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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 $sigma_1$ receptor and emopamil-binding protein, have been characterised molecularly. Only the σ_1 receptor has been shown to bind (Z)*N*-cyclohexyl-*N*-ethyl-3-(3-chloro4-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 $sigma_1$ 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 *Δ*8-cholesterol isomer at the expense of cholesterol in SR31747A-treated cells, suggesting that cholesterol biosynthesis is inhibited by SR31747A at the *Δ*8-*Δ*7 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 $sigma_1$ 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-isomerase 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)tolylguanidine 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 ($sigma_1$ receptor also known as SR31747A-binding protein and emopamil-binding protein) have been characterised molecularly. The $sigma_1$ receptor has been shown to bind (*Z*)*N*-cyclohexyl-*N*-ethyl-3-(3-chloro4-cyclohexylphenyl)propen-2-ylamine hydrochloride (SR31747A) with high affinity. In yeast, SR31747A arrests cell proliferation by inhibiting *Δ*8-*Δ*7 sterol isomerase encoded by the *ERG2* gene [5]. Although yeast sterol isomerase and the $sigma_1$ receptor share considerable sequence similarities, $sigma_1$ 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 $sigma_1$ 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 $sigma_1$ 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.

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Abbreviations. FCS, foetal calf serum; GC-MS, gas chromatography coupled to mass spectroscopy; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA reductase; IC_{50}, concentration causing 50% inhibition; IL2, interleukin 2; IL6, interleukin 6; LDL, low density lipoprotein; MTT, 3-(4,5-dimethylthiazol 2-yl)3,5diphenylformazan; 3.PPP, HOPh-Pip-Pr.

MATERIALS AND METHODS

Chemicals. Bovine insulin, human transferrin, cholesterol, *Δ*7-lathosterol and pentazocine were purchased from Sigma Chemical Company. 1,3-Di-(o)tolylguanidine, HOPh-Pip-Pr and trifluoroperazine were supplied by Interchim. Cyclosporin A (Sandimmun) was kindly provided by Sandoz Laboratories. SR31747A and [^{3}H]SR31747, (specific activity 2109 GBq/mmol), were synthetised by Sanofi Recherche [4]. All

SR31747A stock solutions were prepared in ethanol at 1000× concentration. Lipid-depleted serum was purchased from J. Boy, Reims, France; mevanololactone, sorbitol, Pfablock, leupeptin, pepstatin, epibestatin, soybean trypsin inhibitor, ifenprodil, methotrexate, phenylmethylsulfonyl fluoride, low density-lipoprotein-depleted serum and foetal calf serum were purchased from Sigma Chemical Company. Cytokines interleukin 2 (IL2), IL6, granulocyte-monocyte-colony stimulating factor were purchased from Genzyme. The anti-*c-myc* mouse Ig 9E10 was a gift from B. PAU (CNRS, Montpellier, France). The fluorescein-coupled rabbit-anti-mouse polyclonal antibody was from Silenus. Ignosterol was isolated from a yeast mutant devoid of *ERG24* (kindly given by F. Karst, Poitiers, France). *Δ*8-cholestenol 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. Pecceu (Sanofi Recherche, Labège, France) respectively. Cell lines were grown in their respective 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 gentamicin (50 mg/l). CHO recombinant cells were grown in minimum essential medium +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] supplemented 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 culture wells containing 1 ml culture medium. Briefly, the cells were washed twice with medium without serum, seeded at 10^5 cells/well and incubated in growth medium for the duration of the test. Cell proliferation was determined by the 3-(4,5-dimethylthiazol 2yl)3,5diphenylformazan (MTT) colorimetric assay [12]. Cholesterol was supplied directly in ethanol or with cholesterol coupled to methyl-*β*-cyclodextrin which is a water-soluble complex of cholesterol. In the latter case, it was tested with methyl-*β*-cyclodextrin as control. Lipid-depleted medium is made by supplementation of normal basal medium with an FCS deprived of lipids by treatment with aerosil particles (fused silica) as a dry lipid absorbent agent.

Binding assay. Receptor-binding assays were done according to the method of Paul et al. [4], with some modifications.

Yeast cells were washed twice with cold water, homogenised at 4°C using a French press in 50 mM Tris/HCl, pH 7.5, EDTA (2 mM), sorbitol (0.1 M), in the presence of protease inhibitors (Pfablock 200 μM + pepstatin 1 μM). The pellet was suspended after centrifugation (10 min, 4000 *g*) in the same buffer.

