Antiproliferative effects of SR31747A in animal cell lines are mediated by inhibition of cholesterol biosynthesis at the sterol isomerase step

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SR31747A is a new sigma ligand exhibiting immunosuppressive properties and antiproliferative activity on lymphocyte cells. Only two subtypes of sigma receptor, namely the sigma, receptor and emopamil-binding protein, have been characterised molecularly. Only the σ, receptor has been shown to bind (Z)-N-cyclohexyl-N-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride (SR31747A) with high affinity. It was demonstrated that the SR31747A effect on the inhibition of T-cell proliferation was consistent with a sigma, receptor-mediated event. In this report, binding experiments and sterol isomerase assays, using recombinant yeast strains, indicate that the recently cloned emopamil-binding protein is a new SR31747A-binding protein whose activity is inhibited by SR31747A. Sterol analyses reveal the accumulation of a Δ8-cholesterol isomer at the expense of cholesterol in SR31747A-treated cells, suggesting that cholesterol biosynthesis is inhibited by SR31747A at the Δ8-Δ7 sterol isomerase step in animal cells. This observation is consistent with a sterol isomerase role of the emopamil-binding protein in the cholesterol biosynthetic pathway in animal cells. In contrast, there is no evidence for such a role of the sigma, receptor, in spite of the structural similarity shared by this protein and yeast sterol isomerase. We have found that SR31747A also exerts anti-proliferative effects at nanomolar concentrations on various established cell lines. The antiproliferative activity of SR31747A is reversed by cholesterol. Sterol-iso-merase overproduction enhances resistance of CHO cells. This last observation strongly suggests that sterol isomerase is implicated in the antiproliferative effect of the drug in established cell lines.

Keywords: sterol-isomerase; sigma receptor; cell line; proliferation; SR31747A.

SR31747A is a new sigma ligand displaying immunosuppressive and anti-inflammatory properties [1–4]. SR31747A is one of the most potent competitors of all known σ ligands, such as pentazocine, 1,3-di-(o)tylguanidine and HOPh-Pip-Pr [4]. Few in vitro biological activities have been detected for these ligands. SR31747A was shown to exert in vitro time-dependent and concentration-dependent inhibition of the proliferative response to mitogens of mouse and human mixed lymphocytes [1]. Sigma receptors include several subtypes. Only two subtypes of sigma receptor (sigma, receptor also known as SR31747A-binding protein and emopamil-binding protein) have been characterised molecularly. The sigma, receptor has been shown to bind (Z)-N-cyclohexyl-N-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride (SR31747A) with high affinity. In yeast, SR31747A arrests cell proliferation by inhibiting Δ8-Δ7 sterol isomerase encoded by the ERG2 gene [5]. Although yeast sterol isomerase and the sigma, receptor share considerable sequence similarities, sigma, receptor production does not complement the erg2 defect in yeast and no enzyme activity could be associated with this receptor. In contrast, we could show that mammalian emopamil-binding protein, a protein structurally very different from the sigma, receptor and yeast sterol isomerase, displayed sterol isomerase activity when expressed in yeast [6]. The human emopamil-binding protein encoding cDNA specifies a 27.4-kDa hydrophobic protein containing four transmembrane domains and an endoplasmic reticulum retrieval sequence [7]. In contrast, ERG2 and sigma, receptor genes encode similar proteins with one putative transmembrane domain and two additional hydrophobic regions. In this report, we show that SR31747A is also a human emopamil-binding protein ligand that inhibits cholesterol synthesis at the sterol isomerase step in animal cell lines. We found that the proliferation of the murine M1 cell line is sensitive to SR31747A and noted a correlation with the inhibition of sterol isomerase activity.

