubation, the cells were washed twice with ice-cold PBS and resuspended in 0.5 ml of 10% (w/v) KOH. [^{3}H]cholesterol was added as an internal standard. The samples were treated sequentially with chloroform-methanol (2:1, v/v) and distilled water to obtain the lipidic and water-soluble fractions, as described elsewhere (35). The lipid extract was further subfractionated into the saponifiable and nonsaponifiable fractions. Nonsaponifiable lipids were resuspended in hexane and used for sterol separation by HPLC and radioactivity counting as described previously (36). Recovery of [^{3}H]cholesterol was greater than 65% in all cases. Sterol separation was accomplished by reverse-phase HPLC with a Luna-Pack 5 μm pore size C18 column (250 mm × 4.60 mm; Phenomenex). Lipids were eluted with acetonitrile-water (95:5, v/v) for the first 37 min and then with methanol at a flow rate of 1.2 ml/min. The eluent was monitored simultaneously by ultraviolet-absorption spectroscopy (Beckman 168 variable-wavelength detector; Beckman Instruments) and online radioactivity detection using an LB-506 C-1 radioactivity detector (Berthold). The eluted sterols were identified by comparison of the retention time and the ultraviolet spectrum against those of pure standards.

Cell cycle analysis

Asynchronous HL-60 cells (2.5×10^5 cells/ml) were cultured in DCCM-1 and supplemented with different distal cholesterol biosynthesis inhibitors and/or cholesterol. At the end of the incubation, the cells were washed twice with ice-cold PBS, fixed in 70% cold ethanol, treated with 100 μg/ml RNase A, and labeled with 50 μg/ml propidium iodide for 1 h at 37°C. Cells were analyzed by flow cytometry (FACScalibur; Becton-Dickinson) using selective gating to exclude doublets of cells and subjected to MODFIT analysis (Verity Software House, Inc.).

Statistical analysis

The results are expressed as means ± SEM. Statistical comparisons between groups were done by Student's *t*-test using Statgraphics Plus version 5.0 (Statistical Graphics).

RESULTS

Effects of cholesterol biosynthesis inhibitors on sterol biosynthesis and cholesterol content

We assessed the effects of different cholesterol biosynthesis inhibitors on sterol biosynthesis, as measured by the

incorporation of [^{14}C]acetate. As shown in **Fig. 1**, in untreated cells, radioactivity appearing in nonsaponifiable lipids corresponded mostly to cholesterol. The addition of 60 μM zaragozic acid totally abolished [^{14}C]acetate conversion into any sterol, consistent with the reported inhibitory action of this drug on squalene synthase activity (**Fig. 2**). When the cells were treated with 1.5 μM SKF 104976, 1 μM SR 31747, or 5 μM BM 15766, radioactivity appeared in 24-dihydrolanosterol (peak 2), 7-dihydrozymosterol (peak 3), or 7-dehydrocholesterol (peak 4), respectively (Fig. 1), consistent with the specific inhibition of lanosterol 14α-demethylase, sterol Δ^8-isomerase, or sterol Δ^7-reductase by these drugs (Fig. 2). Given that 7-dihydrozymosterol and cholesterol coeluted in the chromatographic system, to ascertain what compound was actually accumulated by the effect of SR 31747, cells were treated with 1 μM SR 31747 and, simultaneously, 5 μM BM 15766 to prevent cholesterol formation. In these conditions, the chromatographic profile was similar to that in the absence of BM 15766 (data not shown), indicating that the accumulated sterol in cells treated with SR 31747 was actually 7-dihydrozymosterol.