The fraction (50 μg protein/assay) was resuspended in 100 μl 50 mM Tris/HCl, pH 7.4, 2.4 mM EDTA, 0.025% Tween 20, incubated for one hour at 4°C with 2 nM [^{3}H]SR31747. Non specific binding 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% polyethylenimine. The filters were washed twice with 50 mM Tris/HCl, pH 7.4, 2.4 mM EDTA, 0.1% Triton 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 confluence, 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 normal 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 KH_2PO_4, 8.3 mM Na_2HPO_4, 12 H_2O, pH 7.4 (NaCl/P_i) and frozen at −20°C. Sterols 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 Na_2SO_4 column. Dry extracts were kept at −20°C until analysis.

GC analysis. The non-saponifiable lipid fraction was analysed 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 temperature programmed at 200−270°C at 10°/min, at 270−280°C at 0.5°/min and at 280−300°C at 10°/min. All samples were tested without Me_3Si derivatization, prepared for chromatography by adding 1 μg ignosterol before extraction as an internal standard and quantified by the area method comparatively to Ignosterol [14]. Usual methods with Me_3Si derivatization were used as controls but the chromatogram patterns were identical so, subsequent, samples were tested without Me_3Si derivatization.

Gas chromatography/mass spectroscopy analysis. 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 chromatography coupled to mass spectroscopy (GS/MS) on a VG ZAB-2E instrument interfaced with a HP-5890 chromatograph working with a 30m OV1 column (0.32 mm internal diameter). Mass spectra were obtained in electron-impact ionisation. Experimental 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, 5*α*-cholest-8(9)-en-3*β*-ol (*Δ*8-cholestenol) and 5*α*-cholesta-8,24-dien-3-ol (zymosterol), respectively due to their accordance with the corresponding NIST library reference spectra and spectra characteristics standards (*Δ*8-cholestenol, zymosterol, cholesterol).

Yeast strains. All the yeast strains used are congenic derivatives of FL100 [5, 6]. EMY45 is a sterol isomerase deficient strain (MAT*α*, *trp1*, *leu2*, *ura3*, *erg2*:: *TRP1*, *sur4*:: *URA3)*. EMY30 (MAT*α*, *trp1*, *leu2*, *ura3*) is the yeast sterol isomerase-producing control. EMY45 pEMR1235 derives from EMY45 by transformation using the EBP expression vector pEMR1235 [6]. D51-*α* is a yeast mutant impaired in both *ERG2* and *ERG6* genes (MAT*α*, *erg2*:: *TRP1*, *erg6-Δ*, *ura3*, *Trp1*).

Vector construction and expression of murine emopamil-binding protein. The cDNA sequence coding for murine emopamil-binding protein (X97755 in EMBL database) was adapted using synthetic oligonucleotides in such a way that the protein expressed contained a *c-myc* epitope (EQKLISEEDL) at the carboxy terminus. This sequence was inserted into the expression vector 7055 [15] by replacing the interleukin-2 (IL2)-coding sequence (murine emopamil-binding protein expression vector = 865).

Table 1. Binding of [^{3}H]SR31747 to yeast lysates expressing the murine emopamil-binding protein cDNA. Lysate preparation and binding assays were performed as described in Materials and Methods. Each result is the mean of three experiments performed in triplicate and specific binding was calculated as the difference between total bound and non-specific binding. Experiments were performed with 50 µg protein/assay and 1 nM [^{3}H]-SR31747A and 200 nM of SR31747A.

Sample	Specific binding
	dpm
EMY45 (*erg2* disruptant)	0 ± 41
EMY45 pEMR1235 (*erg2* disruptant expressing the M1 murine emopamil-binding protein c-DNA)	7313 ± 320
EMY30 (wild type)	3304 ± 520

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 the 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-3*β*-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/P$_i$, and subsequently fixed with 65% ethanol at −20°C for 10 min. The ethanol treatment was followed by washing with NaCl/P$_i$ containing 1% bovine serum albumin. The fixed cells were treated for 60 min at 4°C with NaCl/P$_i$ containing a mouse monoclonal antibody specific for the *c-myc* epitope (1/500 dilution). Subsequently, the cells were washed with NaCl/P$_i$ containing 1% bovine serum albumin and incubated with a fluoroisothiocyanate-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 [^{3}H]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 [^{3}H]SR31747A showed K_d values of 1.3 nM ± 0.25 nM and B_{max} of 0.4 ± 0.045 pmol/mg (Fig. 1). The B_{max} value was relatively low but in the same order of magnitude in Scatchard analysis of [^{3}H]SR31747A binding to wild-type yeast cell lysate (B_{max} of 2.4 ± 0.56 pmol/mg). K_d values for *ERG2* type were quite different with K_d values of 4.7 nM ± 1.45 nM (data not shown).

