UR-1505, a new salicylate, blocks T cell activation through

Nuclear Factor of Activated T-cells

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Abbreviations: UR-1505: 2-hydroxy-4-2,2,3,3,3-pentafluoropropoxybenzoic acid; NF-AT: Nuclear Factor of Activated T cells; IL: interleukin; IFN: interferon; NF-κB: Nuclear Factor kappa B; AP-1: Activator Protein-1; NSAID: nonsteroidal anti-inflammatory drug; COX: cyclooxygenase; CsA: cyclosporine A; PMA: phorbol 12-myristate 13-acetate; TCR: T cell receptor; JAK: Janus Kinase; TNF: tumour necrosis factor; FACS: fluorescence-activated cell sorting; EMSA: electrophoretic mobility shift assay; TNBS: trinitrobenzenesulphonic acid; CK: casein kinase.
ABSTRACT

UR-1505 is a new molecule, chemically related to salicylic acid with immunomodulator properties, currently under clinical development for atopic dermatitis. The present work describes the immunomodulatory profile of UR-1505. UR-1505 targets T cells inhibiting their proliferation and cytokine production by blocking NF-AT DNA-binding activity. The effects of UR-1505 (100-300 µM) on T cell proliferation appears to be dependent on the stimulus, since UR-1505 inhibited CD3/CD28–induced T-cell proliferation, increased p27Kip levels and induces G1/S cell arrest but, interestingly, did not inhibit the JAK/STAT-induced T-cell proliferation. These data suggest that UR-1505 acts by means of a specific mechanism inhibiting T cell activation depending on TCR signalling pathway. Furthermore, the antiproliferative effects of UR-1505 are not consequence of decreased cell viability. In addition to the inhibition of T-cell proliferation, UR-1505 decreased in a dose dependent manner the production of IL-5 and IFN-γ in activated T cells and this effect was produced at transcriptional level. As T-cell proliferation and cytokine production were regulated through NF-AT, we examined the effect of UR-1505 on this transcription factor. According to its effect on IL-5 and IFN-γ mRNA expression, UR-1505 specifically inhibited NF-AT DNA binding without effect on NF-κB and AP-1 activities. The effect of UR-1505 on NF-AT is not attributable to a blockade of nuclear import. In conclusion, UR-1505 is a new immunomodulator agent that specifically inhibits NF-AT activation. As NF-AT regulates the transcription of most genes involved in lymphocyte activation, its selective inactivation results in both decreased T-cell proliferation and cytokine production.
INTRODUCTION
Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as anti-inflammatory drugs. Among NSAIDs, salicylates are commonly used as anti-inflammatory agents. For not long it has been accepted that the therapeutic effects of NSAIDs, salicylates included, were consequence of the inhibition of cyclooxygenase activity preventing the production of prostaglandins. However, it has been appearing evidences showing that additional mechanisms, independent of COX inhibition, are involved in the anti-inflammatory properties of salicylates (Tegeder et al., 2001). These additional, and COX-independent, effects of salicylates mainly lead to an attenuation of pro-inflammatory gene expression by inhibiting different signal pathways (Tegeder et al., 2001). In that sense, the most well known is the inhibition of NF-κB activation (Koop et al., 1994). Another effect of salicylates on transcription factor activity is the inhibition of c/EBPβ and STAT-6 transcription factors (Perez-G et al., 2002). In addition to their effects on gene expression, acetylsalicylic acid is reported to block T-cell activation (Paccani et al., 2002), which plays a key and central role in autoimmune diseases. However, this effect was observed at supraphysiological concentrations. Knowing the key role of T-cell activation in autoimmune diseases, the blockade of T cell activation and subsequent cytokine production may be an useful treatment for autoimmune diseases. In fact, several immunosuppressive agents such as methotrexate, cyclosporin A (CsA) or tacrolimus are used for the treatment of autoimmune diseases (Gremese et al., 2004; Saxne et al., 2003; Pacor et al., 2004).

UR-1505 (2-hydroxy-4-2,2,3,3,3-pentafluoropropoxybenzoic acid) (Figure 1) is a novel pentafluoropropoxy derivative of salicylic acid, that has been selected from a series of salicylate derivative, according to their activity as inhibitors of lymphocyte activation. These effects may be mediated through the Nuclear Factor of Activated T
Cells (NF-AT) since this transcription factor is known to mediate the expression of the majority of genes expressed in lymphocytes as well as lymphocyte proliferation.

Nuclear Factor of Activated T Cells (NF-AT) family of transcription factors comprise five proteins evolutionary related to Rel/NF-κB family. Four proteins NF-ATc2 (NF-AT1), NF-ATc1 (NF-AT2), NF-ATc3 (NF-AT4) and NF-ATc4 (NF-AT3) are dependent on Ca\textsuperscript{2+}/calmodulin-dependent serine phosphatase calcineurin (Hogan et al., 2006; Rao et al., 1997) while the fifth (NF-AT5) is not regulated by calcineurin. Among these, both NF-AT1 and NF-AT2 are the most expressed NF-AT proteins in mature T cells, regulating the expression of most, if not all, cytokines expressed by T cells. Activation of these members is regulated by their subcellular localization. In resting T cells, without Ca\textsuperscript{2+} signalling, NF-AT proteins are highly phosphorylated, remaining in the cytoplasm. Once activated, cytoplasmic Ca\textsuperscript{2+} levels increase and calcineurin becomes activated and it dephosphorylates NF-AT proteins which produce the exposure of nuclear localization signal and the masking of nuclear export signal, with the consequent nuclear import of NF-AT. In the nucleus NF-AT proteins bind to the DNA and regulate the gene transcription in cooperation with other transcription factors, as AP-1 (Rao et al., 1997; Macian et al., 2005; Serfling et al., 2000).