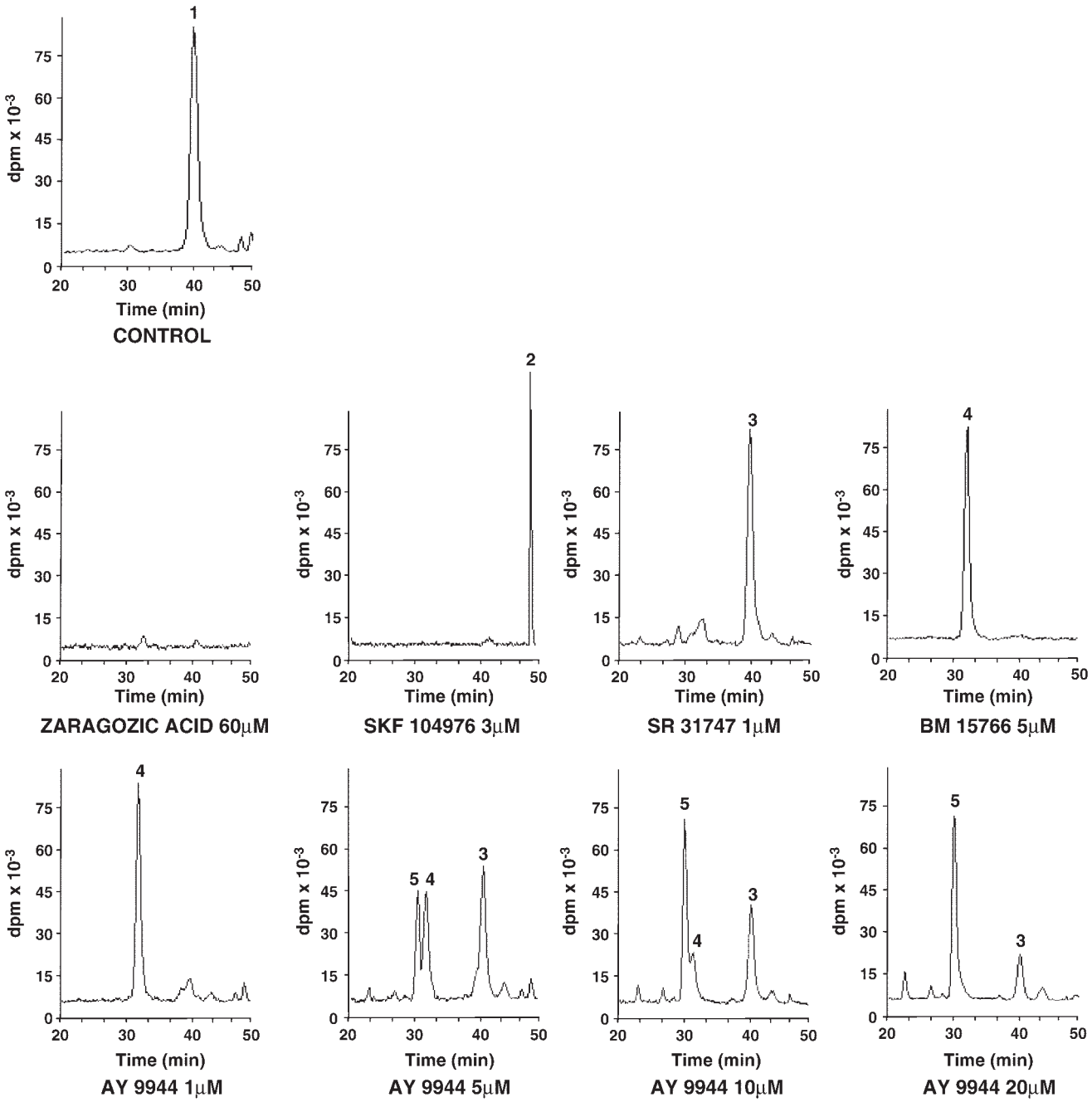

Fig. 1. Effects of zaragozic acid, SKF 104976, SR 31747, BM 15766, and AY 9944 on [^{14}C]acetate incorporation into sterols. HL-60 cells were treated with the different drugs at the indicated concentrations or vehicle (control) and [^{14}C]acetate for 8 h, and radioactivity incorporation into sterols was determined by HPLC. Peak 1, cholesterol; peak 2, 24-dihydrolanosterol; peak 3, 7-dihydrozymosterol; peak 4, 7-dehydrocholesterol; peak 5, 4,4-dimethylcholesta-8(9),14-dien-3β-ol.

