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RP 54745, a potential antirheumatic compound. I. Inhibitor of macrophage stimulation and interleukin-1 production

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Abstract

RP 54745 is an amino-dithiole-one compound found to be active at micromolar concentration on the metabolism of stimulated macrophages, for example, the hexose monophosphate pathway (HMP) and the exocytosis of lysosomal enzymes. LPS-induced interleukin-1 (IL-1) production by murine peritoneal macrophages was also diminished by this compound *in vitro* as well as *in vivo*. This effect was confirmed at the mRNA level; at the concentration of 3×10^{-6} *M*, the IL-1α and β mRNA signals were inhibited, whereas the TNFα mRNA signal was only slightly lessened. These observations were confirmed *in vivo*, with a dose of RP 54745 of 25 mg kg^{-1}. These results led us to consider that RP 54745 might influence certain cells and cytokines implicated in the regulation of the immune system, the disfunctioning of which can lead to inflammatory disorders or autoimmune pathologies.

Introduction

Many cells are implicated in rheumatoid arthritis. Among them are macrophages, which when stimulated, produce substances, such as lysosomal enzymes, proteases, interleukins and prostaglandins, and express activities that contribute to the pathological state.

At the moment, the compounds most efficient in normalizing the majority of these disturbed parameters are steroidal drugs. In order to find an efficient antirheumatic drug without the side effects of the steroids, we selected RP 54745 (Fig. 1), an amino-dithiole-one compound, from among 180 compounds of the same family, for its ability to inhibit macrophage stimulation *in vitro*.

In vitro, RP 54745, at a micromolar concentration, inhibited different parameters that have been exacerbated in stimulated macrophages such as the hexose monophosphate pathway, β-glucuronidase exocytosis and IL-1 production.

In vivo, we studied the effects of the drug on IL-1 and TNF, pluripotent monokines with an important role in immunoregulation and immunopathology [1]. The results demonstrated that RP 54745, at a moderate dose, was also a potent inhibitor of IL-1 production by macrophages *in vivo*, while it had little impact on TNF production. These effects were confirmed at the mRNA level.

Materials and methods

Animals

Female and male 6-week-old OF_1 mice, and female 5-week-old C_3H/Ouj were purchased from IFFA CREDO Breeding Laboratories.

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Cells

Murine peritoneal macrophages, from 6-week-old OF_1 mice, were harvested 4 days after a 1.5 ml thioglycollate broth intraperitoneal injection and cultured in Dulbecco modified Eagle medium (DMEM) with 15% fetal calf serum (FCS) in Petri dishes or multiwell plates, for a 2-h adhesion period, at 37 °C in 5% CO_2 atmosphere. Non-adherent cells were removed by washing and macrophages (greater than 95% pure) were left, at rest, in the same medium for 20 h before stimulation.
Murine thymocytes, from 5-week-old C_3H/Ouj mice, were cultured with 1 $\mu g\,ml^{-1}$ of phytohemagglutinin (PHA) and plated in RPMI 1640 with 5% FCS.

Reagents

RP 54745 was synthesized in four steps; it was patented in Europe (patent number 0 119, 896), US, South Africa, Australia and many other countries. Phosphate acetonide of triamcinolone and dexamethasone were used as reference compounds and were also synthesized at Rhône-Poulenc Laboratories. Test compounds were dissolved in dimethylformamide (DMF) to make 10 m*M* stock solutions, and these were diluted in culture media for *in vitro* tests (final concentration $\leqslant 0.1\%$) or in water with 0.5% Tween 20 for *in vivo* assays. DMF and 0.5% Tween 20 controls, at the appropriate dilutions, were included in each assay to verify the absence of effects.
Thioglycollate Resazurine was obtained from Pasteur Diagnostics. Zymosan, phenolphthalein mono β-glucuronic acid and dimethyl-thiazo diphenyltetrazolium bromide salt (MTT) were obtained from Sigma Chemical Co., PHA was obtained from Wellcome.
Lipopolysaccharide (LPS) of *Escherichia coli* 0127 B8 was supplied by Difco. $^{14}C_1$-glucose was supplied by Commissariat à l'Energie Atomique (CEA). Dynagel was obtained from Baker Chemicals. Human recombinant IL-1β (h.r.IL-1β) was supplied by our Institute of Biotechnology.