Once in the nucleus NF-AT activity is down-regulated by means of serine/threonine kinases that phosphorylate NF-AT proteins promoting their export to the cytoplasm.

NF-AT proteins are not only expressed in T-cells but also in other immune cells types, as B cells (Choi et al., 1994), mast cells (Prieschl et al., 1995) and natural killer cells (Aramburu et al., 1995). NF-AT is essential for T-cell activation and regulates cell-cycle control (Macian et al., 2005) and also regulates the production and expression of cytokines involved in the immune response (IL-2, IL-5, IL-4, IFN-γ, and others) (Serfling et al., 2000).
The anti-inflammatory properties of UR-1505 in the TNBS-induced colitis model has been previously reported (Bailón et al, manuscript in progress) and it is suggested that the anti-inflammatory effect was mediated by blocking of Th1-cell activation. The aim of this study was to determine the effects of UR-1505 on T-cell activation both in T cell proliferation and cytokine production.
MATERIAL AND METHODS

Reagents

RPMI 1640 and other cellular culture products were obtained from GIBCO. All chemicals were obtained from Sigma unless otherwise indicated.

UR-1505 was synthesised at J. Uriach y Compañía, S.A.

ELISA kits for IL-5, CycleTEST Plus DNA Reagent Kit, Transfactor kits for p65 and NF-ATc1 were obtained from BD Biosciences. WST-1 kit was obtained from Roche. NF-AT Activation Kit was obtained from Cellomics (Pittsburg, PA). Cell Proliferation Biotrak ELISA System was from Amersham Biosciences. Bradford reagent was from BioRad (München, Germany). Trizol reagent was from Invitrogen life technologies (Pansley, Scotland, UK). Anti CD28 and anti CD3 were from R&D Systems (Minneapolis, MN).

Cell culture and treatment

Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum in a humidified 5% CO₂ atmosphere at 37°C. Experiments with Jurkat cells were performed in RPMI 2% FBS. Jurkat cells were stimulated with PMA (50 nM) and ionomycin (3 µM) in the presence or absence of UR-1505 (100-300 µM) or CsA (1 µM). Human T-cells were isolated from peripheral blood by means of a Ficoll gradient, followed by a negative selection using MACS immunogenetic beads (Pan T Cell Isolation kit human, Milteny Biotec). A highly pure population of T-cells was obtained (>98%, determined by FACS analysis). Human isolated T cells were, either frozen for further studies, or cultured in RPMI 1640 medium supplemented with 10% FBS. T cells were stimulated with plate-coated anti-CD3 (1µg/ml), anti-CD28 (1 µg/ml) plus rhIL-2
(25 ng/ml) (R&D Systems) in the presence or absence of UR-1505 or CsA. HeLa cells were cultured in MEM supplemented with 20% foetal bovine serum.

**T cell proliferation assays**

We have studied the effect of UR-1505 on two different models of T cell proliferation, depending on the proliferative stimulus. First, human isolated T cells were cultured in 96-well plates (100,000 cells/well) in 100 µl RPMI 10% FBS and were stimulated with coated anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml) plus rhIL-2 (25 ng/ml) for up to 96 hours, this proliferation was dependent on TCR engagement and the co-signal provided by CD28.

Secondly, JAK/STAT-dependent proliferation was studied in human isolated T cells that have been growing in medium containing IL-2 (10ng/ml) for seven days in order to over express IL2 receptors. At day seven, human isolated T cells were plated on 96 microplates and stimulated with IL-2 (1 ng/ml) for another 96 hours, so we have detected the proliferation exclusively caused by 10 ng/ml IL2 through its overexpressed receptor and JAK/STAT.

BrdU was added in both cases about 15 hours before to reach 24, 48, 72 and 96 hours of incubation, at a final concentration of 10 µM. During this labelling period, BrdU is incorporated instead of thymidine into the DNA of growing cells. Quantification of BrdU incorporation to DNA in growing cells is used as a measure of cell proliferation. BrdU was determined by ELISA according to manufacturer instructions.
**Jurkat cell cycle assay**

Jurkat cells in log-phase growth were treated with UR-1505 (10-500 µM). After 24 hours cells were pulsed with BrdU (10 µM, 1 h), harvested by centrifugation, resuspended (1x10⁶ cells/ml) in PBS, fixed in ice-cold 70% ethanol and further processed and stained with 20 µl anti-BrdU-FITC following the manufacturer’s instructions (Becton Dickinson) and with 5µg/ml propidium iodide. Within 3 h after staining, the fractions of cells in G0/G1, S, and G2/M phases of the cell cycle were quantified by fluorescence-activated cell sorting (FACS) analysis performed with a FACSCalibur flow cytometer (BD Biosciences).