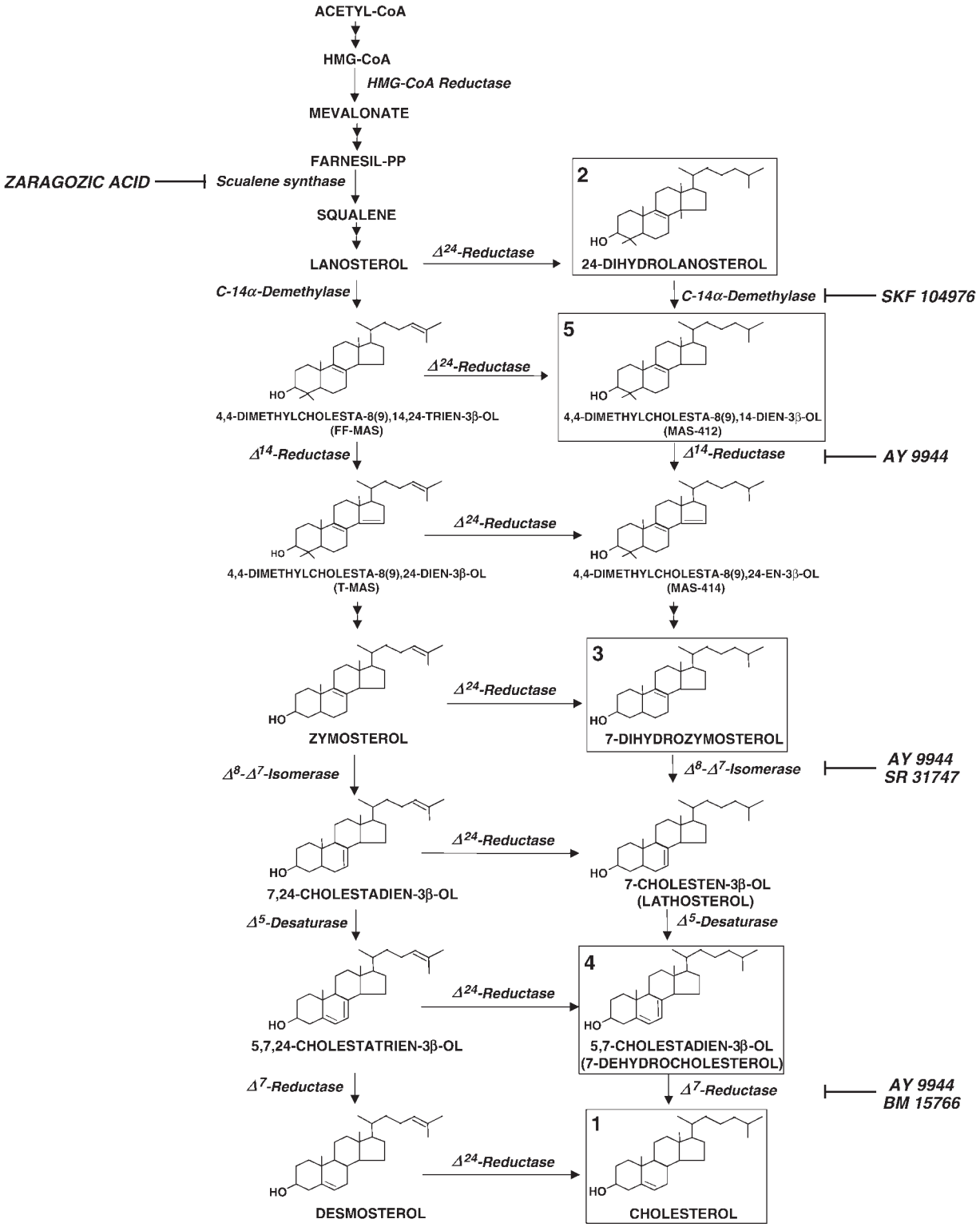

Fig. 2. Scheme of the cholesterol biosynthesis pathway showing the enzymes inhibited by zaragozic acid, SKF 104976, SR 31747, BM 15766, and AY 9944.

The effects of AY 9944 on cholesterol biosynthesis varied widely as a function of the dose of the drug. At the lowest concentration used (1 μM), cholesterol biosynthesis was blocked and radioactivity accumulated in 7-dehydrocholesterol (peak 4) (Fig. 1), indicating the inhibition of sterol Δ^7-reductase (Fig. 2). Treatment with 5 μM AY 9944 resulted in a reduction of the synthesis of 7-dehydrocholesterol (peak 4) and the accumulation of 7-dihydrozymosterol (peak 3) and 4,4-dimethylcholesta-8(9),14-dien-3β-ol (peak 5), changes that were more marked with 10 μM AY 9944 (Fig. 1). Finally, in cells treated with the highest dose of this drug (20 μM), the only radioactive

TABLE 1. Cholesterol content with different drugs

Drug	0 h	24 h	36 h	48 h	60 h	72 h
Control	5.56 ± 0.3	5.14 ± 0.2	5.48 ± 0.4	5.64 ± 0.3	5.36 ± 0.4	5.34 ± 0.4
Zaragozic acid, 60 μM	—	2.56 ± 0.1	2.16 ± 0.2	1.63 ± 0.1	1.52 ± 0.1	1.34 ± 0.1
SKF 104976, 1.5 μM	—	2.61 ± 0.1	1.91 ± 0.1	1.21 ± 0.1	0.91 ± 0.1	0.81 ± 0.1
SR 31747,[a] 1 μM	—	4.72 ± 0.2	5.02 ± 0.3	5.32 ± 0.5	5.07 ± 0.2	4.95 ± 0.2
BM 15766, 5 μM	—	2.74 ± 0.1	2.08 ± 0.1	1.33 ± 0.1	1.16 ± 0.1	0.77 ± 0.1
AY 9944, 1 μM	—	2.95 ± 0.1	2.35 ± 0.1	1.48 ± 0.1	1.04 ± 0.1	0.85 ± 0.1
AY 9944, 20 μM	—	2.81 ± 0.1	2.12 ± 0.1	1.67 ± 0.1	1.48 ± 0.1	1.35 ± 0.1

Effects of zaragozic acid, SKF 104976, SR 31747, BM 15766, and AY 9944 on cholesterol content (μg/mg/cell protein). HL-60 cells were treated with the different drugs or vehicle (control) and cholesterol was determined by HPLC. Data correspond to means ± SEM of three experiments.