Hexose monophosphate pathway (HMP) measurement

Peritoneal murine macrophages were obtained after a 1.5 ml thioglycollate intraperitoneal injection in mice and peritoneal lavages 4 days later. 2×10^6 peritoneal macrophages, after a 2-h period of adherence, were cultured in 35 mm plastic Petri dishes in DMEM with 15% FCS for 17 h at 37°C in 10% CO_2; drugs were added to the medium and left overnight. Cells were stimulated by 0.04% zymosan phagocytosis (20 particles per cell) for 30 min [2], in the presence of $^{14}C_1$-glucose: 18.5 kBq (0.5 μCi). The radioactive hexose was transformed to a pentose by the HMP, and the liberated $^{14}CO_2$ was collected for 15 min in Warburg flasks by means of GFC Whatman filters impregnated with *N* KOH. The radioactivity of the filters was then measured in Dynagel and expressed in disintegrations per minute (dpm). Results were corrected for cell viability and proteins contained in adherent macrophages.

β-glucuronidase activities

Liberation of the lysosomal enzyme contained in the adherent peritoneal macrophages, stimulated by zymosan phagocytosis for 17 h, was obtained as described in the HMP assay [3]. The enzyme was measured in supernatants in the presence of its substrate, phenolphthalein mono β-glucuronic acid 20 m*M*, in 50 m*M* acetate buffer at pH 4.5 after incubating for 17 h at 37 °C. Then 2.5 vol of 200 m*M* glycine buffer pH 10.45 was added in order to induce the coloration of the phenolphthalein produced by the enzymatic cleavage of the substrate. Samples were read at 540 nm, the coloration being stable for at least 1 h.

Interleukin-1-production

1.5×10^6 peritoneal murine macrophages, after a 2-h period of adherence, were cultured in RPMI 1640 with 5% FCS for 20 h at 37 °C in 5% CO_2. *In vitro*, test compounds were added 15 min before the stimulant, 100 $ng\,ml^{-1}$ LPS, and the cells were incubated for 2–24 h. *In vivo*, thioglycollate-stimulated female OF_1 mice were orally treated with 5 or 25 $mg\,kg^{-1}\,day^{-1}$ RP 54745 for 4 days. Harvested macrophages were then stimulated *ex vivo* for 2 h with LPS at concentrations ranging from 0.1–10 $ng\,ml^{-1}$. In some assays, 4 h before macrophage harvesting, mice were treated intraperitoneally with LPS: 0.5 μg per mouse.
At the end of the *in vitro* incubation period, supernatants were removed from cultures, and cells were lysed by three freeze/thaw cycles. Both extracellular and cell-associated IL-1 activities were assayed.

Interleukin-1 bioassay

IL-1 activity was measured according to the LAF-test [4]. Briefly, 1.5×10^6 thymocytes per ml, obtained from C_3H/Ouj mice thymus, were cultured with 1 $\mu g\,ml^{-1}$ PHA for 20 min, plated in multiwell plates with appropriate dilutions of the samples or with a known concentration of IL-1 (2 U ml^{-1}) and incubated for 72 h at 37 °C in 7.5% CO_2. Thymocyte proliferation was determined during the last 5 h of the culture in the presence of 0.02% MTT which was reduced in the cells; insoluble MTT salts were then dissolved with 0.04 *N* HCl in 2-propanol, and the optical density (OD) read at 560/690 nm.

Interleukin-1 and TNF mRNA expression

10^7 peritoneal adherent murine macrophages ± drugs were cultured in 90 mm Petri dishes as described in IL-1 production. After a 6-h LPS stimulation (5 $\mu g\,ml^{-1}$), total cellular RNA was extracted [5], denatured in formaldehyde and run on a 1.2% agarose gel. Separated mRNA molecules were transferred to a nylon membrane for hybridization, analyzed by Northern blot [6], and quantified by densitometry with an Ultroscan. Probes were designated as oligonucleotides complementary to coding sequences between nucleotides 401 and 471 for IL-1β, 415 and 485 for IL-1α, 300 and 369 for TNFα.