The *Δ*8-*Δ*7 sterol isomerase activity of extracts of the *erg2* disruptant expressing the murine emopamil-binding-protein c-DNA 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 (IC_{50}) was 350 nM under these conditions (Fig. 2).

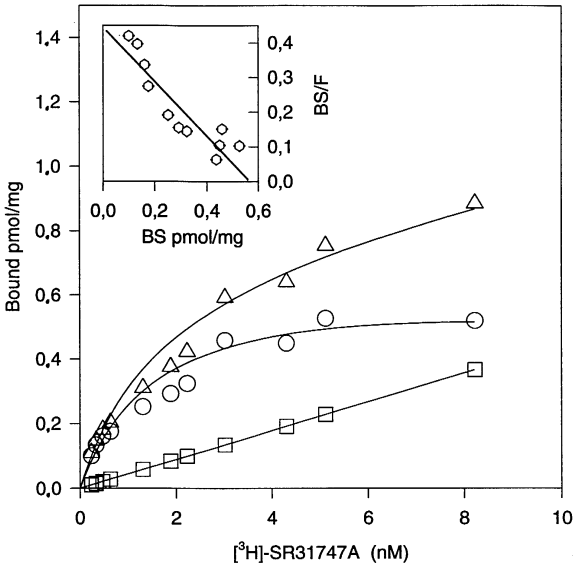

Fig. 1. Saturation experiment on transformed yeast cell extracts. The assays were performed as described in Materials and Methods. Cell lysate (50 µg of (*erg2*, *sur4*) double disruptant expressing murine emopamil-binding cDNA) was incubated with increasing concentrations of [^{3}H]SR31747 at 0.3−9 nM. Non-specific binding was determined by the addition of non-radiolabelled SR31747A (200 fold [^{3}H]SR31747 concentration). Each point is the average of the three independent determinations. For Scatchard transformation of saturation experiment, BS and F were calculated and represent specific bound and free radioligand, respectively. Total bound, triangle; non specific binding, square; specific binding, circle.

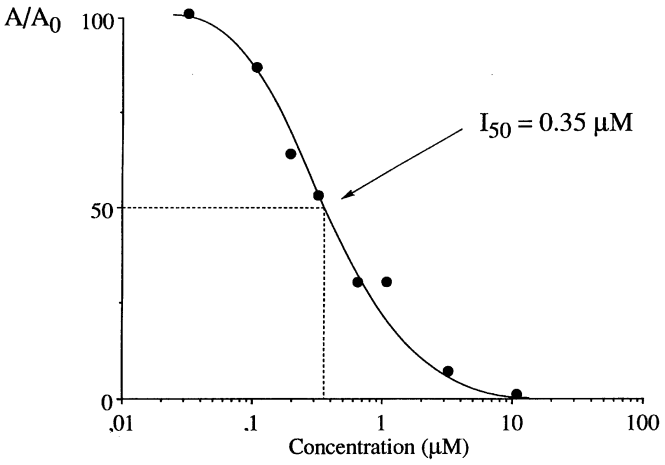

Fig. 2. Effect of SR31747A on *in vitro* enzymatic activity of M1 sterol isomerase. Inhibition of *Δ*8-*Δ*7 sterol isomerase was assayed as described in Materials and Methods. Inhibition is expressed relative to the residual activity (A/Ao) as a function of the SR31747A concentration.