[a] These values correspond to the concentration of cholesterol plus 7-dihydrozymosterol, which could not be separated by HPLC.

sterols were 7-dihydrozymosterol (peak 3) and, especially, 4,4-dimethylcholesta-8(9),14-dien-3β-ol (peak 5) (Fig. 1), which indicates the inhibition of both sterol Δ^{14}-reductase and $\Delta^{8,7}$-isomerase (Fig. 2).

Next, we measured the cholesterol content of cells treated with the drugs for increasing times. As shown in **Table 1**, cholesterol content decreased significantly after 24 h of treatment with the different drugs and continued

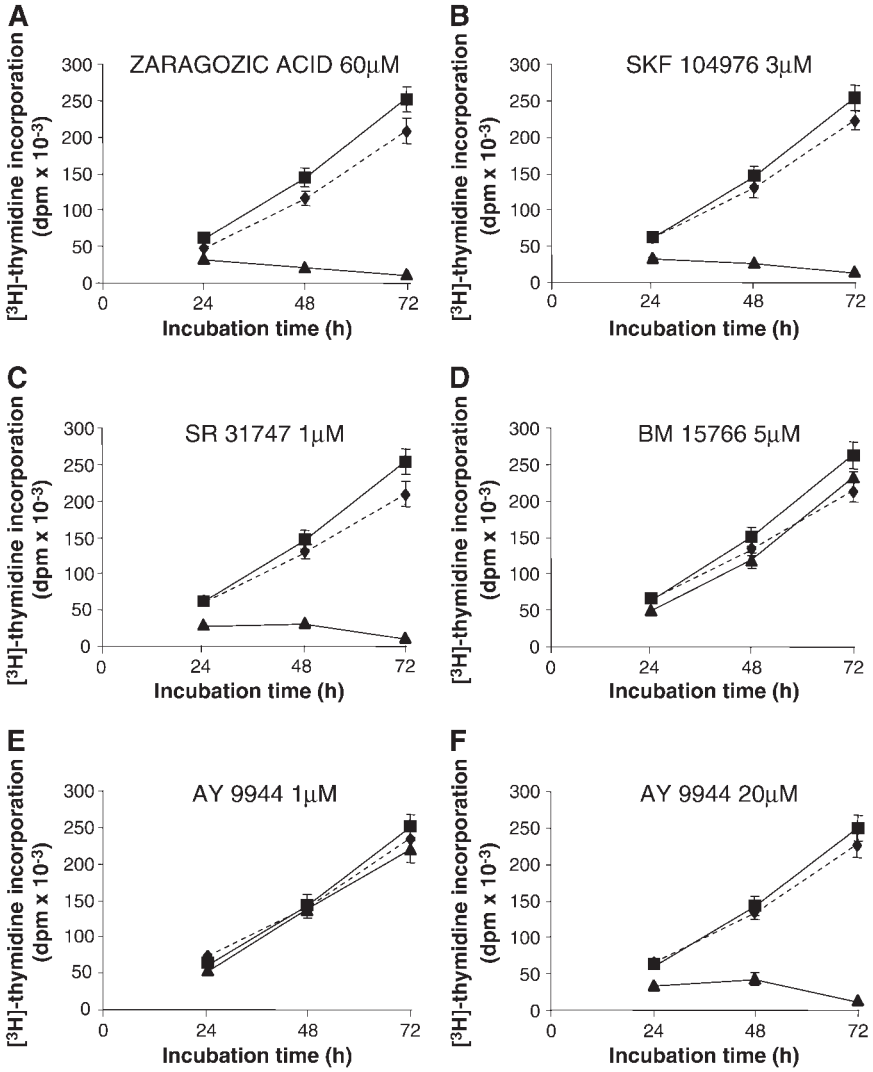

Fig. 3. Time course of the effects of zaragozic acid (A), SKF 104976 (B), SR 31747 (C), BM 15766 (D), and AY 9944 (E, F) on cell proliferation. HL-60 cells were cultured in cholesterol-free medium (DCCM-1) in the absence (control; closed squares) or presence of the different drugs alone (closed triangles) or in combination with 30 μg/ml free cholesterol (closed diamonds). At the indicated times, [^{3}H]thymidine incorporation into DNA was determined. Data correspond to means ± SEM of three experiments.