MATERIALS AND METHODS

Chemicals. Bovine insulin, human transferrin, cholesterol, Δ7-lathosterol and pentazocine were purchased from Sigma Chemical Company. 1,3-Di-(o)tylguanidine, HOPh-Pip-Pr and trifluoroperazine were supplied by Interchim. Cyclosporin A (Sandimmun) was kindly provided by Sandoz Laboratories. SR31747A and [3H]SR31747, (specific activity 2109 GBq/mmol), were synthesised by Sanofi Recherche [4]. All

Abbreviations. FCS, foetal calf serum; GC-MS, gas chromatography coupled to mass spectroscopy; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; IC50, concentration causing 50% inhibition; IL2, interleukin 2; IL6, interleukin 6; LDL, low density lipoprotein; MTT, 3-(4,5-dimethylthiazol 2-yl)3,5-diphenylformazan; 3.PIP, HOPh-Pip-Pr.
SR31747A stock solutions were prepared in ethanol at 1000×
concentration. Lipid-depleted serum was purchased from J. Boy,
Reims, France; mevalonolactone, sorbitol, Pfablock, leupeptin,
peptatin, epistatin, soybean trypsin inhibitor, penprodis,
methotrextate, phenylmethylsulfonyl fluoride, low density-lipo-
protein-depleted serum and fetal calf serum were purchased
from Sigma Chemical Company. Cytokines interleukin 2 (IL2),
IL6, granulocyte-monocyte-colony stimulating factor were
purchased from Genzyme. The anti-r-myc mouse Ig 9E10 was a
gift from B. PAU (CNRS, Montpellier, France). The fluorescein-
coupled rabbit-anti-mouse polyclonal antibody was from Sile-
nus. Ignosterol was isolated from a yeast mutant devoid of
ERG24 (kindly given by F. Karst, Poitiers, France). 
48-choles-
tenol was kindly given by Dr Miettinen (Helsinki, Finland).
Zymosterol was isolated from a yeast mutant D51-α impaired in
both ERG2 and ERG6 genes and was provided by F. Karst
(University of Poitiers, France).

Cells: The Jijoye, U937, HL60, CTLL2, MCF7, COS and M1
cell lines were obtained from the American Type Culture
Collection. CHO strain DXB11, Chinese hamster ovary, were
kindly given by Chasin [8]. B9 and TF1 cells were kindly given
by Aarden [9] and Kitamura [10] respectively. CHO expressing
the central cannabinoid receptor, the corticotropin-releasing-
factor receptor and the neurotensin receptor were kindly given
by B. Calandra, B. Miloux and F. Peuccu (Sanofi Recherche,
Labège, France) respectively. Cell lines were grown in their re-
spective medium: RPMI 1640 containing 10% foetal calf serum
(FCS) for human cell lines and M1; minimum essential medium
alpha +10% FCS for CHO; all media were supplemented with
10% dialysed FCS.

Defined medium. MCF7 cells were grown in RPMI 1640
supplemented with bovine insulin (10 μg/ml) and human
transferrin (10 μg/ml). CHO and M1 cell lines were grown in
defined medium number one for CHO (MDC1) [11] supple-
mented with bovine insulin (1 μg/ml) and human transferrin
(3 μg/ml). For each cell line, we verified that the cells were able
to proliferate in the defined medium (data not shown).

Cell proliferation assay. SR31747A activity was assayed in
24-well plates by adding 1 μl 1000×stock solution directly to
2 culture wells containing 1 ml culture medium. Briefly, the cells
were washed twice with medium without serum, seeded at 10³
cells/well and incubated in growth medium for the duration of
the test. Cell proliferation was determined in the presence of an excess
of SR31747A (200 nM). The membrane-bound radioligand was
separated from the free ligand by filtration on GF/C filters
soaked with 0.5% polyethyleneimine. The filters were washed
twice with 50 mM Tris/HCl, pH 7.4, 2.4 mM EDTA, 0.1% Tri-
ton X-100 at 4°C and the radioactivity was determined.

The protein concentration was determined according to
the method of Bradford [13].

Lipid extraction. Cells were seeded in flasks. At conflu-
ence, they were washed twice with medium without serum and
incubated for one day in medium containing serum without low
density lipoproteins (LDL). 24 h later, the cells were washed
again with medium without serum and incubated for another
day with ligands in the medium containing LDL-depleted serum
(2.5%). Extraction could also be done on cells cultivated in nor-
mal or lipid-depleted media. Briefly, cells were centrifuged at
800 g after detachment by scraping in the case of adherent cells,
washed twice with 150 mM NaCl, 1.5 mM KHP04, 8.3 mM
Na4HPO4, 12 H2O, pH 7.4 (NaCl/P) and frozen at -20°C. Ste-
rols were extracted as described [14]. Briefly, saponification was
performed at 80°C in the presence of KOH (10%) and methanol.
Lipid extraction was performed with heptane as a solvent and
desiccated on a Na2SO4 column. Dry extracts were kept at
-20°C until analysis.