**p27⁰⁴ⁱ⁸ levels in Jurkat cells**

Accumulation of the cyclin-dependent kinase (Cdk) inhibitor p27 was analysed in Jurkat cells. Cells were incubated for 24 hours with 0.1% FBS (quiescent cells) or with 10% FBS (proliferating cells) plus vehicle or UR-1505 (3-30 µM). p27 levels were determined by immunoblot or flow cytometry after cell staining with phycoerythrin conjugate p27 monoclonal antibody following the manufacturer’s instructions (Santa Cruz Biotechnology).

**Viability assays**

Human isolated T cell viability was determined using the Reagent WST-1 (Roche) according to manufacturer instructions.

**Cytokine production**

Human isolated T cells (1x10⁶) were stimulated with anti-CD3, anti-CD28 and rIL-2 for up to 96 hours in the presence or not of UR-1505 (30-300 µM). Every 24 hours
supernatants were obtained and immediately frozen until cytokine determination. Cytokine concentration in the supernatants was determined by flow cytometry (CBA, BD Biosciences).

**Real Time RT-PCR**

Total RNA was obtained from human isolated T cells (4x10⁶ cells) and Jurkat cells (5x10⁶ cells) using TRIzol Reagent and following the manufacturer instructions. cDNA was generated using TaqMan reverse transcription reagents (Applied Biosystems) from 1 µg total RNA. The reaction mix contained, 1X RT buffer, 5.5 mM MgCl₂, 500 µM deoxyNTP mixtures, 2.5 µM Random hexamers, 0.4 U/µl RNase inhibitor and 1.25 U/µl Multiscribe Reverse Transcriptase. PCR was performed in a 20 µl reaction mixture that contains 40 ng of reverse-transcribed cDNA, PCR master mix and the needed amounts of primers and probe included in IL-5 and IFN-γ TaqMan Gene Expression Assays (Applied Biosystems). The PCR and subsequent analysis were done with the ABI PRISM 7700 apparatus (Applied Biosystems).

Cycle threshold (Cₜ) values were calculated for IL-5, IFN-γ and 18S rRNA. The relative IL-5 and IFN-γ transcripts in treated (T) and control samples (C) were expressed as 2⁻ΔΔCₜ, in that ΔΔCₜ= ΔCₜ(T) - ΔCₜ(C) and ΔCₜ=Cₜ (IL5 or IFN-γ)-Cₜ (18S rRNA).

**Nuclear Extract Preparation**

Nuclear extracts from Jurkat cells (4x10⁶) and human isolated T cells (4-6x10⁶) were prepared by lysing cells with Nonidet P-40 followed by differential centrifugation. Briefly, at the end of incubations, cells were collected by centrifugation at 1200 rpm for
10 minutes at room temperature, and washed with ice cold PBS. Then, cells were resuspended in buffer 10 mM Hepes, pH 7.8, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and kept on ice for 15 min. Cells were then lysed with Nonidet P-40 (10%), and the nuclear pellet was recovered after centrifugation at 13000 × g at 4°C for 30 s. The nuclear pellet was resuspended in ice-cold buffer containing 20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and stored at −80 °C. Protein concentration was determined by Bradford assay.

**AP-1 DNA-binding activity**

Activation of transcription factor AP-1 was determined by electrophoretic mobility shift assay (EMSA) using a consensus oligonucleotide of AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') (Promega). Probes were labelled with T4 polynucleotide kinase (Promega) with γ³²P-ATP (3000 Ci/mmol, 250 µCi) (Amersham Biosciences). Briefly, 3 µl H₂O, 2 µl consensus sequence of oligonucleotide (~20 ng), 1 µl of 10x buffer enzyme, 1 µl of T4 enzyme (5 U/µl) and 3 µl of γ³²P-ATP were mixed in an eppendorf tube and incubated at 37°C for 1 hour. Oligonucleotides labelled were purified using Nick columns (Amersham) and stored at −20°C.

Binding reactions included 10 μg of nuclear extracts in incubation buffer (10 mM Tris-HCl, pH 7.5, 40 mM NaCl, 1 mM EDTA and 4% glycerol), 1 μg of poly (dI-dC) (Amersham Biosciences), and labelled oligonucleotide (~30,000 cpm). The mixture was electrophoresed in a 6% non-denaturing polyacrilamide gel for 2-3 hours at 175 V, and the gel was autoradiographed using a Hyperfilm MP (Amersham Biosciences) at −20°C overnight.
NF-AT and NF-κB activities

NF-AT and NF-κB activities were analyzed with the commercially available ELISA kits, TransFactor NF-ATc1 colorimetric kit and TransFactor NFκB p65 colorimetric kit (BD Biosciences). Briefly, nuclear extracts (10 µg protein) from Jurkat cells or human isolated T cells were introduced into the wells of 96-well microtitre plates pre-coated with oligonucleotide containing the NF-AT and NF-κB consensus sequence (5’-GAGGAAAATTTG-3’), (5’-GGGGTATTTCC-3’) in a volume of 50 µl for 1 hour at room temperature. After washing three times, 100 µl of monoclonal antibody against NF-ATc1 or p65 was added to the appropriate wells, and incubated for a further 1 hour at room temperature. Anti-IgG horseradish peroxidase-conjugate in a volume of 100 µl was then added and incubated for another 1 hour at room temperature. Absorbance at 450 nm was measured after the addition of 1 M sulphuric acid. Specificity was verified by competition assays and by mutated oligos.