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 unsaponifiable 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 5*α*-cholest-8(9)-en-3*β*-

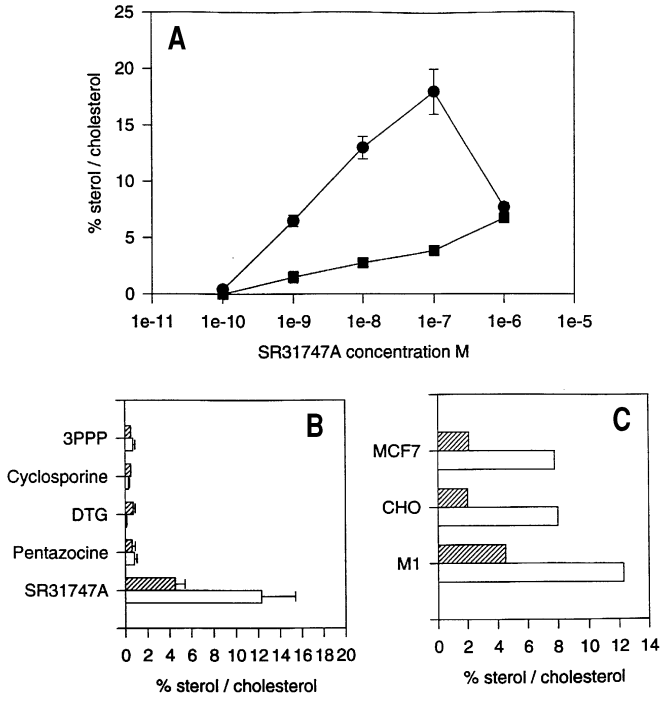

Fig. 3. Analysis of cell extracts by gas chromatography. (A) Effect of SR31747 concentration on the sterol profile of the M1 cell line. The M1 cell line was subcultured in flasks as described in Materials and Methods. Briefly, cells were treated in medium without LDL for 24 hours with SR31747A at different concentrations. The non-saponifiable lipid fraction was analysed on a DB5 capillary column without Me_3Si derivatization. All samples contained 1 μg ignosterol added before extraction as an internal standard. Sterol X [*Δ*8-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 [*Δ*8-cholestenol], open bars; sterolY [zymosterol], filled bars. DTG and 3-PPP are 1,3-di-(o)tolylguanidine and HOPh-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.

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 5*α*-cholesta-8,24-dien-3-ol by GC-MS. These identifications were confirmed by comparison with samples of *Δ*8-cholestenol and zymosterol used as standards. These observations indicate that SR31747A inhibits one of the last steps of the cholesterol biosynthetic pathway. The accumulation of these two sterols points to sterol isomerase inhibition. Other drugs such as cyclosporin A or sigma ligands such as 1,3-di-(o)tolylguanidine and pentazocine do not accumulate these types of cholesterol isomer in cells cultured in medium containing LDL-depleted serum (Fig. 3B). The *Δ*8-cholestenol was detectable in low amounts (⩽1%) in some experiments, in untreated cells and with some ligands. In these cases, further experiments were performed to validate the conclusions in the same type of assay with increasing concentrations of product. At 10 μM, the *Δ*8 sterol was present in the same range but not for the same ligand (⩽1%). It appears clearly that 1% represents the background of the test.

Table 2. Effects of SR31747A on proliferation of M1 cell line in standard medium. Inhibition was calculated at 1 μM SR31747A. Cell lines were cultured in their respective basal medium supplemented with 10% FCS and cytokines if necessary (TF1/GM-CSF, B9/IL6, CTLL2/IL2), for 120 hours at 37°C. Proliferation of the cell lines was assayed by the MTT method as described in Materials and Methods.

Cell	Description	Inhibition
		%
Jijoye	Human B cell line	20
U937	Human monocytic cell line	11
HL60	Human monocytic cell line	23
TF1	Human erythroleukemia cell line	15
MCF7	Human breast adenocarcinoma cell line	30
B9	Mouse plastocytoma cell line	17
CTLL2	Mouse T-cell line	10
I10	Mouse Leydig cell line	2
M1	Mouse myeloblast cell line	61
COS	Monkey kidney cell line	12
CHO	Chinese Hamster ovary	28

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 SR 31747 A 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 media 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-

