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The 5-HT3 receptor antagonist tropisetron inhibits T cell activation by targeting the calcineurin pathway

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Abstract

Tropisetron, an antagonist of serotonin type 3 receptor, has been investigated in chronic inflammatory joint process. Since T cells play a key role in the onset of several inflammatory diseases, we have evaluated the immunosuppressive activity of tropisetron in human T cells, discovering that this compound is a potent inhibitor of early and late events in TCR-mediated T cell activation. Moreover, we found that tropisetron specifically inhibited both IL-2 gene transcription and IL-2 synthesis in stimulated T cells. To further characterize the inhibitory mechanisms of tropisetron at the transcriptional level, we examined the DNA binding and transcriptional activities of NF-κB, NFAT and AP-1 transcription factors in Jurkat T cells. We found that tropisetron inhibited both the binding to DNA and the transcriptional activity of NFAT and AP-1. We also observed that tropisetron is a potent inhibitor of PMA plus ionomycin-induced NF-κB activation but in contrast TNFα-mediated NF-κB activation was not affected by this antagonist. Finally, overexpression of a constitutively active form of calcineurin indicated that this phosphatase may represent one of the main targets for the inhibitory activity of tropisetron. These findings provide new mechanistic insights into the anti-inflammatory activities of tropisetron, which are probably independent of serotonin receptor signalling and highlight their potential to design novel therapeutic strategies to manage inflammatory diseases.

Keywords: Tropisetron; T cells; IL-2; NF-κB; NFAT; Calcineurin

1. Introduction

5-Hydroxytryptamine (5-HT[1], serotonin) is a well-characterized neurotransmitter that plays a crucial role in the regulation of central processes, such as mode, appetite, sleep and other body rhythms. Moreover, 5-HT is found in the immune-inflammatory axis and has been shown to influence the immune response in mammals [1,2]. The pleiotropic activity of 5-HT is due to the molecular complexity of 5-HT receptors (5-HTR) and their wide tissue expression [3]. Multiple serotoninergic receptors have been identified so far and among them the subtype 5-HT_3 receptor (5-HT_3R), which is an ionotropic receptor permeant to cations with high selectivity to Na^+ inward movements, has been found to be expressed in cells of the immune system including T lymphocytes [4,5], and evidence exists that 5-HT can modulate the T cells functionality through activation of 5-HT_3R [4,6]. Interestingly, highly selective 5-HT_3R antagonists, such as tropisetron, have been investigated in chronic inflammatory joint process, although the antiphlogistic mechanisms of action are largely unknown [7,8].

The signal transduction pathways triggered by the activation of the TCR–CD3 complex in T cells lead to the

Abbreviations: AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; 5-HT, serotonin; 5-HTR, serotonin receptor; IKK, IκB kinase; IκB, κB inhibitor; JNK, jun kinase; MAPK, mitogen activated kinase; NF-κB, nuclear factor kappa B; NFAT, nuclear factor of activated cells; TNFα, tumor necrosis factor α

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immediate activation of transcription factors that regulate a variety of activation-associated genes. Many of them are cytokines and surface receptors that play an important role in coordinating the immune response [9]. The signal transduction pathways involved in T cell activation are initiated by the clustering of lipids rafts at the cell surface, with formation of a supramolecular activation complex localized at the antigen-induced immunological synapse [10]. Several studies have demonstrated that the presence of specific signalling proteins such as Cot/Tpl-2, Vav-1, PKCθ and PLCγ1 within lipids rafts control lymphocyte signalling [11,12]. Activated PLCγ1 cleaves phosphatidylinositol 4,5 bisphosphate yielding inositol (1,4,5) triphosphate (IP_3) and diacylglycerol (DAG). While IP_3 mobilizes Ca^{2+} from intracellular stores, DAG mediates activation of the protein kinase C (PKC) family members [13]. As a consequence of an increase in intracellular Ca^{2+} levels, several signalling pathways are activated in T cells [14]. In this sense, calcineurin, a Ca^{2+}-calmodulin dependent protein phosphatase, is activated and subsequently dephosphorylates the nuclear factor of activated T cells (NFAT), allowing its nuclear shuttling [14]. This transcription factor was first described as an inducible regulatory complex critical for transcriptional induction of IL-2 in activated T cells, but was subsequently shown to regulate the transcription of many other genes, including cytokines, cell surface receptors and regulatory enzymes [14,15]. In the nucleus, NFAT binds to the DNA either alone or in conjunction with AP-1 proteins [16]. Nevertheless, the coordinated induction and activation of the transcription factors NFAT, NF-κB and AP-1 is required to regulate cytokine gene expression [17].