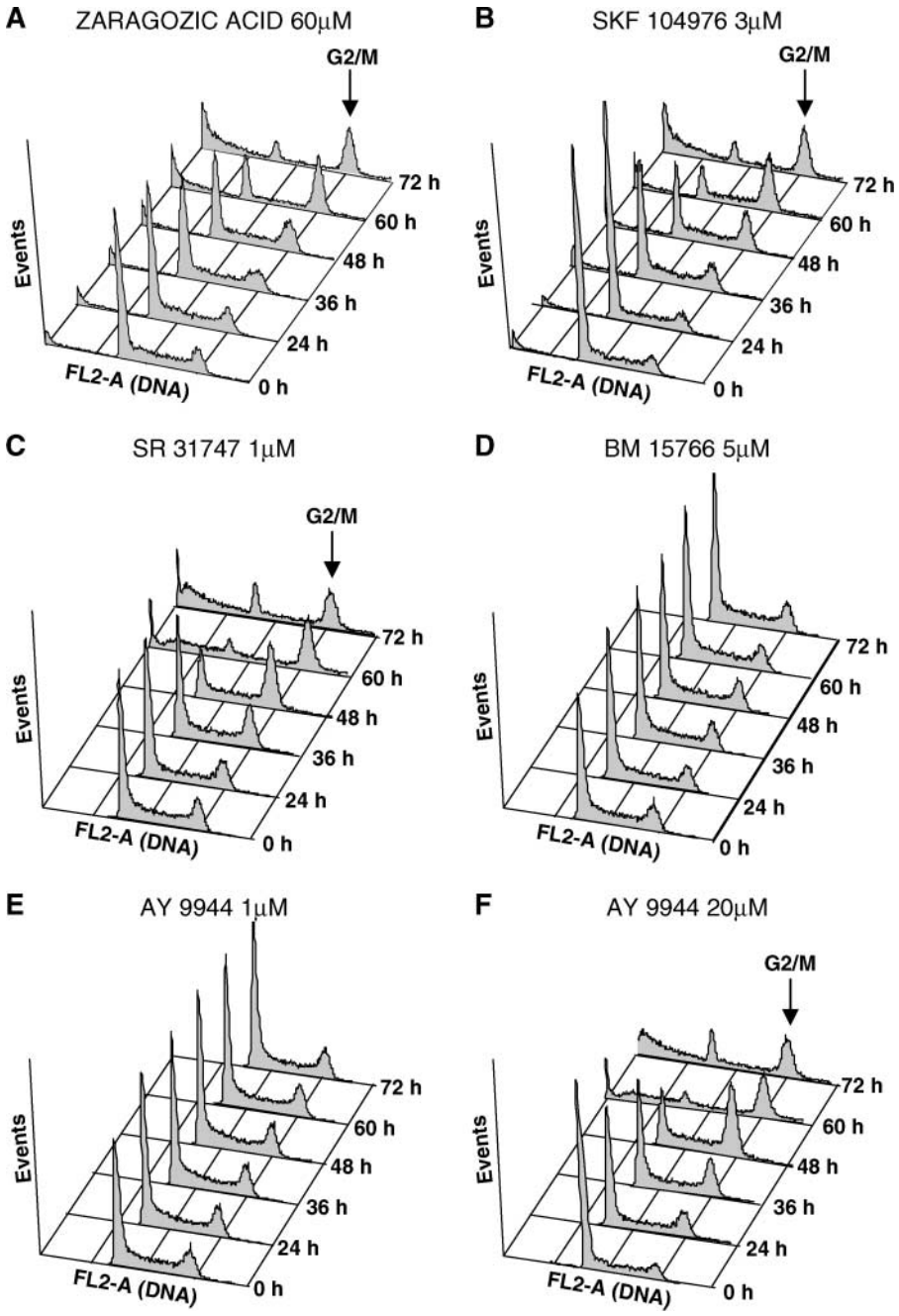

Fig. 4. Effects of zaragozic acid (A), SKF 104976 (B), SR 31747 (C), BM 15766 (D), and AY 9944 (E, F) on cell cycle distribution. HL-60 cells were incubated in DCCM-1 in the presence of the different drugs. At the indicated times, cells were stained with propidium iodide and analyzed by flow cytometry. Data correspond to a representative experiment among three that gave similar results. FL2-A corresponds to the fluroescence emitted by propidium iodide bound to DNA, which is measured by cytometry in channel 2 (FL2). To avoid the eventual presence of doublets, among the parameters given by the cytometer area (FL2-A) is preferred in order to quantify the amount of DNA present in the cell.

to decline at longer incubation times. In general, after treatment with the drugs for 72 h, cholesterol content was ~1 μg of cholesterol per milligram of cell protein, 20% of that in control conditions. In cells treated with SR 31747, the extent of the cholesterol reduction could not be ascertained because, as shown above, cholesterol and 7-dihydrozymosterol were indistinguishable in our HPLC system. It is worth mentioning that the cholesterol content after treating the cells with 1 μM AY 9944 was reduced to a similar extent as after treatment with 20 μM.