Immunocytochemistry

HeLa cells (1x10⁴) were plated on 8-well culture coverslips (BD Biosciences) and cultured overnight. Then, cells were pre-incubated with UR-1505 (300 µM) or CsA (1 µM) for 30 minutes at 37ºC and stimulated or not with ionomycin (1 µM) for another 40 minutes. Immediately, cells were fixed with 3% paraformaldehyde (pre-warmed at 37ºC) for 20 minutes at room temperature. Immunocytochemistry was performed following the manufacturer instructions.
RESULTS

**UR-1505 inhibits TCR-induced T-cell proliferation**

To investigate the effect of UR-1505 on T-cell activation, we first evaluated the effect of UR-1505 on TCR-induced human isolated T cell proliferation. TCR engagement by antigen and CD28 co-signal allow T cells to proliferate through TCR signalling pathway. Therefore, in order to induce cellular proliferation, human isolated T cells were stimulated through T cell receptor with coated anti-CD3 (1µg/ml), and anti-CD28 (1µg/ml) plus IL-2 (25 ng/ml) for up to 96 hours in the presence or absence of UR-1505. In these conditions, human isolated T cells clearly proliferate, as is shown in Figure 2A-C. UR-1505 (10-300 µM) inhibited in a dose dependent manner the CD3/CD28 induced human isolated T cell proliferation whereas salicylic acid treatment for 96 hours (300-500 µM) did not (Figure 2A and 2C). The inhibition achieved by 300 µM UR-1505 was 100%. Additionally, we used CsA as a positive control of T-cell proliferation inhibition because it is known that the inhibitory effect of CsA on T-cell proliferation is due to calcineurin inhibition. Therefore, CsA (5-500 nM) blocks TCR signalling cascade inhibiting T-cell proliferation (Figure 2B).

**UR-1505 did not inhibit JAK/STAT-induced T-cell proliferation**

To assess whether the inhibition of T-cell proliferation was due to an interference of UR-1505 with TCR and CD28 receptor engagement, we examined the effect of UR-1505 on JAK/STAT dependent T cell proliferation. In order to induce cell proliferation, human isolated T cells were exclusively incubated with IL-2, as is detailed in Material and Methods. In these conditions T cell proliferation is not dependent on TCR signalling pathway but is controlled through JAK/STAT signalling pathway.
JAK/STAT dependent T cell proliferation was not affected by UR-1505, since 100 µM failed inhibiting human isolated T cell proliferation and the highest concentration of UR-1505 assayed (300 µM) had only a minor effect on cell proliferation after 96 hours (Figure 2D). It has to be mentioned that 300 µM UR-1505 absolutely abrogated the TCR-dependent T cell proliferation from 24 hours (Figure 2A).

CsA also failed inhibiting T-cell proliferation in these conditions, however a JAK inhibitor reduced IL-2 induced lymphocyte proliferation (data not shown).

**UR-1505 induces cell cycle arrest through p27KIP1 accumulation**

Co-signalling with TCR/CD3 and CD28 promotes progression of T cells through the cell cycle. To this end, a decrease in the levels of Cdk inhibitor p27KIP1 is required to permit cell passage from G1 to S phase of cell cycle.

UR-1505 induced G1 arrest of growing Jurkat cells, inhibiting in a dose dependent manner their progression into S phase of the cell cycle (Figures 3A y B). This G1 arrest seems to be mediated by an UR-1505-promoted accumulation of p27KIP1. As shown in Figure 3C, UR-1505 concentration-dependently increased p27KIP1 levels in log-phase proliferating Jurkat cells. Levels of p27KIP1 in UR-1505 (30 µM) treated cells were similar to those of arrested cells incubated in low (0.1%) FBS.

Moreover, the effects of UR-1505 on p27KIP1 concentration seem to be due to inhibition of degradation of this Cdk inhibitor. As shown in Figure 3D incubation of HeLa cells in 0.1% FBS (48 h) induced cell quiescence and a marked increased in p27KIP1 levels. Changing these cells to a 10% FBS medium, resulted in a time-dependent degradation of p27KIP1, which correlated with the onset of cell proliferation. However, when 10% FBS medium was supplemented with UR-1505 (300 µM), p27KIP1 degradation was inhibited, leaving the cells in a quiescent mode.
UR-1505 did not decrease T cell viability

To discard that the anti proliferative effect of UR-1505 was not due to an increase in cellular toxicity in human isolated T cells, we have examined the human isolated T cell viability by means of WST-1 assay. Starting from 48 hours stimulation with anti-CD3, anti-CD28 plus hrIL-2, we have observed a higher mitochondrial activity of human isolated T cells due to an increase on cell number as a consequence of increased cell proliferation. Interestingly, UR-1505 did not reduce the human isolated T cell viability at concentrations up to 300 µM (Figure 4), since the reduction of mitochondrial activity always was higher than basal values (non stimulated lymphocytes) but not below that.

Effect of UR-1505 on cytokine production in activated T-cells

Isolated human peripheral blood T cells were activated in the same way than that for proliferation and viability studies, that is by cross-linking the TCR with anti-CD3 (1 µg/ml) plus a co-stimulation provided by anti-CD28 (1 µg/ml) antibodies for up to 96 hours in the presence or not of UR-1505 (30-300 µM). Activation of human isolated T-cells resulted in an increased production of IFN-γ, IL-5, IL-4 and TNF-α, while non activated T-cells did not produce detectable levels of any cytokine (Figure 5A). The increased secretion of cytokines was observed from 24 hours (Figure 5B), although it was more evident from 48 hours (Figure 5A).

