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SSR125329A, a high affinity σ receptor ligand with potent anti-inflammatory properties

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Abstract

SSR125329A ([(*Z*)-3-(4-Adamantan-2-yl-3,5-dichloro-phenyl)-allyl]-cyclohexyl-ethyl-amine) is a new ligand exhibiting high affinity for σ_1 and σ_2 receptors and for the human Δ8–Δ7-sterol isomerase. Here we show that this molecule has potent immunoregulatory properties both in vitro and in vivo. SSR125329A inhibited staphylococcal enterotoxin B-induced mouse splenocyte proliferation in vitro, whereas in vivo it enhanced lipopolysaccharide-induced systemic release of interleukin-10 while simultaneously inhibiting tumor necrosis factor-alpha (TNF-α) synthesis. It also prevented graft-versus-host disease in B6D2F1 mice and protected Mrl/lpr mice against the development of its spontaneous rheumatoid-like syndrome. There is high interplay of pro- and anti-inflammatory cytokines in inflammatory processes, particularly in human rheumatoid arthritis. The results of this study provide substantial evidence that σ receptor ligands may represent a new effective approach for rheumatoid arthritis treatment.

Keywords: TNF-α (tumor necrosis factor); Interleukin-10; σ Receptor; Autoimmune disease

1. Introduction

Sigma (σ)-binding sites recognize an array of structural classes of compounds exhibiting potent antipsychotic, neuroprotective and immunoregulatory effects. These ligands include haloperidol, 1,3-di-*o*-tolylguanidine (DTG), (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl) piperidine [(+)-3PPP], and (+)-benzomorphans such as (+)-pentazocine and (+)-*N*-allylnormetazocine. According to biochemical and radioligand-binding data, recognition sites have been classified into at least two types, termed σ_1 and σ_2 (Quirion et al., 1992), that mediate σ receptor ligand activities. While σ_1 has been molecularly characterized, the σ_2 receptor has not yet been cloned. The σ_1 is a 26 kDa protein related to the yeast *Saccharomyces cerevisiae* Δ8–Δ7-sterol isomerase encoded by the *erg2* gene, and both proteins share 30% sequence homology (Silve et al., 1996a). Despite this substantial homology, σ_1 does not mediate any sterol isomerase function. No sequence information is available for σ_2, but the protein has been pharmacologically characterized and its tissular expression studied using radiolabeled specific ligands. The σ receptors are widely expressed throughout the body and have been described in a variety of tissues, including distinct regions of the central nervous system and at the periphery of some endocrine-related structures (Wolfe et al., 1989), in the gastrointestinal tract (Roman et al., 1988), in the liver (Samovilova et al., 1988) and the kidney (Hellewell et al., 1994). The σ_2 receptors are reported to be overexpressed in tumor cells as compared with their normal counterparts, including breast, neural, lung, prostate and melanoma tumors (Vilner et al., 1995; Wheeler et al., 2000). Another binding protein has been identified as sharing pharmacological similarities with σ receptors, and σ receptor ligands bind this protein, i.e. the human sterol isomerase, with high affinity. This enzyme exhibiting Δ8–Δ7 sterol isomerase activity is

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essential for the sterol biosynthesis pathway and catalyzes the conversion of Δ8-sterols to their corresponding Δ7-isomers (Paul et al., 1998). Interestingly, even though the two proteins are not structurally related, σ_1 and the human sterol isomerase are colocalized and associated within the endoplasmic reticulum and the nuclear envelope membrane, and are delocalized together during mitosis (Dussossoy et al., 1999). Besides their neuroactive properties, σ receptor ligands are known to modulate immune responses. The involvement of σ receptors in mediating immunoregulatory properties of σ receptor ligands is supported by data highlighting that σ receptors are significantly expressed in the immune system. Binding studies using specific radiolabeled ligands have shown that both σ_1 and σ_2 receptors are expressed on human peripheral blood mononuclear cells (Wolfe et al., 1989), on mouse leukocytes (Carr et al., 1991) and rat splenocytes (Paul et al., 1994). We have developed a σ receptor ligand, SR31747A (*N*-cylohexyl-*N*-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride), which specifically binds human and rat lymphocytes with high affinity (Paul et al.,1994). This molecule was shown to be the most potent in competing with all known σ receptor ligands specific to subtype 1 or subtype 2 receptors described so far. The molecule also binds the human sterol isomerase with nanomolar affinity (Paul et al., 1998). We conducted investigations with different in vitro and in vivo models and demonstrated that SR31747A modulated immune responses (Casellas et al.,1994). SR31747A was thus shown to significantly inhibit TNF-α production and concomitantly stimulate the expression of the anti-inflammatory interleukin-10 (Derocq et al., 1995; Bourrié et al., 1995, 1996). To date, no other immune modulator has been described to induce such a dual effect, i.e. cyclosporin A, steroids, and even dexamethasone, which is a strong TNF-α repressor, fail to stimulate interleukin-10 production. TNF-α is known to play a key role in the pathogenesis of rheumatoid arthritis, in inflamed joints, where it exhibits a variety of complex inflammatory effects leading to joint destruction. By contrast, interleukin-10 acts as a powerful inhibitor of most mediators involved in this pathology. Current strategies aimed at inhibiting TNF-α activity using neutralizing antibodies to TNF-α and soluble forms of the receptor have demonstrated substantial efficacy and are approved for rheumatoid arthritis therapy (Feldmann and Maini, 2001). Some limitations of such treatments may appear since these large peptides elicit an immune response that reduces their efficacy, thus prompting the need for concomitant treatment with immunosuppressive molecules (e.g. methotrexate). The use of molecules that inhibit TNF-α synthesis is new in rheumatoid arthritis and only a few products have reached the clinical phase of development. Molecules that concomitantly inhibit TNF-α synthesis and induce interleukin-10 expression at the inflammatory site may offer new prospects for treatment. The properties of SR31747A indicate that this molecule could be an interesting candidate. Nevertheless, its range of application may be limited, as it is almost completely metabolized by the cytochrome P450 2D6 (CYP2D6). Human CYP2D6 polymorphism is highly difficult to cope with in clinical trials. This metabolization mediated by CYP2D6 is a major drawback, in that variable plasma concentrations are obtained in patients. We have developed a backup molecule no more metabolized by CYP2D6, whose properties are concomitant inhibition of the TNF-α expression and induction of interleukin-10 synthesis. In the present study, we report on the pharmacological profile of this original σ receptor ligand, SSR125329A ([(*Z*)-3-(4-Adamantan-2-yl-3,5-dichloro-phenyl)-allyl]-cyclohexyl-ethyl-amine), which showed higher affinity for σ_1 and σ_2 subtypes and for the human sterol isomerase than the parent compound SR31747A. In mice, we demonstrate that the molecule drives an anti-inflammatory pathway that is potentially pivotal for rheumatoid arthritis therapy.