Statistics and data analysis

Statistical analysis was performed using the Student's *t*-test, and differences were considered as significant at $p \leqslant 0.05$. Standard curves were obtained by regression analysis of the dose-response data.

Results

Effect of RP 54745 on hexose monophosphate pathway and β-glucuronidase release by murine macrophages

When stimulated with zymosan mice peritoneal macrophages increased their glucose metabolism, which was measured as CO_2 production in the HMP. In the presence of RP 54745, a concentration-dependent inhibitory effect occurred. The IC_{50} value, deduced from the results shown in Fig. 2, was 3 μM.

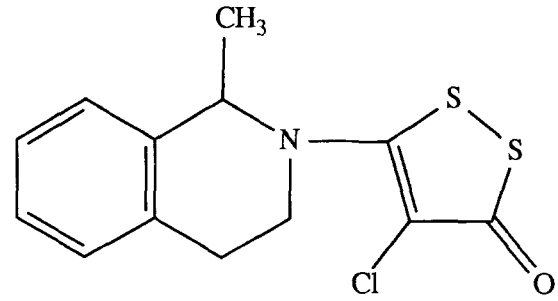

Figure 1
RP 54745, (4-chloro 5-(1-methyl 1,2,3,4-tetrahydro 2-isoquinolyl)1,2-dithiole 3-one).

It was verified that this effect was not a cytotoxic effect, by the measure of its reversibility as shown in Table 1.
Macrophages stimulated with zymosan also released more of the lysosomal enzyme β-glucuronidase.
At a concentration of 2.5 μM, RP 54745 inhibited this β-glucuronidase release by 70% (data not shown).

Effect of RP 54745 on IL-1 production and cytokine mRNA expression by murine macrophages

Murine macrophages stimulated with LPS produced more IL-1 than unstimulated cells [13]. With this type of stimulus, the major part of the IL-1 produced, measured by the LAF test, was cell-associated (about 40 times higher than the extracellular level).
Under these conditions, 10^{-6} *M* RP 54745, added 15 min before LPS was found to be a potent inhibitor of cell-associated IL-1 production. Measured 2 or 4 h after stimulation, IL-1 production was inhibited by 80%; 24 h after stimulation, it was inhibited by 55% (Fig. 3). Extracellular production was also inhibited: by 90% after 4 h and by 70% after 24 h (data not shown).
This effect was confirmed at the mRNA level with murine macrophages cultured under the same conditions as for IL-1 production. IL-1α and IL-1β mRNA signals in LPS-stimulated cells were significantly reduced by RP 54745 (3×10^{-6} *M*); whereas TNFα mRNA seemed hardly affected. By contrast, dexamethasone, a steroid drug (10^{-6} *M*), inhibited the signal of the three mRNAs (Fig. 4).

Table 1
Reversibility of the effect of RP 54745 in the HMP test.

	Dose (μM)	HMP (dpm ± ESM)	Inhibition (%)	p value /Z
Control	0	533 ± 53 475 ± 47		
Zymosan (Z)	0	6550 ± 852 6579 ± 797		
Z + RP 54745	1	6018 ± 389	9	NS
Z − RP 54745		6950 ± 749	−6	NS
Z + RP 54745	2.5	5197 ± 381	23	<0.01
Z − RP 54745		6577 ± 997	0	NS
Z + RP 54745	5	2660 ± 265	65	<0.001
Z − RP 54745		4593 ± 323	33	<0.01

2×10^6 adherent peritoneal macrophages were cultured for 17 h at 37 °C, 10% CO_2 ± drug; after washing, macrophages used to study the reversibility of the effect of RP 54745 were cultured overnight without drug (−RP); then zymosan (0.04%) phagocytosis was performed for 30 min with $^{14}C_1$-glucose and $^{14}CO_2$ was liberated for 15 min. Results are mean values of five Petri dishes and are expressed as disintegrations per minute (dpm).