Stimulation via TCR–CD3 complex alone is sufficient for NFAT activation, but it is insufficient for activation of NF-κB and AP-1. Thus, a second signal mediated by the CD28 co-stimulary receptor is required for the induction of NF-κB and AP-1 in antigen stimulated T cells [18]. The transcription factor NF-κB is one of the key gene regulators involved in the immune/inflammatory response as well as in survival from apoptosis [18]. NF-κB is an inducible transcription factor made up of homo- and heterodimers of p50, p65 (RelA), p52, RelB and c-rel subunits that interact with a family of inhibitory IκB proteins, of which IκBα is the best characterized [19,20]. In most cell types, these proteins sequester NF-κB in the cytoplasm by masking its nuclear localization sequence, and in response to a variety of stimuli, including TCR and CD28 co-stimulation, IκBs are phosphorylated by the IκB kinase complex, followed by their ubiquitination and degradation in the proteasome. The release of IκBs unmasks the NLS and allows NF-κB to enter the nucleus [18,21].

In this paper, we studied the effect of tropisetron, a 5-HT_3R antagonist, on early and late T cell activation events and we have demonstrated that tropisetron inhibits antigen-induced proliferation and IL-2 production in human peripheral T cells. Moreover, we show here for the first time that tropisetron inhibits the signalling pathways that regulate the activation of the transcription factors NFAT, NF-κB and AP-1, which are known to play a critical role in the immune response.

2. Material and methods

2.1. Cell lines and reagents

The 5.1 clone (obtained from Dr. N. Isräel, Institut Pasteur, Paris, France) line is a Jurkat derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-LTR promoter and was maintained in exponential growth in RPMI 1640 (Gibco BRL–Life technologies, Barcelona, Spain) supplemented with 10% heat inactivated foetal calf serum, 2 mM L-glutamine, 1 mM Hepes and antibiotics (completed medium) (Gibco BRL–Life technologies, Barcelona, Spain) and G418 (200 μg/ml). Jurkat cells (ATCC, Rockville, MD, USA) were also maintained in exponential growth in complete medium. The anti-IκBα mAb 10B was a gift from R.T. Hay (St. Andrews, Scotland), the mAb anti-tubulin was purchased from Sigma Co (St. Louis, MO, USA), the rabbit polyclonal and anti-p65 (1226) was a gift from A. Isräel (Institute Pasteur, Paris, France). The anti-phospho-ERK 1 + 2 (sc-7383) was from Santa Cruz Biotechnology (CA, USA), the mAbs anti-phospho-p38 (9211S), anti-phospho-JNK (9255S) and anti-phospho-p65 (3031S) were from New England Biolabs (Hitchin, UK). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA, USA). All other reagents were from Sigma.

2.2. Plasmids

The AP-1-Luc plasmid was constructed by inserting three copies of an SV40 AP-1 binding site into the Xho site of pGL-2 promoter vector (Promega, MA, USA), the NFAT-Luc plasmid contains three copies of the NFAT binding site of the IL-2 promoter fused to the luciferase gene [22]. The KBF-Luc contains three copies of the MHC enhancer κB site upstream of the conalbumin promoter followed by the luciferase gene [23]. The IL-2-Luc (−326 to +45 of the IL-2 promoter) was previously described [22]. The plasmid pEF-BOS trunk-Cot containing the truncated active form of the Cot kinase was obtained from S. Alemany (CSIC, Madrid, Spain). The expression plasmid ΔCaM-AI encodes a truncated form of a murine calcineurin catalytic subunit that has Ca^{2+} independent and constitutive phosphatase [24]. The Gal4-Luc reporter plasmid includes five Gal4 DNA-binding sites fused to the luciferase gene. The Gal4-hNFAT1 contains the first 1–415 amino acids of human NFAT1 fused to the DNA binding domain of yeast Gal4 transcription factor and was previously described [25]. The Gal4-p65 contains the C-terminal region of the human p65 (amino acids 286–551)

fused to the Gal4 binding domain and was a gift from Dr. Schmitz.