2. Materials and methods

2.1. Mice

Six- to eight-week-old BALB/c, C57BL/6 or B6D2F1 female mice were purchased from IFFA-CREDO (L'arbresle, Lyon, France). MRL/lpr mice were obtained from Harlan France. All animals rested for 7 days prior to the onset of treatment. Animal protocols were approved by the animal ethical committee of Sanofi-Synthelabo Recherche.

2.2. Reagents

D-galactosamine, staphylococcal enterotoxin B and lipopolysaccharide from *Escherichia coli* (serotype 055:B5) were purchased from Sigma (St. Louis, MO). SSR125329A was synthesized at the Chemistry Department of Sanofi-Synthelabo Recherche (Toulouse, France). These reagents were dissolved in a phosphate buffered saline (PBS), except for SSR125329A which was dissolved in 5% ethanol, 5% Tween 80 and 90% H_2O.

2.3. Culture medium

The complete cell culture medium used for splenocyte proliferation was RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 2% heat-inactivated foetal serum (Gibco Laboratories), 2 mM L-glutamine, 5×10^{-5} M 2-βmercaptoethanol (Merck), 1 mM sodium pyruvate, 100 UI/ml penicillin, 100 μg/ml streptomycin (Gibco Laboratories), 15 mM PIPES (Sigma), with pH adjusted to 6.6.

2.4. Binding experiments

Receptor binding assays for [^{3}H](+)-pentazocine and [^{3}H]DTG were performed as already described (Paul et al., 1994; De Costa et al., 1989; Weber et al., 1986; Largent et al., 1988) with minor modifications:

- Rat spleens were homogenized at 4 °C in a 50-mM Tris–HCl buffer at pH 8.0 for [^{3}H](+)-pentazocine and pH 7.4 for [^{3}H]DTG. Homogenates were centrifuged at 900 × *g* and the supernatant was recentrifuged at 48,000 × *g* for 10 min. The pellet was washed twice.
- Guinea pig brains were first homogenized in a 0.32-M saccharose solution and centrifuged for 10 min at 900 × *g*. The supernatant was centrifuged again for 20 min at 22,000 × *g*. The pellet, resuspended in a 50-mM Tris–HCl buffer (pH 8.0), was incubated for 30 min at 37 °C and centrifuged again for 20 min at 22,000 × *g*. It was finally resuspended in a 50-mM Tris–HCl buffer (pH 8.0) and frozen at − 80 °C until used.

The membranes were incubated for 150 min at 37 °C with [^{3}H](+)-pentazocine or for 90 min at 20 °C with [^{3}H]DTG (250 μg protein for [^{3}H](+)-pentazocine and 600 μg protein for [^{3}H]DTG) in 1 ml of buffer containing increasing concentrations of compounds to be tested. Ligand concentrations were 0.5 nM for [^{3}H](+)-pentazocine and 2 nM for [^{3}H]DTG. Determinations were done in triplicate.