GC analysis. The non-saponifiable lipid fraction was ana-
ysed by gas chromatography with a Varian 3300 chromatograph
using a 30m DB5 capillary column (0.32 mm internal diameter),
a ross injector, carrier gas helium (3 ml/min), column oven tem-
perature programmed at 200–270°C at 10°C/min, at 270–280°C
at 0.5°C/min and at 280–300°C at 0°C/min. All samples were
tested without Me3 Si derivatization, prepared for chromatogra-
phy by adding 1 μg ignosterol before extraction as an internal
standard and quantified by the area method comparatively to
Ignosterol [14]. Usual methods with Me3 Si derivatization were
used as controls but the chromatogram patterns were identical
so, subsequent, samples were tested without Me3 Si derivatiza-

gation. The cDNA sequence coding for murine emopamil
binding protein. The M1
cell line was treated in large scale culture without or with
SR31747A in low concentrations of normal serum (2.5% FCS)
or lipid-depleted medium. Sterols were analysed by gas chroma-
tography coupled to mass spectrometry (GC/MS) on a VG ZAB
2E instrument interfaced with a HP-5890 chromatograph work-
ing with a 30m OV1 column (0.32 mm internal diameter). Mass
spectra were obtained in electron-impact ionisation. Experimen-
tal mass spectra were automatically compared with reference
spectra included in the NIST spectra library.

Peak 1, peak 2 and peak 3 were assigned to cholesterol, 5α-
cholest-8(9)-en-3β-ol (J8-cholesterol) and 5α-cholesta-8,24-
dien-3-ol (zymosterol), respectively due to their accordance with
the corresponding NIST library reference spectra and spectra
characteristics standards (J8-cholesterol, zymosterol, choles-
terol).

Yeast strains. All the yeast strains used are congenic deriva-
tives of FL100 [5, 6]. EMY45 is a sterol isomerase deficient
strain (MATα, trp1, leu2, ura3, erg2:: TRP1, sur4:: URA3).
EMY30 (MATα, trp1, leu2, ura3) is the yeast sterol isomerase-
producing control. EMY45 pEMB1235 derives from EMY45 by
mutation using the EBP expression vector pEMB1235 [6].
D51-α is a yeast mutant impaired in both ERG2 and ERG6 genes
(MATα, erg2:: TRP1, erg6:: A, ura3, Trp1).

Vector construction and expression of murine emopamil-
binding protein. The cDNA sequence coding for murine emop-
aml-binding protein (X97755 in EMBL database) was adapted
using synthetic oligonucleotides in such a way that the protein
expression contained a c-myc epitope (EQKLISEEDL) at the car-
boxy terminus. This sequence was inserted into the expression
vector 7055 [15] by replacing the interleukin-2 (IL2)-coding se-
quenue (murine emopamil-binding protein expression vector =
865).
CHO-DHFR− cells (DXB11) [8] were transfected and stable transformants were isolated as described earlier [16] and subcultured into minimum essential medium +10% dialysed FCS medium. Transformants were screened for expression of murine emopamil-binding protein by immunofluorescence as described below.

Sterol isomerase assays. They were performed as described elsewhere [5] using cholest-8-en-3β-ol as the substrate.

Immunodetection of murine emopamil-binding protein. Transfected cells or transformants were incubated for two days in slide flasks (Nunc). The cells were washed with NaCl/Pioule containing 1% bovine serum albumin. The fixed cells were treated for 60 min at 4°C with NaCl/Pi containing a mouse monoclonal antibody specific for the c-myc epitope (1/500 dilution). Subsequently, the cells were washed with NaCl/Pi containing 1% bovine serum albumin and incubated with a fluorescein isothiocyanate-labelled rabbit antimouse antibody (1/100 dilution). The cells were examined using a Leitz Dialux microscope.