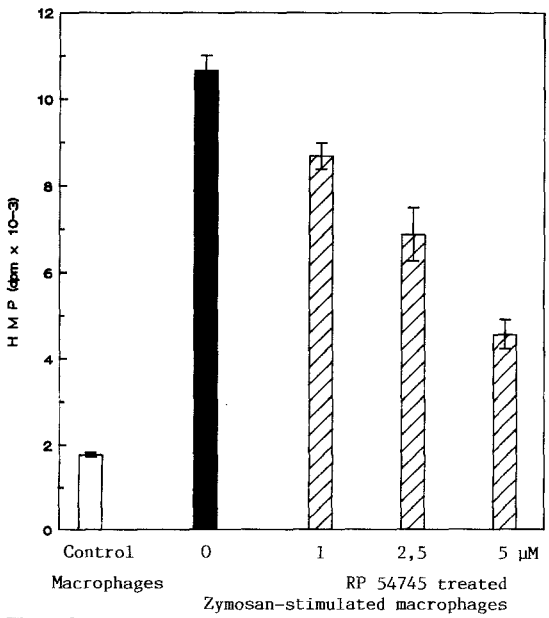

Figure 2
Effect of RP 54745 on the HMP of zymosan-stimulated macrophages. 2×10^6 adherent peritoneal macrophages were cultured for 17 h at 37 °C, 10% CO_2 ± drug; zymosan (0.04%) phagocytosis was performed for 30 min with $^{14}C_1$-glucose and $^{14}CO_2$ was liberated for 15 min. Results are mean values of five Petri dishes and are expressed as disintegrations per minute (dpm).

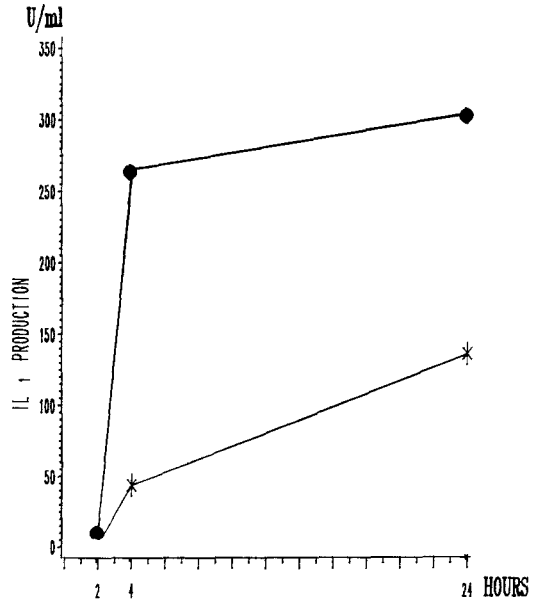

Figure 3
In vitro effect of RP 54745 on LPS-induced IL-1 production. 1.5×10^6 peritoneal macrophages, harvested from thioglycollate-stimulated mice, were cultured for 2 h (adherence) and for 20 h (rest) at 37 °C, 5% CO_2; drug (10^{-6} M) was then added 15 min before LPS stimulus (100 ng ml^{-1}). At the end of incubation, cell-associated IL-1 production was measured in control (●) as well as in treated macrophages (*), according to the LAF test. Results are expressed as units of IL-1 per ml, by plotting OD on a standard IL-1 curve.

Effect of RP 54745 on LPS-induced IL-1 production and mRNA expression in mice

After an *in vivo* treatment with RP 54745, followed by an *ex vivo* LPS stimulation of the peritoneal macrophages harvested, we observed a significant decrease of the IL-1 production, which occurred in a dose-dependent fashion for the cell-associated IL-1 (Fig. 5) and only at the highest dose studied (25 mg kg^{-1} day^{-1} of RP 54745) for the extracellular IL-1 (data not shown).