Receptor binding assays for [^{3}H]SR31747 were conducted as follows: Yeast homogenates were resuspended in 50-mM Tris–HCl buffer (pH 8.0). Membrane proteins (15 μg) and 0.2 nM [^{3}H]SR31747 were incubated for 150 min at 30 °C in 1 ml of the binding buffer (Tris–HCl 50 mM, EDTA 1.5 mM, NaN_3 0.01% and bovine serum albumin 0.1%, pH 7.4) containing the test molecules at different concentrations. Nonspecific binding was determined in the presence of 1 μM SR31747. Membranes were separated from the free radioligand by filtration on GF/B filters soaked with 0.5% polyethylenimide and radioactivity was counted after washing.

2.5. In vitro cell proliferation

Mouse cell suspensions were prepared from spleens using a 80-gauge stainless steel mesh. Red cells were first lysed by a short hypotonic shock and the remaining cells were washed twice in culture medium before use. Cell viability determined by trypan blue exclusion always exceeded 95%. Splenocytes were cultured in 96-well flat-bottomed plates (Falcon Becton Dickinson, Lincoln Park, NJ) in quadruplicate at 4×10^5 cells/well with 1 μg/ml of staphylococcal enterotoxin B for 4 days, then pulsed with 1 μCi/well [^{3}H]thymidine (Amersham, les Ulis, France), and harvested 4 h later on glass fiber papers using a Skatron harvester (Pharmacia-LKB, Piscataway, NJ).

2.6. Cytokine determinations

Five mice were injected intraperitoneally (i.p.) with either SSR125329A or vehicle alone 30 min before the intravenous (i.v.) administration of lipopolysaccharide (20 μg/mouse). Blood samples were collected 1 h after lipopolysaccharide injection. Subsequently, the samples were centrifuged and the serum collected and stored at − 80 °C until used for cytokine determination. TNF-α and interleukin-10 levels were determined using enzyme-linked immunosorbent assay (ELISA) kits from Genzyme (Genzyme Cambridge, MA). Assays were performed as indicated by the manufacturer. The data are expressed as pg/ml following calibration with a reference standard.

2.7. Systemic graft-versus-host disease

Systemic graft-versus-host disease was induced as described (Simonsen, 1962). The effect of drugs was assessed by measuring the degree of splenomegaly induced by i.p. injection of F1 hybrid mice with splenocytes obtained from one of the parental strains. Briefly, spleen cells (7.5×10^7) from parental C57BL/6 mice were injected into hybrid B6D2F1 hosts. The mice were killed 7 days later. Body and spleen weights of recipients (exp) and control animals (contr) were determined. The systemic graft-versus-host disease index (I) was defined as follows:

$$I = \frac{\text{Spleen weight}_{(exp)}}{\text{Body weight}_{(exp)}} \Big/ \frac{\text{Spleen weight}_{(contr)}}{\text{Body weight}_{(contr)}}$$

Experimental mice (exp) were injected i.p. with the test compound and control mice (contr) with the vehicle alone. Cell subsets were determined by flow cytometry analysis 7 days following systemic graft-versus-host disease induction. Spleens were excised, then gently dissociated at 4 °C using a Dounce homogenizer. After dissociation, cells were filtered through gauze to obtain a single cell suspension. Leukocytes were stained and analyzed using a Facstar plus cytometer (Becton Dickinson, Mountain View, CA). Splenocytes from the B6D2F1 host were stained in 100-μl volumes with the anti-H-2K^d antibody (clone SF1-1.1) labeled with *R*-phycoerythrine. B-lymphocytes were stained with the anti-B220/CD45R antibody (clone RA3-6B2) conjugated to allophycocyanine. CD8$^+$ lymphocytes were stained with the anti-CD8 antibody (clone 53–6.7) labeled with fluorescein isothiocyanate (FITC). These antibodies were purchased from Pharmingen (San Diego, CA). After 30 min staining at 4 °C, cells were washed in PBS and labeled splenocytes were immediately analyzed.

Splenocytes were also resuspended in RPMI 1640 containing 10% foetal calf serum. Cell suspension (5×10^6/ml) was distributed in 96-well microplates

Table 1
SSR125329A binding characteristics

Receptors	σ_1	Human sterol isomerase	σ_2
Ligands	[^{3}H](+)-pentazocine	[^{3}H]SR31747	[^{3}H]DTG
Tissues/species	Brain/guinea pig	Yeast	Spleen/rat
SSR125329A	0.40 ± 0.04	0.29 ± 0.02	25 ± 2
SR31747A	4.2 ± 0.2	1.5 ± 0.1	45 ± 7
Haloperidol	0.8 ± 0.1	>1000	43 ± 2
(+)-Pentazocine	4.3 ± 0.2	>1000	2900 ± 500
(−)-Pentazocine	62 ± 3	>1000	75 ± 10
DTG	38 ± 1	>1000	38 ± 3
Tamoxifen	37 ± 6	3.6 ± 0.4	>1000
7-Ketocholestanol	>1000	30 ± 0.2	>1000
SKF-525A	115 ± 14	150 ± 9	>1000

Binding studies were performed on membrane extracts from cells or yeast expressing σ_1, σ_2 or the human sterol isomerase and IC_{50} values are expressed in nM.