Treatment with UR-1505 (30-300 µM) reduced the cytokine production by activated T cells (Figures 5A and 5B). The most important inhibitory effect was observed for IFN-γ and IL-5. Interestingly, the inhibition of IFN-γ and IL-5 production showed the same pattern than the inhibition of cell proliferation in human isolated T cells.
In Figure 5B we have focused on the inhibitory effect of UR-1505 on cytokine secretion at 24 hours. As shown, at this early time point there was already inhibition of IL-5 and IFN-γ by UR-1505 (100-300 µM).

**UR-1505 decreases IL-5 and IFN-γ gene expression**

As IL-5 and IFN-γ were the most inhibited cytokines by UR-1505 and in order to determine if the inhibition of IL-5 and IFN-γ production was consequence of a decreased gene expression, we analysed the effect of UR-1505 on IL-5 and IFN-γ mRNAs levels in T cells. For this, T cells; human isolated T cells and Jurkat cells were pre-incubated with UR-1505 (300 µM), CsA (1 µM) or tacrolimus (1 µM) for 30 minutes and then both types of T cells were stimulated. The stimulus was different for each type of T cell; human isolated T cells were stimulated by cross-linking the TCR with anti-CD3 plus a co-stimulation provided by anti-CD28 antibodies while Jurkat cells were stimulated pharmacologically for 24 hours with PMA (50 nM) plus ionomycin (3 µM), which mimics the engagement of TCR with anti-CD3 and anti-CD28. Both stimuli are equivalent because ionomycin increases intracellular Ca^{2+} levels and PMA activates MAPK, the same signalling pathways that are activated after TCR engagement with anti CD3 and the co-signal allowed by anti-CD28. At the end of experiments, total RNA was obtained and reversed transcribed to cDNA and IFN-γ and IL-5 gene expression was quantified by Real Time PCR. As shown in Figure 6A and 6B, UR-1505 (300 µM) reduced the gene expression of both genes in activated T cells. The inhibition on IL-5 and IFN-γ was observed both in Jurkat (Figure 6A) and human isolated T cells (Figure 6B). As expected, CsA (1µM) and tacrolimus (1 µM) inhibited the expression of both genes, in Jurkat cells and in human isolated T cells, respectively.
UR-1505 specifically inhibits NF-AT DNA-binding activity both in human isolated T-cells and in Jurkat cells

Most of the genes involved in the immune response, including IL-5 and IFN-γ, are transcriptionally regulated by NF-AT, AP-1 and NF-κB binding sites located in their promoter regions. Furthermore, these transcription factors, especially NF-AT, play a key role during T cell activation. Thus, we have studied the effect of UR-1505 on NF-AT, AP-1 and NF-κB DNA-binding in T cells. For this we have used two cellular models; human isolated T-cells activated with anti CD3/anti CD28 (both at 1 µg/ml) for 6 hours and Jurkat cells pharmacologically stimulated for 4 hours with PMA (50 nM) plus ionomycin (3 µM). T cells have been pre-incubated or not with UR-1505 (100-300 µM) or CsA (1 µM) 30 minutes before stimulation.

Activated human isolated T cells and pharmacologically stimulated Jurkat cells showed increased DNA binding for all three transcription factors (NF-AT, AP-1 and NF-κB) (Figure 7A and 7B).

UR-1505 (100-300 µM) reduced NF-AT DNA binding whereas NF-κB and AP-1 DNA-binding activities remained unaffected both in Jurkat cells and in human isolated T cells (Figure 7A and 7B). CsA is a well known immunosuppressive drug that targets NF-AT activity through calcineurin inhibition. In our model, CsA (1 µM) strongly inhibited NF-AT DNA binding and, in addition, also reduced partially NF-κB DNA-binding.

UR-1505 has not effect on ionomycin induced-NF-AT translocation to the nucleus

The finding of a diminished NF-AT DNA-binding does not allow the distinguish between a block of NF-AT import (like CsA does) or an enhanced export. In order to ascertain whether UR-1505 modulates NF-AT import we have performed immunocitochemistry experiments with HeLa cells. This human cell line has been
widely used for NF-AT translocation experiments. As shown in figure 8, NF-AT localization was cytosolic in non-stimulated HeLa cells, while an important NF-AT translocation to the nucleus was observed after stimulation with ionomycin (1 µM). As expected, the calcineurin inhibitor CsA (1 µM) prevented the ionomycin-induced NF-AT translocation into the nucleus. However, UR-1505 (300 µM) failed to inhibit NF-AT nuclear translocation, since the fluorescence had been detected principally in the nucleus (Figure 8). Indeed, salicylates are known to not inhibit NF-AT translocation to the nucleus (Aceves et al., 2004).

**Nuclear export blockade has not effect on UR-1505 induced NF-AT inhibition**

To determine if the inhibitory effect of UR-1505 on NF-AT DNA-binding was consequence of an increased export, we used leptomycin B as a general inhibitor of exportin (CRM1)-dependent export. As shown in Figure 9, treatment with leptomycin B did not abolish the inhibitory effect of UR-1505 on NF-AT DNA-binding suggesting that UR-1505 did not increase NF-AT nuclear export.