RESULTS

SR31747A binds the murine emopamil-binding protein expressed in yeast and inhibits the in vitro enzymatic activity of this enzyme. We have previously isolated a murine emopamil-binding-protein-encoding cDNA from a M1 cell line by complementation of the ERG2 defect in yeast and demonstrated its sterol isomerase enzymatic activity [6]. We checked if this murine enzyme was a SR31747A-binding protein in yeast cells devoid of the ERG2 gene product. No [3H]SR31747A specific binding could be detected in untransformed yeast cells devoid of the endogenous ERG2 gene product as described elsewhere [17]. In contrast, transforming yeast cells with the murine emopamil-binding-protein-encoding cDNA in a yeast expression vector restored SR31747A binding sites (Table 1). Saturation experiments on transformed yeast cell lysates with [3H]SR31747A showed Ko values of 1.3 nM ± 0.25 nM and Bmax of 0.4 ± 0.045 pmol/mg (Fig. 1). The Bmax value was relatively low but in the same order of magnitude in Scatchard analysis of [3H]SR31747A binding to wild-type yeast cell lysate (Bmax of 2.4 ± 0.56 pmol/mg). Ko values for ERG2 type were quite different with Ko values of 4.7 ± 1.45 nM (data not shown).

The A8–A7 sterol isomerase activity of extracts of the erg2 disruptant expressing the murine emopamil-binding-protein cDNA was assayed in the presence and in the absence of SR31747A. As expected, enzymatic activity was inhibited in the presence of SR31747A. The SR31747A concentration required for obtaining 50% inhibition (IC50) was 350 nM under these conditions (Fig. 2).

SR31747A-induced changes in cell sterol composition. To test if our drug inhibited cholesterol biosynthesis at the sterol isomerase step, sterols of SR31747A-treated M1 cells grown in low concentration of FCS were analysed by GC-MS. Chromatograms of unsonifiable lipid extracts from M1 cells revealed a single large peak corresponding to cholesterol. In contrast, SR31747A-treated cells accumulated an additional sterol which was identified by mass spectrometry as 5a-cholest-8(9)-en-3β-
ol, a substrate of sterol isomerase [18]. The accumulation of this sterol, not found in untreated M1 cells, was particularly pronounced in cultures kept in high concentrations of SR31747A and in cholesterol-free medium (medium containing LDL-depleted serum). This accumulation was dose-dependent; it increased at 1–100 nM SR31747A (Fig. 3A). A concomitant reduction in cholesterol was observed and estimated at 16% (data not shown). Cells treated with a high concentration of SR31747A (1 μM) accumulated an additional sterol (called sterol Y). Sterol Y was identified as 5α,3β-cholestenol, circle; sterol Y [zymosterol], square. (B) The effect of different ligands on sterol accumulation in the M1 cell line. Cells were treated in medium without LDL for 24 hours with different ligands at 100 nM. Sterol X [5β-cholestenol], open bars; sterol Y [zymosterol], filled bars. DTG and 3-PPP are 1,3,di-(o)tolylguanidine and HOPh-Pip-Pip-Pr, respectively. (C) SR31747A effects on different cell lines. Cells were treated in medium without LDL for 24 hours with SR31747A at 100 nM.

M1 cells are sensitive to the antiproliferative activity of SR31747A. In previous studies, Casellas et al. [1] demonstrated that the sigma ligand SR31747A elicited a suppressive effect on immune responses, possibly through sigma-binding sites expressed on lymphocytes. In vitro, the sigma ligand induced inhibition of proliferative response to mitogens of mouse and human lymphocytes. SR31747A suppressed cell proliferation in a concentration-dependent and time-dependent manner. Whereas a slight or no effect on cell growth was apparent at 48 h, prolonged incubation resulted in an efficient inhibition. These authors did not find any inhibitory effect of SR31747A on the proliferation of different established cell lines, even after a long exposure (120 h) to the drug at high concentrations (1 μM), under standard conditions. Only a weak effect on M1 cells, at micromolar concentrations of the drug, could be observed for the M1 cell line under these conditions of culture. M1 cells displayed 61% growth inhibition in the presence of 1 μM SR31747A in medium containing 10% FCS (120 h). We tested a number of other cell lines and confirmed the results obtained by Casellas et al. [1]. Table 2 shows the results obtained for the cell lines tested.