(100 μl per well) and stimulated with 5 μg/ml lipopolysaccharide for 48 h at 37 °C in an atmosphere of 95% air and 5% CO_2, and then pulsed with 1 μCi [^{3}H]thymidine for 4 h. Cells were subsequently harvested on glass fiber filters and counted to determine cell-associated radioactivity.

Spontaneous interferon-gamma release by these splenocytes was also measured using an ELISA kit (R&D Systems, Minneapolis, MN).

2.8. Spontaneous rheumatoid arthritis-like pathology of the MRL/lpr mice

Mice obtained from Harlan France were kept on a standard diet with water ad libidum. Female mice (13 week old) were injected intradermaly into a thoracic and an inguinal site with 0.1 ml complete Freund adjuvant, supplemented with 5 mg/ml heat-inactivated *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI). Arthritis development was checked for 4 weeks and hindpaw swelling determined with a micrometer. This parameter was determined by a treatment-blind observer. Twenty-eight days after complete Freund adjuvant priming, hindpaws were collected and radiologically examined under a mammography X-ray unit: Siemens, focal spot 0.6 × 0.6 mm. Film: X-Omat MA Kodak (9 × 12 cm); exposures at a distance of 55 cm at 28 kV and 160 mA. X-ray photographs were carefully examined using a macroscopic table associated with a microscope.

2.9. Statistical analysis

Statistical significance was determined by the RS/1 multicompare procedure using a one-way analysis of variance and a Dunnett's test for multiple comparisons with a common control group or a Mann–Whitney *U*-test.

3. Results

3.1. SSR125329A is a potent ligand for σ_1 and σ_2 subunits and the human sterol isomerase

To evaluate the affinity of SSR125329A, binding studies were performed on membrane extracts from cells or yeast expressing σ_1, σ_2 or the human sterol isomerase. The σ_1 binding was analysed on guinea pig brain extracts with [^{3}H](+)-pentazocine, while the σ_2 binding was analysed on rat spleen cell extracts with [^{3}H]1,3-di-*o*-tolylguanidine (DTG). The human sterol isomerase binding analysis was performed on yeast expressing the protein (Silve et al., 1996b; Soustre et al, 2000) with [^{3}H]SR31747. SR31747A is displaced by tamoxifen on the human sterol isomerase with a similar efficacy as cold SR31747A itself (IC_{50}: 3.6 ± 0.4 nM for tamoxifen and 1.5 ± 0.1 nM for SR31747A) (Table 1).

SSR125329A [(*Z*)-3-(4-Adamantan-2-yl-3,5-dichlorophenyl)-allyl]-cyclohexyl-ethyl-amine) (Fig. 1) was found to inhibit the binding of [^{3}H](+)-pentazocine to σ_1 receptors, of [^{3}H]DTG to σ_2 receptors and of [^{3}H]SR31747 to the human sterol isomerase, with an IC_{50} of 0.4 ± 0.04, 25 ± 2 and 0.29 ± 0.02 nM, respectively. It is the only available ligand that had such a potency for these three binding sites. It differed from (+)-pentazocine, a pure σ_1 receptor ligand, and from haloperidol, DTG and (−)-pentazocine, which had nanomolar affinity for σ_1 and σ_2 subunits, but little affinity for the human sterol isomerase. Tamoxifen, 7-ketocholestanol and the cytochrome P450 inhibitor SKF-525A showed high affinity for the human sterol isomerase (3.6 ± 0.4; 30 ± 0.2 and 150 ± 9 nM, respectively). Contrary to 7-ketocholestanol which is a pure sterol isomerase binder, tamoxifen and SKF-525A showed significant σ_1 receptor binding but no affinity for the σ_2 receptor.

3.2. SSR125329A inhibits staphylococcal enterotoxin B-driven mouse splenocyte proliferation

In splenocytes isolated from naive mice, 1 μg/ml staphylococcal enterotoxin B induced high proliferation, as measured by [^{3}H]thymidine incorporation on day 4 of culture. The σ receptor ligand SSR125329A and the immunosuppressive agent cyclosporin A, added at the time of staphylococcal enterotoxin B stimulation, produced dose-dependent inhibition of this proliferative response

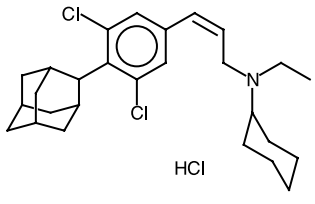

Fig. 1. Chemical structure of SSR125329A.

(Fig. 2). [^{3}H]thymidine incorporation was decreased to 50% of control in the presence of 65 nM SSR125329A and 10 nM cyclosporin A. In contrast, the four σ receptors ligands haloperidol, DTG, (+)-3PPP and (+)-pentazocine did not show any significant antiproliferative properties.