**Effect of UR-1505 on the p38, GSK and CK1 activities**

The effect of UR-1505 on the kinases involved in nuclear export of NF-AT was evaluated (Upstate Discovery, Dundee, UK). As shown in table 1, UR-1505 (300 µM) did not affect the p38, GSK or CK1 activities.
DISCUSSION

In this study we have shown that UR-1505, a new immunomodulator agent chemically related to salicylic acid, blocks T-cell activation, which may be an useful strategy for the treatment of autoimmune diseases, knowing the key role of T-cell activation in the onset of these diseases (Goudy et al., 2005; Walter et al., 2005; Chow et al., 2005; Howard et al., 2005). It is important that the prevention of T-cell activation produced by UR-1505 is done in a lower range of concentrations (0.1 –0.3 mM) than that reported previously for acetylsalicylic acid (2-4 mM) (Paccani et al., 2002). Our results indicate that blockade of T-cell activation by UR-1505 results in the inhibition of both T-cell proliferation and cytokine production and both effects appears to be mediated through TCR/NFAT signalling pathway.

UR-1505 inhibits T cell proliferation acting on some point(s) of the TCR signalling cascade since UR-1505 inhibits in a dose dependent manner the T cell proliferation induced by TCR activation with anti CD3 and anti CD28 but does not inhibit the proliferation induced by JAK-STAT (IL-2-dependent cell proliferation). More important is the fact that UR-1505 does not inhibit cell proliferation in macrophages (Fernández de Arriba et al., 2005). On the other hand, salicylic acid was not able to inhibit T cell proliferation independently of the stimulus at any concentration assayed.

It has been reported that tacrolimus, another well known inhibitor of TCR signalling cascade, blocks proliferation of endothelial cells through upregulation of p27 (Matter et al., 2006). In that sense, our data shown that UR-1505 induces cell cycle arrest at G1/S increasing p27KIP1 levels in proliferating Jurkat cells, and the accumulation of p27 produces arrest in the G1 phase (Mohapatra et al., 2001). It should be emphasized that the antiproliferative effect of UR-1505 is not consequence of a decreased T cell viability.
Activated T cells, in addition to proliferate, produce a great array of cytokines. The enhanced production of cytokines is reduced by pre-treatment with UR-1505 being the most important inhibitory effect observed on IL-5 and IFN-γ production, both with a key role in the immune response. This inhibition is produced at transcriptional level since the mRNAs of both genes are reduced after pre-treatment with UR-1505. To understand the mechanisms involved in the inhibition of cytokine gene expression, we have studied the effect of UR-1505 on NF-AT, NF-κB and AP-1 DNA-binding activities in T cells. Our results revealed that UR-1505 inhibits specifically NF-AT DNA-binding in T cells without effect on other transcription factors, such as NF-κB and AP-1. The selective NF-AT inhibition explains the decreased expression of IL-5 and IFN-γ, because their expression is modulated through NF-AT sites in their promoter regions (Lee et al., 1995; Hogan et al., 2006). Indeed, two NF-AT inhibitors as CsA and tacrolimus, completely abrogates the IL-5 and IFN-γ gene expression. Inhibition of NF-AT appears to be a common point in the prevention of both T cell proliferation and cytokine gene expression by UR-1505 since it is known that NF-AT modulates the expression of hundreds of genes in T cells (Macian et al., 2005; Peng et al., 2001) and promotes the transition from G1 to S phase once TCR is engaged (Appleman et al., 2002; Caetano et al., 2002; Feske et al., 2001; Lipskaia et al., 2004). In addition, UR-1505 inhibits T cell proliferation and cytokine expression in a similar range of concentrations than that needed to prevent NF-AT activation. Indeed, UR-1505 follows a similar pattern than CsA because only inhibits TCR dependent cell proliferation (and does not inhibit JAK/STAT dependent proliferation) and prevents NF-AT DNA binding, without effect on AP-1 DNA binding activity. UR-1505 does not inhibit NF-κB although our results shown that CsA has a partial inhibitory effect on NF-
κB DNA-binding, according to other authors who previously reported that CsA inhibits NF-κB by inhibition of proteosome activity (Meyer et al., 1997).

Our results are in agreement with previous data reporting that salicylates inhibit cell proliferation through inhibition of Ca2+/calcineurin/NFAT pathway (Núñez et al., 2006) since this pathway is activated after TCR engagement. On the other hand, Aceves et al. have shown that salicylates prevent NF-AT dependent gene expression (Aceves et al., 2004). Therefore, NF-AT is a target for salicylates and inhibition of NF-AT results in decreased cell proliferation and cytokine production.

The inhibition of NF-AT produced by UR-1505 may also explain the previously reported specificity of UR-1505 inhibiting splenocyte proliferation without effect on murine bone marrow derived macrophage proliferation (Fernández de Arriba et al., 2005) since NF-AT plays a central role in T lymphocytes, while NF-κB appears to be more relevant in macrophages.