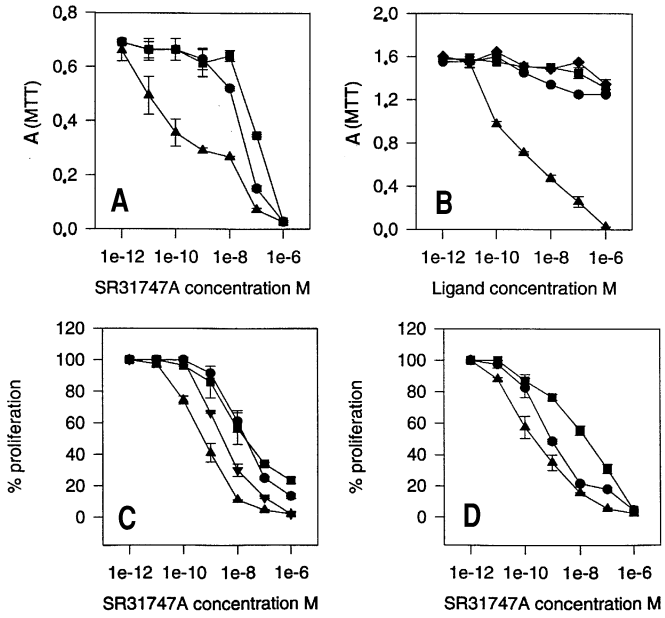

Fig. 4. Antiproliferative assays. Proliferation of the cell lines was determined after different conditions of culture as described in Materials and Methods and expressed as an absorbance or relative proliferation. (A) Effect of SR31747A and serum concentration on M1 cell proliferation. Medium containing 10% (square), 5% (circle), 2.5% (triangle) FCS − 7 day culture. (B) Effect of different drugs on M1 cell line proliferation in lipid-depleted medium. Proliferation of the cell line was determined after a three-day culture. SR31747A (triangle) was compared with different sigma ligands 3-PPP (circle), DTG (square), ± pentazocine (diamond-shaped). DTG and 3-PPP, 1,3-di-(o)tolylguanidine and HOPh-Pip-Pr, respectively. (C) The effect of cholesterol on SR31747 activity. M1 cell line cultured in lipid-depleted medium for three days. No cholesterol (triangle), 0.5 μg/ml cholesterol (downward pointing triangle), 1 μg/ml cholesterol (circle), 2 μg/ml cholesterol (square). (D) SR31747 anti-proliferative effect on different cell lines in defined medium. Proliferation of the cell lines was assayed after five days of culture in serum-free media as described in Materials and Methods. For each cell line, it was verified that the cell proliferates in defined medium (data not shown). M1 (triangle), CHO (circle), MCF7 (square).

creased after two days). Lipid-depleted medium is essentially deprived of lipids such as cholesterol and 80% LDL remains in the medium.

The use of dialysed serum instead of normal FCS did not alter the response to SR31747A, whereas lipid depletion resulted in an increased sensitivity to this drug, with regard to both the 50% inhibition concentration (IC_{50}) and the time required for maximal inhibition. The IC_{50} concentration was determined to be 1 nM in the three-day culture. We confirmed the accumulation of 5α-cholesta-8(9)-en-3β-ol when the cells were grown in lipid-depleted medium (data not shown). Lipid depletion did not increase the sensitivity of M1 to other sigma ligands (Fig. 4B). Anti-proliferative agents such as cyclosporin A inhibited M1 cell proliferation [19] but the sensitivity was the same in standard medium (10% FCS) as in lipid-depleted medium (IC50 of 100 nM; data not shown).

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. 4C). 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 LDL.

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.

SR31747A	LDL concentration	Inhibition
nM	μg/ml	%
10	0	76
10	0.2	76
10	2	73
10	20	42
10	200	20

SR31747A inhibited cell proliferation unless the media were supplemented with LDLs (at 20 μg/ml or more; Table 3). Δ7-Lathosterol is the product of the Δ8-cholestenol isomerisation reaction. We verified that we could reverse the SR31747A-induced anti-proliferative effect by adding this sterol in M1 cells culture (data not shown). However cholesterol is much more potent than Δ7-lathosterol, perhaps because of problems of the intracellular transport of this lipid.

Since the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase step is known to be rate-limiting in the cholesterol biosynthetic pathway [20], we tested the possibility of reversing the SR31747A effect with the product of (HMG-CoA) reductase, namely mevalonate. No effect of mevalonate was found, even at 0.5 mM (data not shown), suggesting no inhibition of this enzyme in SR31747A-treated cells. Jbilo et al. [17] observed that pentazocine reversed the SR31747A-induced inhibition of lymphocyte proliferation. No such protective effect of pentazocine, 1,3-di-(o)tolylguanidine and HOPh-Pip-Pr was observed in our case (data not shown).