3.3. SSR125329A differentially regulates interleukin-10 and TNF-α production in lipopolysaccharide-treated BALB/c mice

We examined the effect of SSR125329A on interleukin-10 and TNF-α production after 20 μg lipopolysaccharide injection in BALB/c mice. Lipopolysaccharide injections resulted in the secretion of high amounts of TNF-α, peaking at 1-h post injection. A low level of interleukin-10 was also detected at the same time. Concentrations of the two cytokines in the serum of lipopolysaccharide-treated mice were also measured following the administration of various SSR125329A doses, ranging from 1 to 75 mg/kg. As shown in Fig. 3, SSR125329A treatment resulted in a marked dose-related increase in interleukin-10 release (mean ± S.D.: 2950 ± 420 pg/ml in mice treated with vehicle alone vs. 7050 ± 500 pg/ml in mice treated with 75 mg/kg SSR125329A, $P<0.01$) (Fig. 3A). In contrast, TNF-α levels were markedly reduced in SSR125329A-treated mice (mean ± S.D.: 22100 ± 2400 pg/ml in the vehicle-treated group vs. 1000 ± 205 pg/ml in the 75 mg/kg SSR125329A-treated group, $P<0.01$) (Fig. 3B). The ED_{50} obtained in these conditions was 22 mg/kg dose, with a 95% confidence interval ranging from 17 to 26 mg/kg.

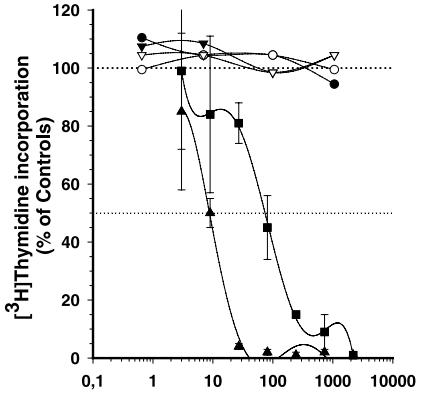

Fig. 2. SSR125329A inhibits staphylococcal enterotoxin B-driven BALB/c mice splenocyte proliferation. Spleen cells were isolated from naive BALB/c mice and cultured (4×10^5/well) for 72 h in the presence of 1 μg/ml staphylococcal enterotoxin B and the test molecules. Incubation was then carried on for 72 h, and [^{3}H]thymidine was incorporated during the last 4 h. The test molecules were SSR125329A (■–■), cyclosporin A (▲–▲), haloperidol (●–●), DTG (○–○), (+)-3PPP (▼–▼), (+)-pentazocine (▽–▽). Control [^{3}H]thymidine incorporation in staphylococcal enterotoxin B-activated splenocytes was 145,400 ± 12,500 cpm. Vertical bars indicate standard deviations from quadruplicate cultures.

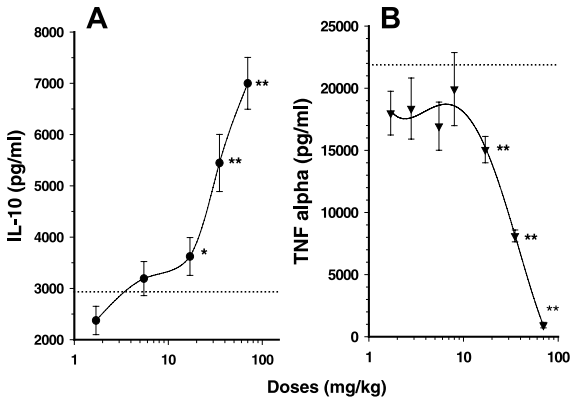

Fig. 3. SSR125329A pretreatment increases interleukin-10 secretion and inhibits TNF-α in lipopolysaccharide-treated BALB/c mice. Groups of five mice were treated i.p. with SSR125329A 30 min before lipopolysaccharide (20 μg/mouse, i.v.). Interleukin-10 (panel A) and TNF-α (panel B) levels were measured in the serum 1 h after lipopolysaccharide challenge. Data are means ± S.D., * and ** denote $P<0.05$ and $P<0.01$ in Dunnett's test.

3.4. SSR125329A inhibits systemic the systemic graft-versus-host disease induced in B6D2F1 mice

The administration of splenocytes originating from the parental C57BL/6 strain into non-irradiated immune-competent F1 hybrid B6D2F1 (C57BL/6 × DBA/2) induced a systemic graft-versus-host disease. Th1 cytokine interferon-gamma and CD8 responses likely drive this form of acute disease, activating macrophages to produce TNF-α and interleukin-1, which mediate the graft-versus-host-associated pathology (Allen et al., 1993). B6D2F1 mice suffering from this disease showed high splenomegaly, which is an easy and reliable indicator of severity. The disease index was 4.5 ± 1.0 at day 7 and the number of cells per spleen grew from $58\pm11\times10^6$ in normal mice up to $110\pm24\times10^6$ in graft-versus-host disease-affected mice. Splenomegaly was lower after treatment with 75 mg/kg SSR125329A, i.e. the disease index was significantly reduced (2.0 ± 0.7, $P<0.01$/vehicle-treated group), and the number of spleen cells remained within the normal range ($54\pm17\times10^6$, $P<0.01$/vehicle-treated group). The ID_{50} of SSR125329A, evaluated through the disease index, was 28 mg/kg, which was in the same range as the ID_{50} determined for TNF-α inhibition. B cells from normal mice that were stimulated to proliferate by lipopolysaccharide treatment, incorporated high levels of [^{3}H]thymidine in vitro (66351 ± 15813 cpm). By contrast, B cells from graft-versus-host disease-affected mice were strongly immunocompromised and did not incorporate [^{3}H]thymidine (852 ± 442 cpm). B cells from disease-affected mice treated with 75 mg/kg SSR125329A showed an almost normal [^{3}H]thymidine incorporation (52906 ± 6224 cpm, $P<0.01$/vehicle-treated group) (Fig. 4A). Moreover, splenocytes from disease-affected mice, cultured in vitro for 48 h, spontaneously released high levels of interferon-gamma (5347 ± 772 pg/ml), contrary to those of normal mice or