Increased sensitivity of the M1 cell line to SR31747A in medium containing low concentrations of serum. To check if the SR31747A effect on long-term culture resulted from nutrient limitation, we studied the kinetics of the M1 growth response to SR31747A under different serum concentrations (10, 5 or 2.5%) and for various periods of culture (2–7 days). The SR31747A effect was serum concentration and time dependent. Lowering the FCS concentration induced a higher sensitivity to SR31747A (Fig. 4A). In the presence of 10 nM SR31747, the maximal inhibition was obtained in cultures grown for 6 days (data not shown). Other sigma ligands displayed no anti-proliferative activity on M1 cells at these concentrations (data not shown), which is in agreement with data shown by Casellas et al. [1] for lymphocytes.

Depleting lipids from serum increases the sensitivity of M1 cells for SR31747A. The sensitivity to SR31747A of cells grown in media supplemented with serum lacking different components, namely LDL, lipid or low molecular-mass molecules (dialysed FCS), was assayed. Serum without LDL was discarded as it did not support long-term cultures of M1 (viability de-
Anti-proliferative agents such as cyclosporin A inhibited M₁ proliferation in an increased sensitivity to this drug, with regard to both the dose and response to SR31747A, whereas lipid depletion resulted in a decreased level of resistance. As myeloid M₁ cells are not easily transfectable, we tested this hypothesis in CHO cells. These latter, as well as MCF7 cells, are sensitive to the antiproliferative effect of SR31747A (Fig. 4D) and accumulated 5α-cholest-8(9)-en-3β-ol in the presence of the drug (Fig. 3C).

Different CHO clones that stably expressed c-myc-tagged murine emopamil-binding protein (clones CHO-865-1 and CHO-865-2) were used to investigate the effect of SR31747A on the M₁ cell line. Hanner et al. [7] proposed the existence of a superfamily of microsomal high-affinity drug acceptors comprising binding for pentazocine, haloperidol, 1,3-di-(o)tolylguanidine, and emopamil-binding-site ligands such as trifluoperazine and ifenprodil. Here, it was found that M₁ biological activities (inhibition of M₁ proliferation and 8-cholesterol isomer accumulation) of these products are in agreement with the pharmacology proposed for the emopamil-binding site [7]. Most potent drugs related to emopamil, trifluoperazine and ifenprodil, are active in the M₁ binding site, whereas LDL remains in the medium.

Cholesterol supplementation protects M₁ cells from proliferation inhibition by SR31747A. We studied the effect of supplementing lipid-depleted medium with cholesterol on the sensitivity of M₁ cells to SR31747A. Cholesterol at concentrations as low as 0.5 µg/ml reversed the effect of SR31747A (Fig. 4A). As cholesterol was utilised by the cells from LDL, M₁ cells were seeded into either 2.5% FCS or lipid-depleted medium containing 10 nM SR31747A in the absence or presence of CHO cells expressing the murine emopamil-binding protein cDNA overexpression should confer an increased level of resistance. As myeloid M₁ cells are not easily transfec-

Table 3. LDL effect on SR31747A anti-proliferative activity. Proliferation of the M₁ cell line was assayed as described in Materials and Methods with lipid-depleted serum in 3-day-old cultures. Assays were performed in triplicate.