2.3. T cell proliferation assays and IL-2 synthesis

Human peripheral blood mononuclear cells (PBMC), from healthy adult volunteer donors, were isolated by centrifugation of venous blood on Ficoll-Hypaque® density gradients (Amersham Biosciences, Piscataway, NJ, USA). 10^5 cells were cultured in triplicate in 96-well round bottom microtiter plates (Nunc, Roskilde, Denmark) in 200 μl of complete medium and stimulated with Staphylococcal enterotoxin B (SEB) (1 μg/ml)) in the presence or absence of increasing concentrations of tropisetron. SEB-activation model was used since this superantigen is presented by B cells and macrophages and activates T cells through TCR and co-stimulators. The cultures were carried out for 3 days and pulsed with 0.5 μCi [^{3}H]-TdR/well (Perkin-Elmer) for the last 12 h of culture. Radioactivity incorporated into DNA was measured by liquid scintillation counting and expressed as DPM. To measure IL-2 synthesis PBMC (10^6 ml^{-1}) were preincubated with tropisetron for 30 min in complete medium. Thereafter, cells were treated with SEB (1 μg/ml) for 18 h. After culture, supernatants were harvested and centrifuged for 10 min at 10,000 × g, and the levels of IL-2 in the supernatant were measured by ELISA (R&D Systems, Wiesbaden–Nordestedt, Germany) according to the manufacturer's instructions. Experiments were carried out in triplicate.

2.4. Cytofluorimetric analyses of cell surface antigen and cell cycle

For cell cycle analyses and measurement of CD25 (IL-2Rα chain) expression, peripheral mononuclear cells (10^6 ml^{-1}) were stimulated with SEB (1 μg/ml) in 24 well plates in a total volume of 2 ml of complete medium for 48 h in the presence or absence of different concentrations of tropisetron. CD25 cell surface fluorescence was measured by using a specific mAb (Pharmigen, San Diego, CA, USA) and analysed by flow cytometry in an EPIC XL flow cytometer (Coulter, Hialeah, FL, USA). For DNA profile analysis, cells were washed in PBS, fixed in ethanol (70%, for 24 h at 4 °C), followed by RNA digestion (RNAse-A, 50 U/ml) and propidium iodide (PI, 20 μg/ml) staining, and analyzed by cytofluorimetry. Ten thousand gated events were collected per sample and the percentage of cells in every phase of the cell cycle determined.

2.5. Transient transfections and luciferase assays

Jurkat cells (10^7 ml^{-1}) were transiently transfected with the indicated plasmids. The transfections were performed using Lipofectamine™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations for 24 h. After incubation with tropisetron for 30 min, transfected cells were stimulated for 6 h as indicated. To determine NF-κB-dependent transcription of the HIV-LTR promoter, 5.1 cells were preincubated for 30 min with tropisetron as indicated, followed by stimulation with TNFα (2 ng/ml) for 6 h. Then, the cells were lysed in 25 mM Tris–phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and 7% glycerol. Luciferase activity was measured using an Autolumat LB 953 (Berthold Technologies Bad Wilbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA) and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation expressed as a fold induction over untreated cells. All the experiments were repeated at least four times.

2.6. Western blots

Jurkat cells (1×10^6 cells/ml) were stimulated with either TNFα (2 ng/ml) or PMA (20 ng/ml) plus ionomycin (0.5 ug/ml) for p65, JNK, ERK1 + 2, p38 and IκBα proteins in the presence or absence of tropisetron as indicated. Cells were then washed with PBS and proteins extracted in 50 μl of lysis buffer (20 mM Hepes pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaFl, 1 mM DTT, leupeptin 1 μg/ml, pepstatin 0.5 μg/ml, aprotinin 0.5 μg/ml and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA) and 30 μg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% non-fat dry milk overnight at 4 °C, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (Amersham Biosciences).

2.7. Isolation of nuclear extracts and mobility shift assays

Jurkat cells or 5.1 cells (10^6 ml^{-1}) were treated with the agonists in complete medium as indicated. Cells were then washed twice with cold PBS, and proteins from total cell extracts (for NF-κB and AP-1 binding) or nuclear extracts (for NFAT binding) were isolated as previously described [26]. Protein concentration was determined by the Bradford method (Bio-Rad, Richmond, CA, USA). For the electrophoretic mobility shift assay (EMSA), double stranded oligonucleotides containing the consensus sites for NF-κB, 5′-AGTTGAGGGGACTTTCCCAGG-3′ (Promega, Madison, WI, USA), NFAT 5′-GATCGGAGGAAAAACTGTTTCATACAGAAGGCGT-3′ (distal NFAT site of human IL-2 promoter) and AP-1 5′-CGCTTGATGAGTCAGCCGGAA-3′ (Promega, Madison, WI, USA) were end-labelled with [γ-^{32}P]ATP. The binding reaction mix-