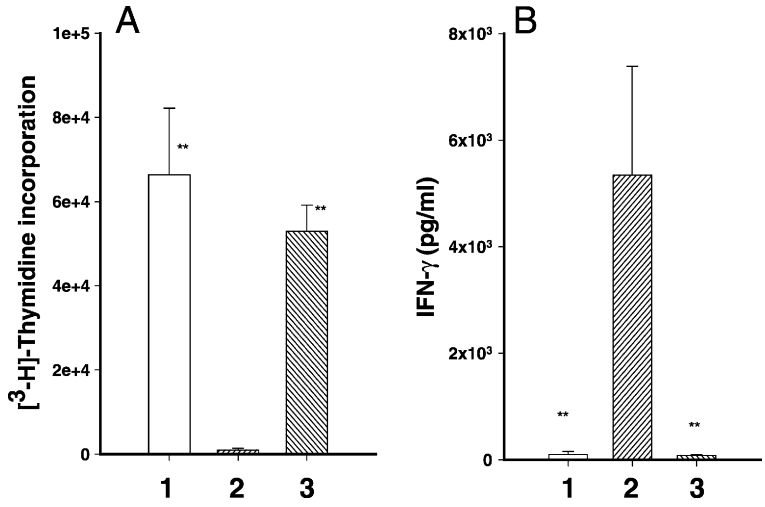

Fig. 4. Lipopolysaccharide-proliferative response (A) and spontaneous interferon-gamma release (B) by splenocytes of graft-versus-host disease-induced mice. Splenocytes isolated from normal B6D2F1 mice (1), graft-versus-host disease-induced mice treated i.p. with the vehicle alone (2), or with 75 mg/kg SSR125329A (3), were cultured for 48 h with 5 μg/ml lipopolysaccharide and assessed for their proliferative response (A), or without lipopolysaccharide and assessed for their spontaneous interferon-gamma release (B). Data are means ± S.D. ** denotes $P < 0.01$ in Dunnett's test.

disease-affected mice treated with 75 mg/kg SSR12539A (97 ± 57 and 150 ± 256 pg/ml, $P < 0.01$/vehicle-treated group, respectively) (Fig. 4B). Finally, during the first weeks of the pathology, there was a marked change in the spleen cell population. Initially, host B cells disappear (Schattenfroh et al., 1995), and then T cells were affected in the following weeks (Hakim et al., 1991). The marked changes in B and $CD8^+$ host spleen cells in disease-affected mice treated with SSR125329A were examined. B cells normally represent 57 ± 1% of all splenocytes. At day 7 of the pathology, the B cell population fell to 31 ± 3%, but remained almost normal when these mice were treated with 75 mg/kg SSR125329A (49 ± 4%, $P < 0.01$/vehicle-treated group) (Fig. 5A–D). Probably due to this decrease, the host T cell population, particularly $CD8^+$ cells, relatively increased. $CD8^+$ cells represented 14 ± 1% of all splenocytes in normal B6D2F1 mice. Host $CD8^+$ cells grew up to 23 ± 2% in the disease-affected mice at day 7 of the pathology, whereas when these mice were treated with 75 mg/kg SSR125329A the percentage of host $CD8^+$ cells