As in the previous test, mice were orally treated with RP 54745 but here they were also stimulated *in vivo* with an LPS challenge; LPS administration increased, about 2.5 times, extracellular as well as cell-associated IL-1 produced by macrophages. RP 54745 treatment, at doses of 5 and 25 mg kg^{-1} day^{-1}, significantly inhibited the LPS-stimulated production of IL-1. Extracellular and cell-associated IL-1 production were inhibited to the same extent. Figure 6 shows the results obtained for the cell-associated IL-1 production.

These *in vivo* results were confirmed at the mRNA level: RP 54745, at an oral dose of 25 mg kg^{-1} day^{-1} for 5 days, significantly reduced IL-1β mRNA expression (by 71%), and slightly reduced TNFα mRNA expression (by 22%). These results were obtained by taking into account the density of the β actin signal (Table 2).

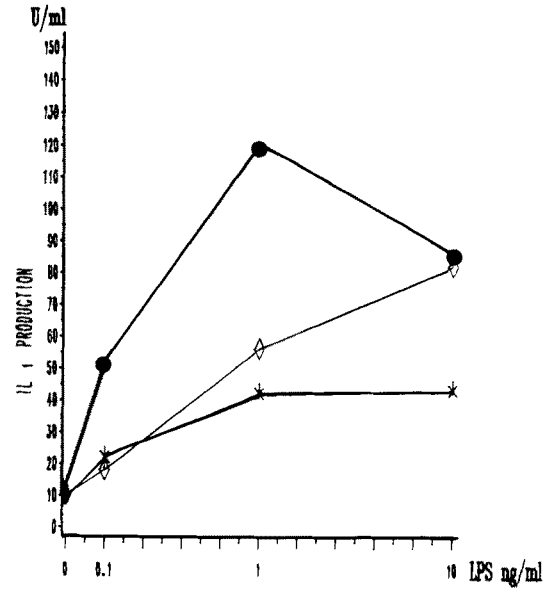

Figure 5
In vivo effect of RP 54745 on *ex vivo* IL-1 production. 1.5×10^6 peritoneal macrophages, harvested from mice previously stimulated with thioglycollate and orally treated, for 4 days, with drug at 5 mg kg^{-1} (◇) or 25 mg kg^{-1} (*) or with vehicle (●), were cultured for 2 h (adherence) and for 20 h (rest) at 37 °C, 5% CO_2. After a 2-h stimulation with LPS, cell-associated IL-1 produced was measured according to the LAF test. Results are expressed in units of IL-1 per ml, by plotting OD on a standard IL-1 curve.

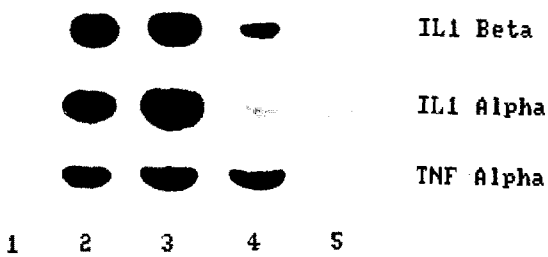

Figure 4
Effect of RP 54745 and dexamethasone on IL-1 and TNFα mRNA expression. 10^7 peritoneal macrophages, harvested from thioglycollate-stimulated mice, were cultured for 2 h (adherence) and for 20 h (rest) at 37 °C, 5% CO_2. After a 6 h LPS stimulation (5 μg ml^{-1}), RNA was extracted, denatured in formaldehyde and run on agarose gels. Separated mRNA molecules were transferred to a nylon membrane and hybridized with probes for IL-1α, Il-1β and TNFα. Membranes were washed and then exposed to a sensitive film at −70 °C for 4 days. Lane 1 = Control; Lane 2 = LPS (5 μg ml^{-1}); Lane 3 = LPS + RP54745 (3×10^{-7} *M*); Lane 4 = LPS + RP54745 (3×10^{-6} *M*); Lane 5 = LPS + dexamethasone (10^{-6} *M*).

Table 2
In vivo effect of RP 54745 on IL-1β and TNFα mRNA expression.