The precise mechanism through UR-1505 inhibits NF-AT activation is not completely known yet. NF-AT may be regulated at different levels: phosphorylation/dephosphorylation, subcellular localization, DNA binding activity and transactivation (Rao et al., 1997; Aceves et al., 2004). Among these possibilities, our data indicate that UR-1505 acts at the level of DNA binding and/or transactivation, because UR-1505 failed inhibiting NF-AT translocation into the nucleus in response to ionomycin stimulation of HeLa cells. This precludes an inhibitory effect of UR-1505 on calcineurin activity. By contrast CsA, a genuine calcineurin inhibitor, blocks the ionomycin induced NF-AT nuclear translocation in HeLa cells. Therefore, although the pattern of UR-1505 and CsA as inhibitors of T cell activation is similar, the mechanisms involved are different. Furthermore, these results are in agreement with Aceves et al (Aceves et al., 2004) who reported that salicylates inhibit NF-AT dependent
transcription without effect on ionomycin-induced NF-AT nuclear localization. Besides nuclear import, another important point of regulation of NF-AT is the reversible phosphorylation of NF-AT proteins by specific kinases affecting its nuclear export rates. In that sense, it is known that phosphorylation of NF-AT by p38, GSK, CK1 in some residues leads to NF-AT export to cytoplasm and decreased NF-AT DNA binding (Kiani et al., 2000; Chow et al., 1997; Beals et al., 1997). Therefore, inhibition of NF-AT DNA binding by UR-1505 may be consequence of an increased kinase activity that enhanced NF-AT nuclear export.

In that sense, we have determined that UR-1505 has no effect on in vitro kinase activity of p38, GSK-3 and CK1. Indeed, blockade of nuclear export by leptomycin B has not consequence on decreased NF-AT DNA-binding suggesting that the mechanism of UR-1505 is not mediated by an increase of NF-AT export.

Although in most cases NF-AT rephosphorylation induces its inactivation through nuclear export, there are increasing data showing that phosphorylation in specific residues belonging to the NF-AT regulatory domain increases its transcriptional activity improving their DNA binding by means of conformational changes and/or by recruiting cofactors as p300. In that sense Okamura et al have reported an inducible phosphorylation in the transactivation domain of NF-ATc2 after stimulation of T cells with PMA plus ionomycin which enhances its transcriptional activity (Okamura et al., 2000). Furthermore, Pim kinase I (Rainio et al., 2002), JNK (Ortega-Pérez et al., 2005) and Cot kinase (de Gregorio et al., 2001) phosphorylate NF-ATc1 and NF-ATc2 increasing their transcriptional activities. Therefore, a reasonable possibility is that UR-1505 may inhibit NF-AT DNA-binding reducing the activity of the inducible kinase that increases NF-AT transactivation (Okamura et al., 2000). Studies are in progress to further identify the kinases targeted by UR-1505.
In conclusion, our results showed that UR-1505 inhibits T-cell activation, phenomenon characterized by inhibition of both T-cell proliferation and cytokine production, and both effects appears to be the consequence of NF-AT inhibition. Furthermore, our data indicate that the mechanism of action of UR-1505 is different from the classical calcineurin inhibitors. Since the adverse effects of CsA and tacrolimus, nephrotoxicity, neurotoxicity, diabetogenicity and gastrointestinal toxicity, are due to the calcineurin inhibition in cells outside the immune system (Fung et al., 1991; Ho et al., 1996; Dumont et al., 1992), UR-1505 may be safer than calcineurin inhibitors. UR-1505 clinical development is in progress for atopic dermatitis. Clinical results in this indication and others will show whether UR-1505 could be a safer and effective alternative to calcineurin inhibitors.
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REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1. Chemical structure of UR-1505.

Figure 2. UR-1505 inhibits specifically TCR-induced T cell proliferation, having a minor effect on JAK/STAT-induced T cell proliferation. (A) Human isolated T cells were stimulated with coated anti-CD3 (1 µg/ml) plus anti-CD28 (1 µg/ml) plus rIL-2 (25 ng/ml) for up to 96 hours and the effect of increasing concentrations of UR-1505 (10-300 μM) on T-cell proliferation was assayed. B and C, Effect of CsA for up to 96 h (5 and 500 nM) and salicylic acid after 96 hours (300-500 μM) on TCR induced T cell proliferation. Finally, the effect of UR-1505 (10-300 μM) and salicylic acid (30-1000 μM) after 96 hours on JAK/STAT-dependent T cell proliferation was assayed in human isolated T cells as is detailed in M&Ms (D). Data shown are the mean±SEM of three different experiments done in triplicate. * p<0.001 vs activated T cells, # p<0.01 vs activated T cells

Figure 3. Effect of UR-1505 on cell cycle and p27KIP1 levels.

(A) Percentage of cells in the S phase of cell cycle in asynchronously growing Jurkat cells treated with UR-1505 (0.3 mM) or vehicle for 24h. After incubation, cells were pulsed with BrdU (1 h) and then stained with anti-BrdU-FITC and propidium iodide followed by FACS analysis. (B) Effect of UR-1505 in the percentage of Jurkat cells in the S phase of cell cycle. Experimental details as above. (C) Representative histograms showing the effects of UR-1505 on p27 levels in Jurkat cells. Cells were incubated for 24 h with 0.1% FBS (quiescent cells), 10% FBS (proliferating cells) or 10% FBS plus UR-1505. After 24 h cells were stained with phycoerythrin conjugated p27 monoclonal
antibody or with an isotypic control followed by FACS analysis. (D) Immunoblots showing the inhibition of p27 degradation by UR-1505 in HeLa cells. Cells were changed from a quiescent environment (0.1% FBS, 48 h) to a proliferating one (10% FBS) in presence of UR-1505 (0.3 mM) or vehicle. At different times, cells were harvested and immunoblottings were performed with antibodies against p27. Column graph shows the quantification of two independent experiments (mean±SD).