Effects of cholesterol biosynthesis inhibitors on cell proliferation and cell cycle progression

To study the effects of distal cholesterol biosynthesis inhibitors on cell proliferation, HL-60 cells were incubated in a cholesterol-free medium and treated with the differ-

ent drugs. Zaragozic acid (**Fig. 3A**), SKF 104976 (Fig. 3B), and SR 31747 (Fig. 3C) caused a rapid and intense inhibition of cell proliferation, as measured by [^{3}H]thymidine incorporation into DNA, being practically nil at 72 h of treatment. These results showed that dihydrolanosterol and 7-dihydrozymosterol, which accumulated after treatment with SKF 104976 and SR 31747 (Fig. 1), respectively, were ineffective in supporting cell proliferation. The simultaneous addition of free cholesterol to the medium prevented the inhibition of cell proliferation exerted by these drugs, indicating that the inhibition was attributable to the cholesterol deficiency produced by these drugs (Fig. 3A–C).

Treatment with BM 15766 for 72 h did not affect cell proliferation (Fig. 3D), despite the fact that the cholesterol cell content was markedly reduced (Table 1). Similar results were obtained in cells treated with 1 μM AY 9944 (Fig. 3E), which selectively inhibited sterol Δ^7-reductase (Fig. 1). This suggests that 7-dehydrocholesterol, which is the only sterol that accumulated in these conditions, contributed to cell proliferation.

By contrast, 20 μM AY 9944, which caused the accumulation of 4,4-dimethylcholesta-8(9),14-dien-3β-ol and 7-dihydrozymosterol instead of 7-dehydrocholesterol, did suppress cell proliferation (Fig. 3F). As expected, this inhibition was abrogated by supplementing the medium with free cholesterol.

We next studied the effects of cholesterol biosynthesis inhibitors on cell cycle distribution at different incubation times. Treatment with zaragozic acid (**Fig. 4A**), SKF 104976 (Fig. 4B), SR 31747 (Fig. 4C), or AY 9944 at the highest concentration used (Fig. 4F) resulted in a progressive accumulation of cells in G2/M phase. In contrast, cell cycle distribution was not affected by treatment with BM 15766 (Fig. 4D) or 1 μM AY 9944 (Fig. 4F), as in the control condition (data not shown).

Taken together, these results show that, despite the fact that the cholesterol cell content was reduced to a similar extent with all of the inhibitors, cell cycle was arrested only when sterol biosynthesis was blocked at the reaction catalyzed by sterol $\Delta^{8,7}$-isomerase or upstream.

Comparison of the effects of cholesterol and 7-dehydrocholesterol on cell proliferation

These results suggested that 7-dehydrocholesterol may support cell proliferation. To directly examine this possibility, we compared the effects of 7-dehydrocholesterol and cholesterol on DNA synthesis in HL-60 cells. In the absence of any drug in the medium, supplementation with cholesterol did not affect cell proliferation (**Fig. 5A**). In contrast, 7-dehydrocholesterol showed some cytotoxicity, especially at concentrations greater than 1 μg/ml (Fig. 5B). In cells treated with SKF 104976, free cholesterol added to the medium abrogated the inhibition of cell proliferation in a dose-dependent manner (Fig. 5A). The addition of up to 1 μg/ml 7-dehydrocholesterol also restored DNA synthesis, but less efficiently than cholesterol (Fig. 5). Moreover, 2.5 μg/ml 7-dehydrocholesterol markedly inhibited cell proliferation (Fig. 5B). To prevent the

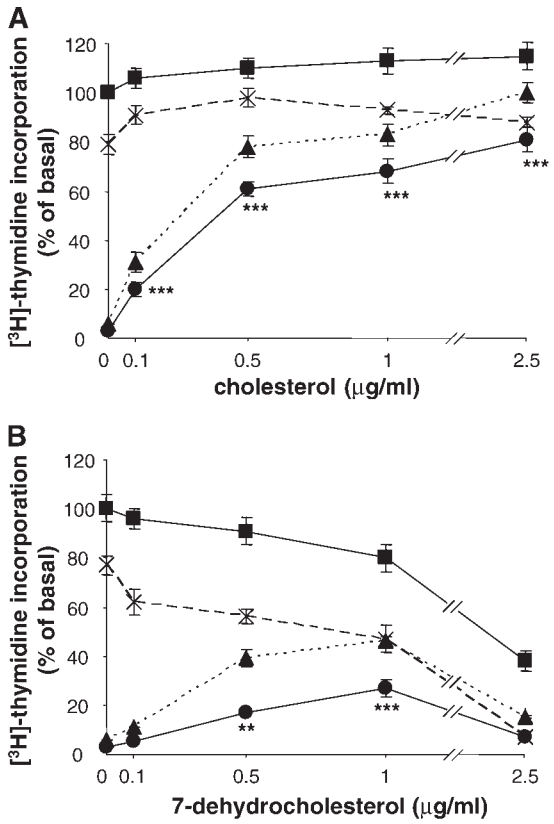

Fig. 5. Comparative effects of cholesterol and 7-dehydrocholesterol on cell proliferation. HL-60 cells were cultured in DCCM-1 in the absence (control; closed squares) or presence of 3 μM SKF 104976 (closed triangles), 5 μM BM 15766 (crosses), or 3 μM SKF 104976 plus 5 μM BM 15766 (closed circles) supplemented with increasing concentrations of cholesterol (A) or 7-dehydrocholesterol (B). At 72 h, [^{3}H]thymidine incorporation into DNA was determined. Data correspond to means ± SEM of three experiments. Statistical comparisons between treatment with cholesterol or 7-dehydrocholesterol versus the control condition (in the absence of sterols) by Student's *t*-test: ** $P < 0.01$, *** $P < 0.001$.