	RP dose mg kg^{-1} p.o	IL-1β (% of maximal mRNA expression)	TNFα
Control	0	4.8	5.8
LPS	0	100	100
RP 54745	25	3.5	3.4
RP 54745 + LPS	25	28.9	77.8

10^7 peritoneal macrophages were harvested from mice previously stimulated with thioglycollate, treated with drugs (p.o.) and then with LPS (0.5 μg i.p.). RNA was extracted from adherent cells, denatured in formaldehyde and run on agarose gels; after transfer to a nylon membrane, separated mRNA molecules were hybridized with different probes for IL-1, TNFα and actin, and subjected to Northern blot analysis. Results are expressed as a percentage of the maximal mRNA expression in LPS-treated animals.

Discussion

Human rheumatoid arthritis (RA), as well as the various animal models used to study it, consists of complex processes. These processes are influenced by a variety of cellular and humoral factors exerting on one another multistage regulating effects. The etiology of RA is not fully understood, but the immunological mechanism underlying the evolution of the chronic inflammatory process leading to joint destruction are now better known [7]. Cellular processes have a great importance. Activated macrophages, fibroblasts and synovial cells, chondrocytes, and T and B lymphocytes are among the main cell populations implicated in inflammatory events. RA, as are other autoimmune diseases, is regulated by a number of distinct T cell populations, some of which exacerbate disease and others of which prevent it [8].

Cellular secretions, such as cytokines, also play a major role in the process of inflammation, which results in articular degradation and in chronicity of the pathology. The effects of several cytokines such as IL-1, TNFα, IL-6, IL-8, and GM-CSF are the best studied. These cytokines are all produced in the rheumatoid articulation and can mediate both stimulatory and inhibitory effects in the synovium [9]; they are capable of inducing cell–cell interactions that result in the release of tissue-damaging enzymes (e.g. lysosomal enzymes, collagenase) and other cytokines (IL-2, IL-4) and in fibroblast proliferation [10]. For these reasons, cytokines are potential targets for therapeutic intervention. The IL-1 produced in stimulated macrophages or macrophage-like cells, following cellular interaction within the synovium, is one of the most potent inflammatory mediators involved in the destruction of the articular cartilage. Moreover, the efficacy of some antirheumatic drugs, including the steroid compounds, can in part be explained by their effect on IL-1 activity or production [11].

In these conditions, the decrease of IL-1 production was an attractive pharmacological target. From the results described in this paper, RP 54745, could have potential in pharmacological domains where IL-1 is produced in excess. RP 54745 is active on macrophages, the main IL-1 producing cells, and is able to decrease several consequences of the cellular stimulation. *In vitro*, micromolar concentrations of RP 54745 inhibited some manifestations of stimulated macrophages, such as oxidative metabolism, lysosomal enzyme release, and IL-1 production,

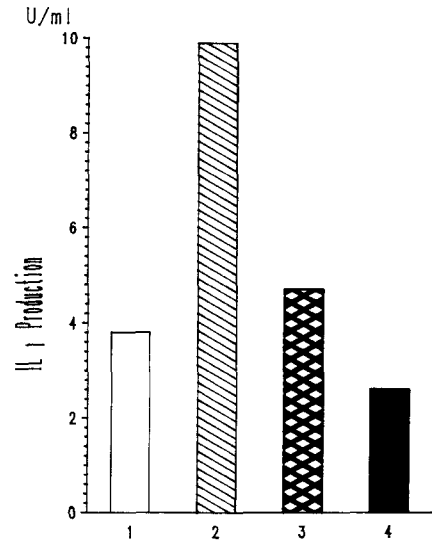

Figure 6
In vivo effect of RP 54745 on *in vivo* IL-1 production. 1.5×10^6 peritoneal macrophages, harvested from mice previously stimulated with thioglycollate (TG), treated with drug (p.o.) and then with LPS (0.5 μg i.p.), were cultured for 2 h (adherence) and for 20 h (rest) at 37 °C, 5% CO_2. Cell-associated IL-1 production was then measured in each group. Results are expressed in U ml^{-1}. Group 1 = TG mice; Group 2 = TG + LPS mice; Group 3 = TG + RP 54745 (5 mg kg^{-1} day^{-1} × 4 days) + LPS; Group 4 = TG + RP 54745 (25 mg kg^{-1} day^{-1} × 4 days) + LPS.

while even at 10^{-5} *M*, it did not affect the binding of IL-1β either to $EL_{4-6.1}$ cells from a murine thymoma or to Raji cells from a human B lymphoma (data not shown).