**Figure 4. UR-1505 does not decrease T cell viability.** Human isolated T cells were stimulated with anti-CD3 (1µg/ml), anti-CD28 (1µg/ml) plus IL-2 (25ng/ml) for up to 96 hours in the presence or not of UR-1505 (10-300 µM). Every 24 hours mitochondrial activity was measured by WST-1 Reagent (Roche). Results shown are a representative experiment performed in triplicate.

**Figure 5. UR-1505 reduces cytokine production in T-cells.** (A) Human isolated T-cells were stimulated with anti-CD3 (1µg/ml), anti-CD28 (1µg/ml) plus IL-2 (25ng/ml) for up to 96 hours in the presence or not of UR-1505 (30-300 µM) and cultures supernatants collected every 24 hours for IL-4, TNF-α, IL-5 and IFN-γ determination. (B) Effect of UR-1505 (30-300 µM) on IFN-γ and IL-5 production by antiCD3/CD28 stimulated human isolated T cells after 24 hours. Results shown are a representative experiment performed in triplicate. * p<0.05 vs activated T cells

**Figure 6. Inhibition of IL-5 and IFN-γ gene expression by UR-1505.** IL-5 and IFN-γ gene expression were determined by Real-time RT-PCR analysis in Jurkat cells stimulated pharmacologically with PMA (50 nM) + ionomycin (3 µM) for 24 hours (A) and in human isolated T cells stimulated with anti CD3 and anti CD28 both at 1 µg/ml.
for 24 hours (B). In both cases T cells were pre-treated with UR-1505 (300 μM) or CsA (1 μM) in the case of Jurkat or tacrolimus (1 μM) in the case of human isolated T cells 30 minutes before stimulation. Relative amounts of IL-5 and IFN-γ mRNAs were calculated and normalized with the endogenous control 18S rRNA. Data shown (2-ΔΔCT) are the mean ±SEM of three different experiments done in duplicate. *< p<0.05 vs activated T cells

**Figure 7. UR-1505 decreases NF-AT DNA binding without effect on NF-κB and AP-1 in T cells.** (A) Jurkat cells were incubated with the indicated concentrations of UR-1505 and CsA (1μM) for 30 min and then stimulated pharmacologically with PMA (50 nM) plus ionomycin (3μM) for 4 hours. (B) Human isolated T cells were stimulated with anti CD3 (1 µg/ml), anti CD28 (1 µg/ml) plus IL2 (25 ng/ml) for 6 hours and incubated in the presence or not of 300 μM UR-1505.

The nuclear NF-AT and NF-κB DNA binding were detected by ELISA assays and AP-1 was examined by EMSA.

Data shown are the mean±SEM of three-five different experiments. For AP-1 experiments, a representative EMSA is shown. * p<0.01, # p<0.001 vs activated T cells
Figure 8. UR-1505 has not effect on ionomycin-induced NF-AT nuclear import in HeLa cells.

HeLa cells were attached overnight and then were pre-incubated with 300 µM UR-1505 or 1 µM CsA for 30 minutes, then were stimulated with 1 µM ionomycin for 40 minutes. NF-AT localization was studied by immunocytochemistry. The images shown are representative of three separate experiments.

Figure 9. Nuclear export blockade has not effect on UR-1505 inhibition of NF-AT DNA-binding.

Jurkat cells were treated in the presence or not of leptomycin B (20 ng/ml), with UR-1505 (300 µM) for 30 minutes and then stimulated with PMA+ionomycin for 4 hours. At the end, nuclear extracts were obtained and NF-AT DNA binding determined. Results are the mean±SEM of three separate experiments.
**Table 1. Effect of UR-1505 on the activity of p38, GSK and CK1 kinases.**

The effect of UR-1505 at 300 µM, expressed as a percentage of activity compared to that of control incubations (100%) was measured. ATP was used at 100 µM in all assays.

<table>
<thead>
<tr>
<th>Kinase</th>
<th>% control</th>
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<tbody>
<tr>
<td>GSK</td>
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</tr>
<tr>
<td>p38α</td>
<td>90</td>
</tr>
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<tr>
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</tr>
<tr>
<td>CK1</td>
<td>92</td>
</tr>
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</table>
Figure 1
Figure 2
Figure 3
Figure 4

Viability

![Graph showing viabilities at different times and concentrations of UR1505](attachment:image.png)

- **activated**
- **activ+10μM UR1505**
- **activ+30μM UR1505**
- **activ+100μM UR1505**
- **activ+300μM UR1505**
- **basal**
Figure 5
Figure 5
Figure 7
Figure 7

<table>
<thead>
<tr>
<th></th>
<th>NF-AT</th>
<th>NF-κB</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti CD3+anti CD28</td>
<td>- + +</td>
<td>- + +</td>
</tr>
<tr>
<td>UR-1505, µM</td>
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<td>- - 300</td>
</tr>
</tbody>
</table>

B

AP-1

Free probe
Figure 8
Effect of nuclear export blockade on NF-AT DNA binding

Figure 9