ture contained 3 μg of nuclear extract (or 15 μg of total extracts), 0.5 μg poly(dI–dC) (Amersham Biosciences), 20 mM Hepes pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μg/ml BSA, 4% Ficoll and 100,000 cpm of end-labelled DNA fragments in a total volume of 20 μl. When indicated, 0.5 μl of rabbit anti-p65 or preimmune serum was added to the standard reaction before the addition of the radiolabelled probe. For cold competition, a 100-fold excess of the double stranded oligonucleotide competitor was added to the binding reaction. After 30 min incubation at 4 °C, the mixture was electrophoresed through a native 6% polyacrylamide (4% in the case of NFAT) gel containing 89 mM Tris–base, 89 mM boric acid and 2 mM EDTA. Gels were pre-electrophoresed for 30 min at 225 V and then for 2 h after loading the samples. These gels were dried and exposed to X-ray film at −80 °C.

3. Results

3.1. Effects of tropisetron on T cell activation

Recent clinical data have shown that tropisetron, a 5-HT_3R antagonist, is effective for the treatment of inflammatory and pain joint processes [7,8]. Since 5-HT_3R is expressed in human peripheral T cells we studied the effects of this 5-HT_3R antagonist on several T cell activation events and we found that DNA synthesis measured by [^{3}H]-TdR uptake in SEB and in PMA plus ionomycin-stimulated T cells was markedly inhibited by tropisetron in a concentration-dependent manner (Table 1). Due to cell activation, primary T cells up-regulate several surface molecules such as the α chain of the IL-2R (CD25), produce IL-2 and progress to the S phase and G_2/M phases of the cell cycle. Thus, resting non stimulated peripheral T cells remained largely in the G_0/G_1 phase of the cell cycle and expressed low levels of CD25. Three days following activation by SEB, a significant percentage of T cells were expressing the CD25 activation marker at the cell surface (48%) and progressing through the S, G_2 and M phases of the cell cycle (18.2% of the cells). As expected, the percentage of cells progressing through the cell cycle was higher in cells treated with PMA plus ionomycin (49.3% of the cells), and pre-treatment with tropisetron diminished the percentage of CD25$^+$ cells and cycling cells in both SEB and in PMA plus ionomycin-stimulated cells. Similar results were obtained with the analogue ondasetron although to a lesser extent (Table 1). These results clearly indicate that the inhibitory effects of tropisetron were not mediated by targeting early events in TCR-mediated signalling. Interestingly, no significant differences were found in the percentage of hypodiploid cells (sub G_0/G_1) observed in cells stimulated in the presence or absence of tropisetron, and these results indicate that, at the doses used, tropisetron did not induce cytotoxicity or apoptosis in primary T cells.

3.2. Effects of tropisetron on IL-2 synthesis and promoter activity

IL-2 represents one of the major growth factors for the clonal expansion of activated T cells. Thus, we studied the effects of tropisetron on SEB-induced IL-2 production in primary T cells and in Fig. 1A it is shown that this compound was able to inhibit the release of IL-2 with an IC50 of approximately 15 μg/ml (≅50 μM). Interestingly, no significant inhibitory activity on IL-2 production was found using granisetron, another 5-HT3 receptor antagonist (data not shown). Since IL-2 gene expression is regulated mainly at the transcriptional level, we investigated the regulation of IL-2 promoter activity in Jurkat T cells transiently transfected with the luciferase reporter plasmid IL-2-Luc. After transfection, cells were preincubated with tropisetron for 30 min, activated with PMA (20 ng/ml) plus ionomycin (1 μM) for 6 h, and tested for luciferase activity. Tropisetron efficiently inhibited PMA plus ionomycin-induced luciferase expression driven by the IL-2 promoter in a dose-dependent manner (Fig. 1B). The inhibitory effects of tropisetron were not due to interference with the transcriptional machinery or with

Table 1
Human peripheral T cells were stimulated with SEB (1 μg/ml) or PMA plus Ionomycin (Io) in the presence or absence of increasing concentrations of either tropisetron or ondasetron for 72 h