The cellular target of RP 54745 is not known at present, but these preliminary results give several interesting insights to its mechanism of action. RP 54745 is not acting by preventing zymosan action directly, since it also prevents the action of other different stimulating agents as LPS. Its inhibitory effects concern several activated cellular events but not all to the same extent; for example, it decreases IL-1α and IL-1β production more than TNFα production, an observation that was confirmed at the mRNA level. This selective effect of RP 54745 was against a toxic process; this was confirmed by its reversible action on the stimulated hexose monophosphate pathway. The action of RP 54745 shows that IL-1 and TNFα can have an independent regulation mechanism on LPS-induced cytokines production *in vitro* and *in vivo* as shown by many authors [12]. Moreover, the *in vitro* inhibitory effect of RP 54745 on IL-1 mRNA expression

is also observed *ex vivo* as well as *in vivo*. Concerning this effect, we distinguished between the two types of IL-1: IL-1β, the secreted form, and IL-1α, the cell-associated form. According to the mRNA measurements, the expression of both of these forms is inhibited by RP 54745; however, in experiments on the biological response to LPS *in vitro*, the greatest inhibition of IL-1 production is that of cell-associated IL-1. This effect could be due to the use of LPS as the stimulant in this *in vitro* model [13]; indeed, when LPS is administered *in vivo*, equal amounts of cell-associated and extracellular IL-1 are produced and RP 54745 is an inhibitor of both. Our approach does not permit clarification of whether RP 54745 is acting at the transcriptional or (and) post-transcriptional level. However, its effect and its specificity on IL-1 mRNA expression versus TNFα makes RP 54745 different from other compounds that decrease IL-1 mRNA expression, such as corticosteroids and cycloheximide, or that only decrease IL-1 release. Indeed, RP 54745 differs from glucocorticoids which decrease IL-1 (α and β) and TNFα at gene expression and post-transcriptional levels [14]; it also differs from cycloheximide which diminishes IL-1β but increases IL-1α and TNFα expression and production [15].

Furthermore, RP 54745, at 10^{-5} *M*, does not inhibit cyclooxygenase (CO) and, at 5×10^{-6} *M*, slightly reduces 5-lipoxygenase (LO) (the effect of RP 54745 was measured on these enzymes isolated from RBL_1 sonicated cells; data not shown). Thus, RP 54745 differs from some other compounds that decrease IL-1 secretion, without effect on mRNA expression, such as:

- CP 66248, an oxo-indole compound described by Otterness et al. [16], which inhibits cyclooxygenase and 5-lipoxygenase activities;
- SKF 86002, an imidazo-thiazole compound described by Lee et al. [17], which is also a CO/LO inhibitor, as are the classical nonsteroidal anti-inflammatory drugs (NSAIDs);
- IX 207-887, a benzocycloheptathiophene acid described by Schnyder et al. [18], which is not a CO inhibitor but inhibits monokine release at the post-transcriptional level.

On the other hand, RP 54745 could have a mechanism of action related to that of a new compound, (Z)-3-(5 ethyl-4-hydroxy-3-methoxy-1-naphthalenyl)-2-methyl-2-propenoic acid, which was described as an IL-1 inhibitor at the transcriptional level without CO inhibitory properties [19].

In summary, the results of this study suggest that RP 54745 could be an original compound with some analogous *in vitro* properties with corticosteroids. Like the corticosteroids, RP 54745 inhibits the hexose monophosphate pathway [20], a property which can lead to an interesting drug as menadione [21, 22]. It also diminished lysosomal enzyme exocytosis and IL-1α and IL-1β expression which are both, directly or indirectly, implicated in degenerative processes.

In the light of these results, it was important to study the properties of RP 54745 *in vivo* in different experimental murine models of induced or spontaneous arthritis.

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