<table>
<thead>
<tr>
<th>SR31747A</th>
<th>LDL concentration</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td>nM</td>
<td>µg/ml</td>
<td>%</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>76</td>
</tr>
<tr>
<td>10</td>
<td>0.2</td>
<td>76</td>
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<tr>
<td>10</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>42</td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>20</td>
</tr>
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</table>

SR31747A inhibited cell proliferation unless the media were supplemented with LDLs (at 20 µg/ml or more; Table 3). 87-Lathosterol is the product of the Δ8-cholesterol isomerisation reaction. We verified that we could reverse the SR31747A-induced anti-proliferative effect by adding this sterol in M₁ cells (data not shown). However cholesterol is much more potent than Δ7-lathosterol, perhaps because of problems of the intracellular transport of this lipid.
Table 4. emopamil-binding protein related ligands and M1 biological activities. M1 proliferation was assayed as described in Materials and Methods in lipid-depleted medium in a three-day-old culture. Analysis of cell extracts by gas chromatography was performed as described in Materials and Methods. Briefly, cells were treated in medium without LDL for 24 hours with ligands at 100 nM. The non-saponifiable lipid fraction was analysed on a DBS capillary column without Me3Si derivatization. All samples contained 1 µg igonsterol added before extraction as an internal standard.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Inhibition of M1 proliferation (IC50)</th>
<th>Sterol X/cholesterol (100 nM SR31747A)</th>
<th>nM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR31747A</td>
<td>1</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>60</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iifenprodil</td>
<td>250</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>&gt;100000</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTG</td>
<td>&gt;100000</td>
<td>&lt;1</td>
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</table>

DISCUSSION

Several lines of evidence indicate that SR31747A is a ligand of emopamil-binding protein, a protein that inhibits cholesterol synthesis at the sterol isomerase step in animal cells.

First, we have shown that [1H]SR31747 binds with high affinity (Kd = 1.3 nM) to yeast lysates expressing the murine emopamil-binding protein, whereas no binding is detected in the lysate of the yeast sterol isomerase disruptant.

Second, SR31747A has been found to inhibit sterol isomerase activity of the emopamil-binding protein in in vitro assays. The IC50 value is about 350 nM under these conditions.

Third, SR31747A added to cultures of M1 cells and to other cell lines blocks the conversion of lanosterol to cholesterol and, depending on its concentration, causes the accumulation of Δ8-sterol intermediates in the cells. These sterols have been identified as 5α-cholest-8-(9)-en-3β-ol (Δ8-cholesterol) and 5α-cholesta-8,24-dien-3-ol (zymosterol) by GC-MS analysis, which is consistent with inhibition at the sterol isomerase step. 5α-Cholest-8-(9)-en-3β-ol accumulated at an SR31747A concentration as low as 1 nM of SR31747A. Zymosterol has been proposed to be the physiological substrate of sterol isomerase [22]. However, it is also a substrate of C24 reductase, which explains why sterol isomerase blockade provokes the accumulation of Δ8-cholesterol instead of zymosterol [23]. Therefore, it can be deduced that this drug is not an efficient inhibitor of C24 reductase. In contrast, 5α-cholesta-8,24-dien-3β-ol accumulated at high concentrations of SR31747A. The accumulation of high levels of Δ8-cholesterol results in the inhibition of C24 reductase by the excess product and the accumulation of the second step (zymosterol). We cannot easily calculate an in vivo IC50 for the enzymatic activity because we did not measure the neo-synthesis of cholesterol; however it seems to be in the range of a few nanomoles (Fig. 3A) which is not far from the estimation of the Kd binding value. However, the IC50 value deduced from the in vitro assay is much higher. This result is not surprising in the light of the previous results obtained by others [21] and by ourselves [6] with the yeast sterol isomerase. Indeed, a similar discrepancy has already been observed with various inhibitors of the yeast sterol isomerase, including SR31747A. In the same, in vitro enzymatic assays, such as the one used here, SR31747A inhibited the yeast sterol isomerase with a IC50 value of 0.35 µM whereas the Kd was in the nanomolar range. However, in this particular case, additional data could also be obtained from experiments on SR31747A-induced sterol isomerase inhibition in entire cells [6]; interestingly, the IC50 value deduced from these in vivo experiments was in the nanomolar range, thus no discrepancy was observed between the Kd determined by radioligand binding and the IC50 value deduced from sterol isomeration inhibition studies when performed in entire cells. Therefore, the values given by the in vitro enzyme assay are clearly not representative of the in vivo situation, possibly because of the relatively high amount of substrate (75 µM) which is added in this assay.