Emopamil binding protein ligands display similar biological activities as 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-cholestenol isomer accumulation) of these products are in agreement with the pharmacology proposed for the emopamil-binding site [7]. Most potent drugs related to emopamil, trifluoroperazine and ifenprodil, are active in the M1 cell proliferation assay in serum-lipid-depleted medium with IC_{50} values of 60 nM and 250 nM, respectively, whereas ethanol which is a diluent and 1,3-di-(o)tolylguanidine were inactive (Table 4). This table shows that trifluoroperazine and ifenprodil are also efficient at blocking sterol isomerase because of 8-cholestenol isomer accumulation, whereas the others are ineffective.

Overexpression of murine emopamil-binding protein increases resistance to SR31747A-induced proliferation inhibition. If the antiproliferative effect of SR31747A is a consequence of sterol isomerase inhibition, murine emopamil-binding-protein cDNA overexpression should confer an increased 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

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 DB5 capillary column without Me_3Si derivatization. All samples contained 1 μg ignosterol added before extraction as an internal standard.

Substance	Inhibition of M1 proliferation (IC_{50})	Sterol X/cholesterol (100 nM SR31747A)
	nM	%
SR31747A	1	24
Trifluoroperazine	60	5.6
Ifenprodil	250	4.2
Ethanol	>100 000	<1
DTG	>100 000	<1

CHO-865-50 and their subclones) were selected by immunodetection in minimum essential medium +10% dialysed FCS medium. Since parental untransfected CHO-DHFR$^-$ cells are not able to grow in this medium, transfected clones were chosen that did not express the *c-myc*-tagged enzyme, as a negative control. Negative controls were a CHO clone obtained from this transfection experiment (CHO-865-42 and its subclones) that did not show any expression of the enzyme, as verified by immunodetection and Northern-blot analysis (data not shown) and other clones obtained from different transfection experiment (CHO expressing the central cannabinoid receptor, CHO expressing the corticotropin-releasing-factor receptor, CHO expressing the neurotensin receptor). The SR31747A proliferation inhibition effect was tested in CHO cells grown in serum-free medium. The IC_{50} value obtained was 198 ± 19 nM for different subclones of CHO-865-1 and of CHO-865-50 (10 subclones tested), whereas it was only 24 ± 2 nM for all tested negative controls (representing overall 10 different clones). Analysis of the sensitivity to methotrexate [16] for different clones showed that overproduction of the enzyme did not modify the sensitivity of the CHO cells, confirming a specific effect of the tagged M1 murine emopamil-binding protein on the SR31747A response (data not shown). This shows that overproduction of emopamil-binding protein conferred SR31747A resistance to CHO cells.

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 [^{3}H]SR31747 binds with high affinity ($K_d = 1.3$ 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 IC_{50} 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-cholestenol) 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-cholestenol 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-cholestenol results in the inhibition of C24 reductase by the excess product and the accumulation of the second sterol (zymosterol). We cannot easily calculate an *in vivo* IC_{50} for the enzymatic activity because we did not measure the neosynthesis 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 K_d binding value. However, the IC_{50} 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 *in vitro* enzymatic assays, such as the one used here, SR31747A inhibited the yeast sterol isomerase with a IC_{50} value of 0.35 μM, whereas the K_d 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 IC_{50} value deduced from these *in vivo* experiments was in the nanomolar range, thus no discrepancy was observed between the K_d determined by radioligand binding and the IC_{50} value deduced from sterol isomerisation 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 trifluoroperazine 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)tolylguanidine, 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 $sigma_1$ 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 IC_{50} value is nanomolar for M1 cells grown in serum-lipid-depleted medium. Trifluoroperazine 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 K_d value of [^{3}H]SR31747 on yeast lysates expressing the murine emopamil-binding protein is in the same order of magnitude as the IC_{50} 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, Δ7-lathosterol or by the overexpression of emopamil-binding protein are consistent with this hypothesis.

In mammalian cells, SR31747A is known to bind the $sigma_1$ 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 $sigma_1$ ligand (+)-pentazocine which led Jbilo et al. [17] to conclude that the $sigma_1$ 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 $sigma_1$ 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-iminolanosterol [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 Δ8-cholestenol in long-term treated patients [23]. Experiments are in progress to study the effect of SR31747A on LDL-cholesterol levels and its anti-tumoral 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 $sigma_1$ 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 mSI 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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