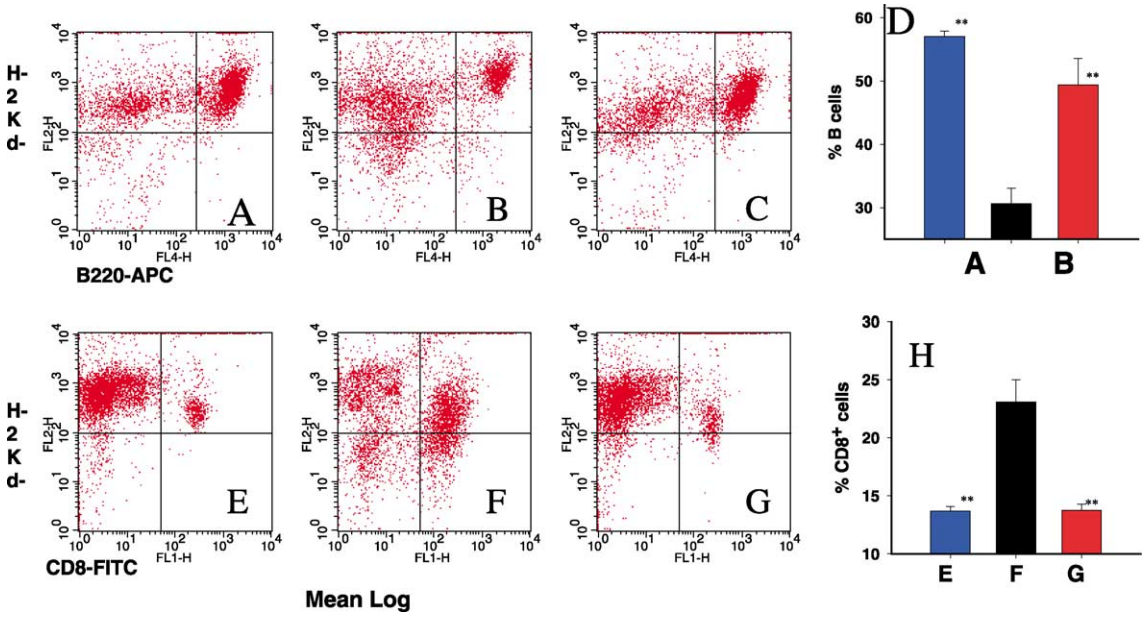

Fig. 5. Effect of SSR125329A on variations in the host B and $CD8^+$ spleen cell subsets in graft-versus-host disease-affected mice. The change in host lymphocyte subsets, originating from either normal B6D2F1 mice injected with syngeneic B6D2F1 splenocytes (A and E), graft-versus-host disease-induced mice treated i.p. with the vehicle alone (B and F) or with 75 mg/kg SSR125329A (C and G), were examined by flow cytometry 8 days after disease induction. % of B cells (D) and $CD8^+$ cells (H) over total splenocytes are means ± S.D., ** denotes $P < 0.01$ in Dunnett's test.

remained normal (14 ± 1%, $P < 0.01$/vehicle-treated group) (Fig. 5E–H).

3.5. SSR125329A inhibits rheumatoid arthritis-like syndrome in MRL/lpr mice

Fas-defective MRL/lpr mice spontaneously develop a hindlimb arthropathy with destruction of articular and meniscal cartilages by a pannus tissue. This rheumatoid-like disease is severe and homogeneous when these mice are intradermally primed with complete Freund adjuvant (Ratkay et al., 1993). The histomorphologic similarities with human rheumatoid synovitis make this mouse strain an excellent model for studying human rheumatoid arthritis (Gong et al., 1997; Waterfield et al., 1999). Administration of 40 mg/kg SSR125329A or 2 mg/kg methotrexate every other day from the day of the complete Freund adjuvant injection was effective in reducing the disease development. Micrometric analysis of hindlimb footpads (Fig. 6) indicated that the disease started 7 days after the complete Freund adjuvant administration and was very significant on day 21 in mice of the vehicle-treated group. By contrast, mice of the SSR125329A or methotrexate groups were significantly less affected at day 21. A radiographic analysis of the ankle region of the hindlimb footpads, performed at day 28 after the Freund adjuvant administration, clearly showed that like young healthy mice (13 weeks old), SSR125329A-treated mice did not show any sign of cartilage destruction (Fig. 7A and C), whereas cartilage destruction associated with a bone pinching (Fig. 7B, arrow) was clearly observable in most mice of the vehicle-treated group.

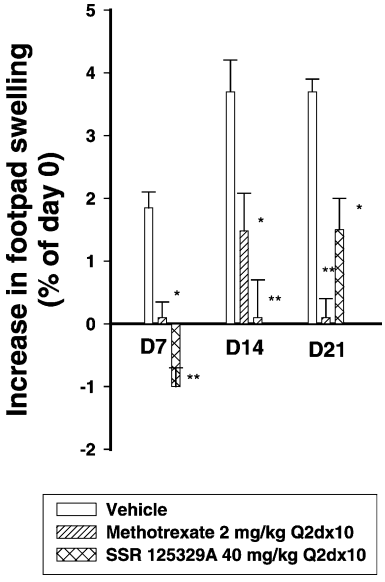

Fig. 6. Effect of SSR125329A on the development of arthritis in complete Freund adjuvant-treated MRL/lpr mice. Mice were treated i.p. every other day from the day of complete Freund adjuvant administration. Footpad swelling was examined by a double-blind observer with a micrometer. Results are the means ± S.D. of 10 individual mice per treatment group. * and ** denote $P < 0.05$ or $P < 0.01$ in Mann–Whitney U-test.