	Proliferation [^{3}H]-TdR-uptake	IL-2R$^+$ cells (%)	Cell cycle phases (%)		
			Sub G_0/G_1	G_0/G_1	S–G_2/M
Control	31246 ± 9770	9 ± 3	0.9 ± 1.4	97.4 ± 0.4	1.2 ± 0.7
SEB	365179 ± 21146	48 ± 9	5 ± 0.3	76.6 ± 1.2	18.2 ± 1
SEB + tropisetron 10 μg/ml	313795 ± 18826	40 ± 6	3.2 ± 0.1	85 ± 0.5	9.7 ± 0.6
SEB + tropisetron 25 μg/ml	37140 ± 3902	23 ± 5	5.4 ± 1.2	86.7 ± 4.5	7.8 ± 5
SEB + tropisetron 50 μg/ml	8344 ± 1500	15 ± 2	8 ± 3.8	88.8 ± 3.5	2.4 ± 0.8
SEB + ondasetron 25 μg/ml	98176 ± 15504	50 ± 4.1	N.D.	N.D.	N.D.
SEB + ondasetron 50 μg/ml	80761 ± 12382	29.7 ± 5	N.D.	N.D.	N.D.
PMA + Io	485030 ± 30847	89.9 ± 3.6	1.32 ± 0.7	55.4 ± 3.2	49.3 ± 2
PMA + Io + tropisetron 25 μg/ml	33269 ± 15415	50.6 ± 5.3	2.25 ± 1.2	78 ± 2.3	15 ± 5
PMA + Io + tropisetron 50 μg/ml	4253 ± 1104	28.4 ± 2.1	3.1 ± 0.3	87.1 ± 1.4	8.5 ± 1

[^{3}H]-TdR incorporation was measured by liquid scintillation counting and represented as the mean of DPM ± S.E. of three different experiments. IL-2R (CD25) expression and cell cycle analyses was analysed by flow cytometry and expressed as percentage of cells.