eventual conversion of 7-dehydrocholesterol into cholesterol and, thus, to block cholesterol provision from any source, the cells were treated with SKF 104976 and BM 15766 simultaneously. This resulted in a complete suppression of cell proliferation (Fig. 5). In these conditions, the addition of free cholesterol restored DNA synthesis, whereas 7-dehydrocholesterol had a limited effect (65% vs. 24%, respectively, with 1 μg/ml of each sterol).

Finally, we analyzed the inhibitory effect of 7-dehydrocholesterol on cell proliferation. Because it had been demonstrated by others that 7-dehydroclolesterol is a strong inhibitor of HMG-CoA reductase (37), we studied whether the addition of mevalonate could prevent the inhibition of cell proliferation induced by 7-dehydrocholesterol. As shown in **Fig. 6**, the addition of mevalonate was unable to restore cell growth. In contrast, when cells were treated with Trolox, a potent antioxidant, the inhibition of cell proliferation was partially prevented, both in the absence and in the presence of SKF 104976 and BM 15766. This suggests that some oxidized derivatives of 7-dehydrocholesterol could induce growth retardation in cell cultures.

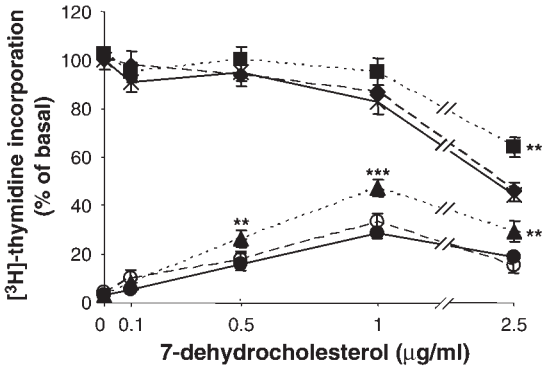

Fig. 6. Effects of 7-dehydrocholesterol, mevalonic acid, and Trolox on cell proliferation. HL-60 cells were cultured in DCCM-1 in the absence (control; crosses) or presence of 0.2 mM mevalonic acid (closed diamonds), 15 μM Trolox (closed squares), 3 μM SKF 104976 plus 5 μM BM 15766 (closed circles), 3 μM SKF 104976 plus 5 μM BM 15766 and 0.2 mM mevalonic acid (open circles), or 3 μM SKF 104976 plus 5 μM BM 15766 and 15 μM Trolox (closed triangles) supplemented with increasing concentrations of 7-dehydrocholesterol. At 72 h, [^{3}H]thymidine incorporation into DNA was determined. Data correspond to means ± SEM of three experiments. Statistical comparisons between the addition of Trolox and the other treatments in the absence of this antioxidant by Student's *t*-test: ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

The requirement of cholesterol for the growth and division of mammalian cells has been known for many years (6, 38). In our present work, we studied the effects of different distal inhibitors of cholesterol biosynthesis on cell proliferation and cell cycle progression. In this context, determining whether or not intermediate sterols of cholesterol biosynthesis could substitute for cholesterol in human cell proliferation may help to define the role of different sterols in this process. For this, we used the inhibitors zaragozic acid, SKF 104976, SR 31747, BM 15766, and AY 9944, whose action on cholesterol biosynthesis enzymes have been reported by others (39–45).

Treatment with SKF 104976 and SR 31747 significantly reduced cell proliferation. These results indicated that sterols accumulating in these conditions (i.e., dihydrolanosterol and 7-dihydrozymosterol, respectively) are not able to sustain cell growth at a normal rate in the absence of cholesterol in the medium. The fact that the content of total sterols in cells treated with SR 31747 was similar to that in control cells indicates that the inhibition of cell proliferation induced by this inhibitor was not attributable to the deficiency of total sterols. These findings are in agreement with previous results by others in different animal cells treated with SR 31747 (40) or mammalian cell lines defective in lanosterol demethylation (10). Similarly, treatment with zaragozic acid, a squalene synthase inhibitor that prevents the synthesis of any sterol through the cholesterol biosynthesis pathway, caused a rapid inhibition of cell proliferation. All of these drugs arrested the cell cycle in G2/M. These results with SR 31747 and zaragozic acid extend our previous observations with SKF 104976 in both HL-60 and MOLT-4 cells (8, 11) and indicate that blocking cholesterol biosynthesis upstream of the reaction catalyzed by sterol $\Delta^{8,7}$-isomerase results in cell cycle arrest in G2/M selectively.