Fourth, we have verified that other emopamil-binding-protein ligands such as trifluoperazine and ifenprodil also lead to the accumulation of 5α-cholest-8-(9)-en-3β-ol in the M1 cell line. In contrast, sigma receptor ligands that do not bind emopamil-binding protein with high affinity, such as 1, di-(o)tolyl-guanidine, pentazocine and HOPh-Pip-Pr, do not provoke accumulation of this intermediate. This observation is consistent with a sterol isomerase role of emopamil-binding protein in the cholesterol biosynthetic pathway in M1 cells. In contrast, there is no evidence for such a role with sigma, receptor, at least in M1 cells, in spite of the structural similarity shared by this protein and yeast sterol isomerase.

SR31747A is also effective in inhibiting proliferation of various established mammalian cell lines under lipid-depleted culture conditions. The IC50 value is nanomolar for M1 cells grown in serum-lipid-depleted medium. Trifluoperazine and ifenprodil have also been shown to display growth inhibitory activities. However, these emopamil-binding-protein ligands are less active than SR31747A in inducing inhibition of sterol isomerase and cell proliferation. Interestingly, the sigma receptor ligands that do not provoke any accumulation of this sterol intermediate do not induce any growth inhibition either.

Several lines of evidence suggest that emopamil-binding-protein enzyme inhibition is responsible for the proliferation inhibition in cell lines. First, the Kd value of [1H]SR31747 on yeast lysates expressing the murine emopamil-binding protein is in the same order of magnitude as the IC50 obtained in the M1 cells antiproliferation assay (1.3 nM and 1 nM, respectively). Second, the efficacy of emopamil-binding-protein ligands in inducing sterol-intermediate accumulation correlates nicely with their growth inhibition efficiency. In addition, the necessity of serum-
lipid-depleted medium for the SR31747A antiproliferative effect, its reversion by the addition of cholesterol, AT-lathosterol or by the overexpression of emopamil-binding protein are consistent with this hypothesis.

In mammalian cells, SR31747A is known to bind the sigma receptor [17] and emopamil-binding protein (this study). It was demonstrated that the *in vitro* inhibitory effect of SR31747A on T-cell proliferation was achieved even in the presence of complete serum. Moreover, this activity was blocked by the competitive sigma ligand (+)-pentazocine which led Jbilo et al. [17] to conclude that the sigma receptor could mediate the immunosuppressive effect of this drug. In contrast, we have shown that SR31747A inhibits cell lines only in serum-lipid-depleted medium, apparently as a consequence of sterol isomerase inhibition, and that this anti-proliferative activity is not affected by the addition of (+)-pentazocine. Thus, emopamil-binding protein and not sigma receptor seems to mediate the effect of SR31747A on established cell lines. Inhibition of cholesterol biosynthesis, whether at the early HMG CoA reductase step (by lovastatin or compactin [24]), or at final steps (by 24(R,S),25-minimolanosterol [25], BM 17.766 [26]), has been shown to induce growth inhibition of tumour cells. Although we did not show that SR31747A inhibits liver sterol isomerase *in vivo*, such an inhibition can be expected as liver sterol isomerase is pharmacologically indistinguishable from the yeast and mammalian enzymes [21]. Furthermore, it is worth noting that tamoxifen is another M1 sterol isomerase inhibitor, which competitively inhibits SR31747A binding to this enzyme [27]. Tamoxifen has been shown to lower plasma cholesterol levels and concomitantly provokes the accumulation of Aβ-cholesterol in long-term treated patients [23]. Experiments are in progress to study the effect of SR31747A on LDL-cholesterol levels and its anti-tumoural activity *in vivo*.

In contrast to its general antiproliferation activity, the immunomodulatory effect of SR31747A could be mediated by a pentazocine-binding protein, such as the sigma receptor. If the two effects of the drug are mediated by distinct acceptor proteins, it should be possible to select drugs related to SR31747A that show dissociation of cholesterol biosynthesis inhibition and immunomodulatory activities.

The recently postulated roles of emopamil-binding protein or mS1 in neuroprotection [5, 6], and the suggestion that this enzyme is implicated in antiproliferative activities on mammalian cell lines might be very important for future clinical applications.

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