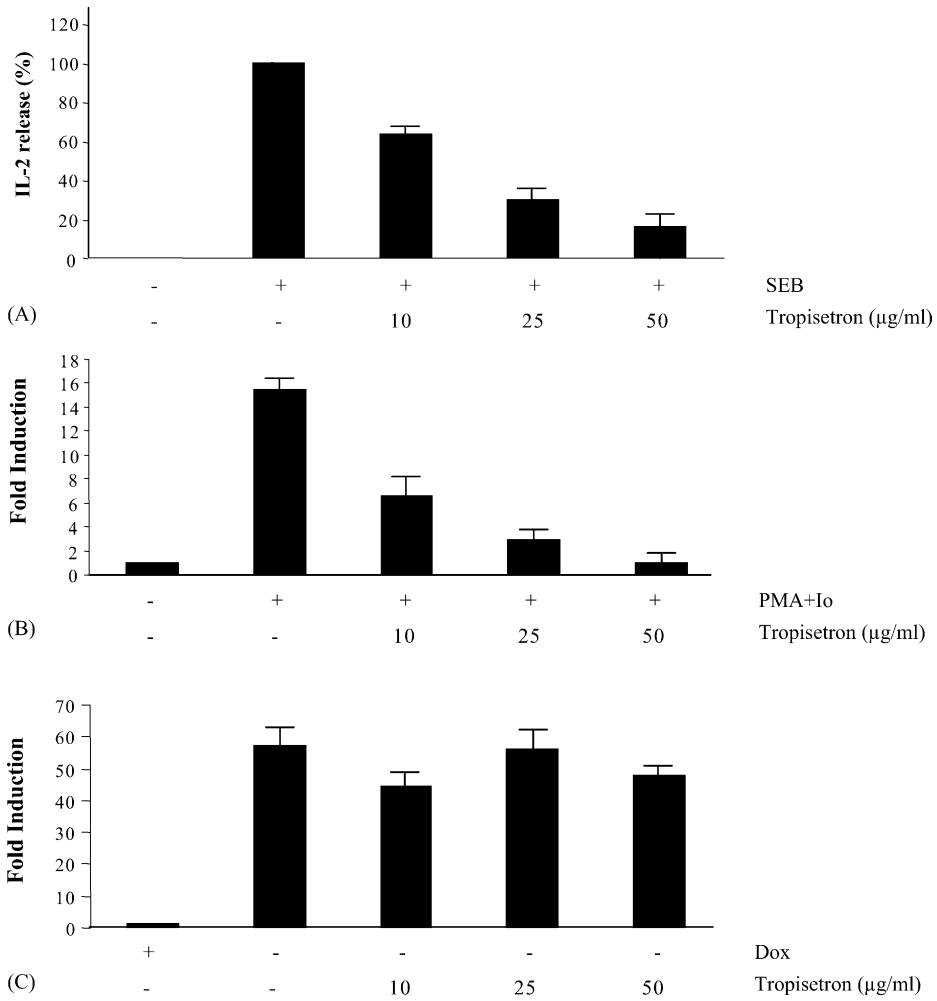

Fig. 1. Effects of tropisetron on IL-2 gene regulation. (A) Tropisetron inhibits IL-2 production by primary T cells. PBMC (10^6 ml^{-1}) were preincubated with increasing concentrations of tropisetron for 30 min and treated with SEB (1 μg/ml) for 18 h. Levels of IL-2 in the supernatant were measured by ELISA according to the manufacturer's instructions. Experiments were carried out in triplicate. (B) Tropisetron inhibits IL-2 gene promoter activity. Jurkat T cells transfected with IL-2 promoter luciferase reporter plasmid were treated for 30 min with increasing concentrations of CAPE, and then stimulated with PMA (20 ng/ml) plus ionomycin (1 μM) for 6 h, and luciferase activity measured in the cell lysates. Results are the means ± S.E. of three determinations expressed as fold induction (experimental RLU-background RLU/basal RLU-background RLU. (C) Jurkat TET-on-Luc assay. Jurkat T cells transfected with pTet-on and pUHC13-3 plasmids and 24 h later treated with doxycycline for 6 h and the luciferase activity measured as indicated.

the in vitro activity of the luciferase enzyme, since the inducible expression of luciferase mediated by doxycycline in Jurkat cells transiently co-transfected with the pTet-on and pUHC13-3 plasmids was not affected by tropisetron (Fig. 1C). Moreover, we did not find cytotoxicity in Jurkat cells treated for 24 h with the indicated doses of tropisetron (data not shown). Altogether, these results clearly indicate that tropisetron inhibits IL-2 production by interfering with the transcriptional machinery that regulates IL-2 gene transcription.

3.3. Tropisetron inhibits AP-1, NFAT and NF-κB transcriptional activities

The transcriptional activity of the IL-2 gene depends on the coordinated activation of several transcription factors, including NFAT, NF-κB and AP-1 families. Therefore, we evaluated the effect of tropisetron on the transcriptional activity of those factors by using luciferase reporter constructs under the control of minimal promoters containing binding sites of each of them. Activation by PMA plus ionomycin increased the luciferase gene expression driven by these promoters in Jurkat cells, and we found that tropisetron effectively inhibited each of these promoters in a dose-dependent manner, NFAT being the most sensitive transcription factor to the inhibitory activity of tropisetron (Fig. 2). Transcriptional activation of NFAT requires its translocation to the nucleus, where it binds to specific consensus sites in the promoter region of IL-2 gene [22]. To study whether tropisetron inhibits NFAT DNA-binding activity we performed EMSA with nuclear extracts of Jurkat cells stimulated with PMA plus ionomycin in the presence or absence of increasing concentrations of tropisetron. Using the distal NFAT site of the IL-2 promoter we found a major complex that was retarded in PMA plus ionomycin treated cells and the binding to DNA of this