The effects of AY 9944 were more complex. AY 9944 interferes in several enzymatic double bond reductions in sterol biosynthesis (46, 47). Our present observations in human cells show that this drug inhibits several enzymes with different levels of potency, in the following order: sterol Δ^7-reductase > $\Delta^{8,7}$-isomerase > Δ^{14}-reductase. At a relatively high concentration (20 μM), when the cholesterol biosynthesis pathway appeared to be blocked at the reaction catalyzed by sterol $\Delta^{8,7}$-isomerase, AY 9944 arrested the cell cycle in G2/M, which demonstrates that 7-dihydrozymosterol and 4,4-dimethylcholesta-8(9),14-dien-3β-ol, which accumulate in this condition, could not substitute for cholesterol in cell cycle progression. At the lowest dose used (1 μM), only sterol Δ^7-reductase was inhibited and cells proliferated at a normal rate. Similar results were obtained with BM 15766, a specific inhibitor of sterol Δ^7-reductase. Thus, the results suggested that 7-dehydrocholesterol contributed to cell proliferation. Supplementing the medium with 7-dehydrocholesterol had been shown previously by others to support the growth of cholesterol-auxotroph cells (9, 14). In these cells, however, 7-dehydrocholesterol was efficiently converted to cholesterol; thus, the specific role of that sterol intermediate in cell proliferation could not be evaluated. Therefore, we treated the cells simultaneously with SKF 104976 and BM 15766 to block both 7-dehydrocholesterol biosynthesis and its eventual conversion to cholesterol. In this condition, supplementing the medium with small amounts of 7-dehydrocholesterol partially restored cell proliferation, demonstrating the ability of this sterol to support cell growth. It is worth noting that despite the fact that cells were incubated in a cholesterol-free medium and cholesterol biosynthesis was totally blocked, the cholesterol cell content was never nil. Therefore, the requirement of a certain amount of cholesterol in addition to 7-dehydrocholesterol to sustain cell growth cannot be ruled out. In agreement with this, we and others have demonstrated that phytosterols may support the proliferation of animal cells, provided that a suboptimal amount of cholesterol is also available (9, 14, 28).

In humans, the deficiency of sterol Δ^7-reductase causes the SLO syndrome, which is characterized by antenatal growth retardation, mental retardation, and severe malformations (18). These malformations have been related to a defect in the patterning of the morphogen Sonic hedgehog, which requires cholesterol for its full maturation and expression (18, 48). A mouse model in which the gene encoding sterol Δ^7-reductase has been deleted displays many phenotypic similarities with the SLO syndrome (19, 20). In addition, the use of sterol Δ^7-reductase inhibitors, such as AY 9944 and BM 15766, also provides a model of this disorder in rats (21–23, 49). In affected fetuses, the accumulation of 7-dehydrocholesterol and reduced concentrations of cholesterol coexist (18), and the contribution of each of these biochemical alterations to

the pathogenesis of the SLO syndrome has merited some attention (25, 26). Feeding with a cholesterol-rich diet was shown to efficiently restore the normal phenotype in rats treated with AY 9944, which led to the suggestion that the lack of cholesterol is the principal cause of the SLO syndrome (24, 25). These findings, however, do not exclude a direct role of 7-dehydrocholesterol in the pathogenesis of the disease, because cholesterol feeding downregulates the synthesis of this sterol. In this respect, 7-dehydrocholesterol has been demonstrated to strongly downregulate HMG-CoA reductase expression (37), which may hamper the provision of important isoprenoids. Moreover, 7-dehydrocholesterol may be oxidized to a sterol hydroperoxide that adversely affects cells (50). In cultured rat embryos, 7-dehydrocholesterol was actually shown to aggravate growth retardation induced by AY 9944 unless the antioxidant α-tocopherol was added to the medium (26).

In our present work, we found that 7-dehydrocholesterol was cytotoxic for HL-60 cells, especially when used at concentrations greater than 1 μg/ml. This could not be prevented by supplementing the medium with mevalonate, which excludes the possibility that the cause of cytotoxicity was the inhibition of HMG-CoA reductase. Interestingly, addition of an antioxidant to the medium partially prevented the inhibition of cell proliferation induced by 7-dehydrocholesterol.

Taken together, our present results show that 7-dehydrocholesterol may sustain cell proliferation in cells whose cholesterol biosynthesis is handicapped. However, an excess of this sterol may be deleterious, which seems to be partially attributable to a peroxidized derivative. In contrast, and pending studies about the role of lathosterol, other sterol intermediates that precede 7-dehydrocholesterol in the cholesterol biosynthesis pathway clearly do not substitute for cholesterol in cell proliferation. This shows the stringency of the sterol requirement for growth in human cells.

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