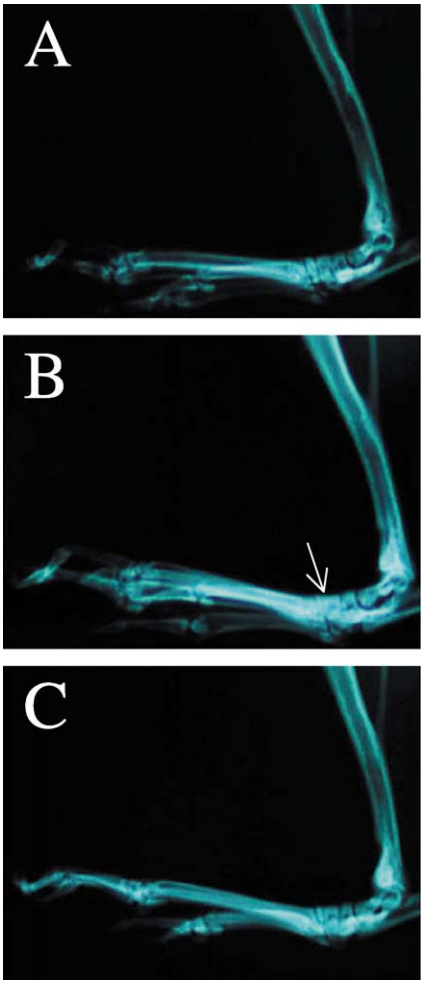

Fig. 7. Radiographic analysis of ankle regions of complete Freund adjuvant-treated MRL/lpr mice. MRL/lpr mice (13 weeks old) (A) did not show any sign of arthritis onset. Conversely, 18 weeks old mice challenged with complete Freund adjuvant at 13 weeks old (B) showed clear cartilage erosions and bone pinching in the ankle region (arrow). After i.p. treatment with 40 mg/kg SSR125329A, every other day from the day of complete Freund adjuvant injection, there were no signs of erosion or pinching detected (C).

4. Discussion

σ_1 and σ_2 are two proteins that pertain to the σ receptor family, and high affinity ligands have been described for each site. There is now considerable evidence that the σ_1 site may be closely related with the human sterol isomerase, an antiestrogen-binding microsomal fraction exhibiting high affinity for tamoxifen. The σ receptor ligands are reported to have immunosuppressive and anti-inflammatory properties (Casellas et al., 1994). The σ receptor ligand SR31747A was reported

to stimulate lipopolysaccharide- or staphylococcal enterotoxin B-induced interleukin-10 serum release, while concomitantly inhibiting TNF-α production (Derocq et al., 1995; Bourrié et al., 1995, 1996). These two cytokines are critical in many inflammatory and autoimmune diseases, with their imbalance altering the inflammatory process (Katsikis et al., 1994; Feldmann and Maini, 1999). Therefore, σ receptor ligands could be of interest for pathologies in which these two cytokines are highly involved, e.g. rheumatoid arthritis and autoimmune gastrointestinal diseases. No molecules having high and selective affinity for the three related binding units σ_1, σ_2 and human sterol isomerase have been analyzed to date for their pharmacological properties. SSR125329A, a new σ receptor ligand with an original chemical structure including an adamantyl ring, is a very potent σ_1, σ_2 receptor ligand and also binds the human sterol isomerase. It recognizes σ_1 and σ_2 with an affinity 2-fold higher than that of haloperidol, and binds the human sterol isomerase with an affinity 12-fold higher than that of tamoxifen. This compound has high selectivity since it has no affinity for more than 80 receptors, including adrenergic, cannabinoid, dopamine, glucocorticoids, histamine, muscarinic, and serotonin receptors. In this study, SSR125329A was found to possess potent anti-inflammatory and anti-proliferative properties. Thus, SSR125329A dramatically increased interleukin-10 synthesis during lipopolysaccharide-induced shock in mice. The amplification of interleukin-10 synthesis under SSR125329A treatment came along with a substantial decrease in TNF-α synthesis. Interleukin-10 has been described to inhibit alloreactivity in vitro and low interleukin-10 production is associated in allogeneic bone marrow transplantation with an increased risk of severe graft-versus-host disease (Korholz et al., 1997). Moreover, interleukin-10 treatment reduces graft-versus-host disease-induced splenomegaly and splenocyte production of interferon-gamma in mice (Smith et al., 1995). We thus investigated whether SSR125329A would be active in reducing experimental acute graft-versus-host disease. We indeed found that SSR125329A caused a significant reduction in splenomegaly and a significant reduction in the spontaneous interferon-gamma production that normally occurs in splenocytes of graft-versus-host disease-affected mice. Moreover, the accompanying signs of a severe pathology (impaired splenocyte proliferation, host B cell loss) were dramatically decreased. Finally, as interleukin-10 seems to be a key cytokine in human rheumatoid arthritis (Katsikis et al., 1994), we evaluated the activity of SSR125329A in the spontaneous rheumatoid-like disease occurring in MRL/lpr mice injected with complete Freund adjuvant. We observed that SSR125329A was significantly active in reducing the disease development and a radiographic analysis revealed a low frequency of cartilage lesions. Altogether, our results strengthen the notion that σ receptors and the human sterol isomerase could constitute new targets for anti-inflammatory pharmacological reagents useful for the treatment of immune disorders. The modulating effect of SSR125329A on inflammatory responses, together with its ability to bind the three binding sites (σ_1, σ_2 and human sterol isomerase), makes this compound an original new ligand which deserves further analysis of its pharmacological spectrum.

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