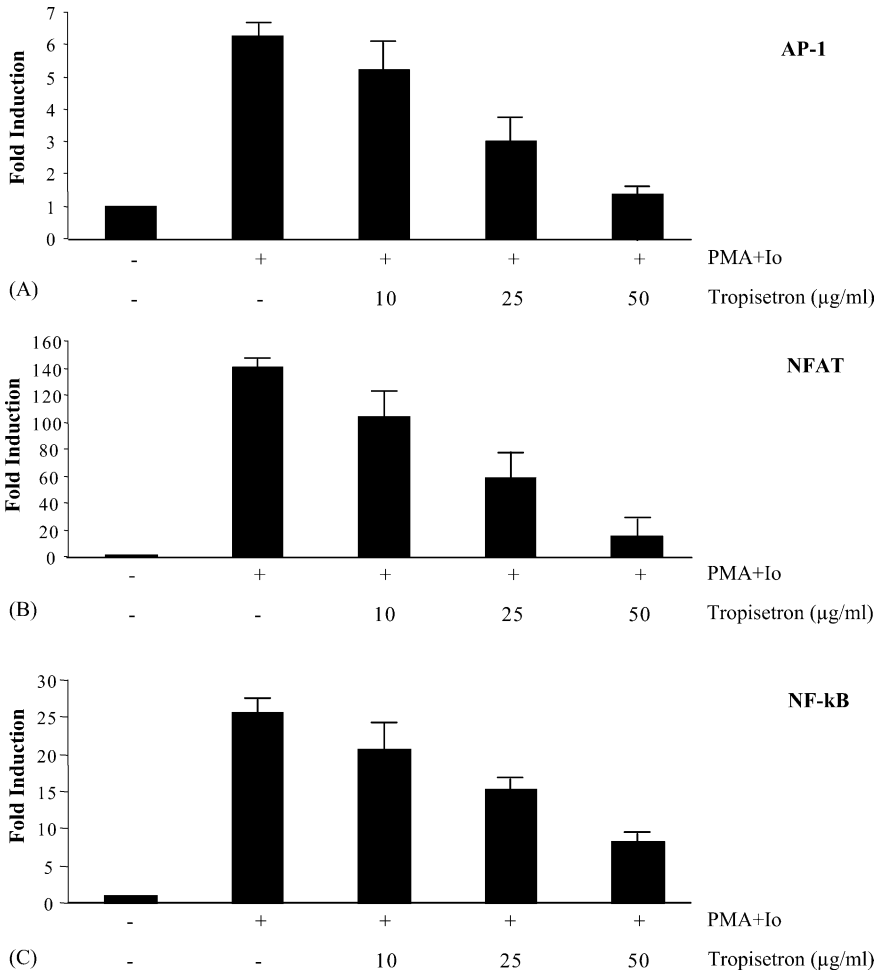

Fig. 2. Regulation of transcription factor-mediated transactivation by tropisetron. Jurkat T cells were transiently transfected with the luciferase reporter plasmid AP-1-Luc (A), NFAT-luc (B) or KBF-Luc (C), as described in Section 2. Cells were preincubated for 30 min with tropisetron at the indicated concentrations, before stimulation with PMA (20 ng/ml) plus Ionomycin (1 μM) for 6 h. Luciferase activity was measured and the results are the means ± S.E. of three determinations expressed as fold induction (observed experimental RLU/basal RLU in absence of any stimuli).

complex was clearly inhibited in the nuclear extracts of tropisetron-treated cells (Fig. 3A). This complex was previously characterized as NFAT1 by supershift experiments with an anti-NFAT1 antiserum and by cold competition experiments [26]. Next, we studied the effects of tropisetron on the major signalling pathways that regulate NFAT activation in T cells. Cot/Tpl-2 is a protein serine/threonine kinase, classified as a MAPK kinase kinase, implicated in the signal transduction pathways leading to the activation of several inducible transcription factors including NFAT [25]. Therefore, to analyse the effects of tropisetron on Cot-mediated NFAT activation we co-transfected Jurkat cells with a construct encoding an active form of this kinase (pEFBOS trunk-Cot) along with the chimeric vector pGal4-NFAT1 and the reporter plasmid Gal4-Luc. As shown in Fig. 3B, tropisetron weakly prevented the transactivation function of NFAT1 induced by Cot/Tpl-2 in this T cell line. Since the Cot/Tpl-2 kinase can also activate NFAT through calcineurin-dependent and -independent pathways [25], we were interested in investigating whether tropisetron was also able to inhibit NFAT-dependent transactivation induced by the phosphatase calcineurin. In Fig. 3C, it is shown that NFAT transcriptional activity induced by over-expression of an active form of the phosphatase calcineurin, ΔCAM-AI, [24] was greatly inhibited by tropisetron in a dose-dependent manner.

3.4. Effects of tropisetron on AP-1 binding to DNA and MAPKs phosphorylation

It has been shown that in the nucleus activated NFAT binds to the AP-1 family of transcription factors to increase the rate of transcription of target genes [27]. Moreover, a crosstalk between signalling pathways that activates both NFAT and AP-1 has been demonstrated in T lymphocytes [28]. To study the effects of tropisetron on AP-1 activation, we stimulated Jurkat cells with PMA plus ionomycin in the presence of tropisetron and total cell extracts obtained for EMSA and western blot analyses. In Fig. 4A, it is shown that increasing concentrations of tropisetron effectively inhibited PMA plus ionomycin induced AP-1-DNA binding. In addition, by using specific antibodies that recognize

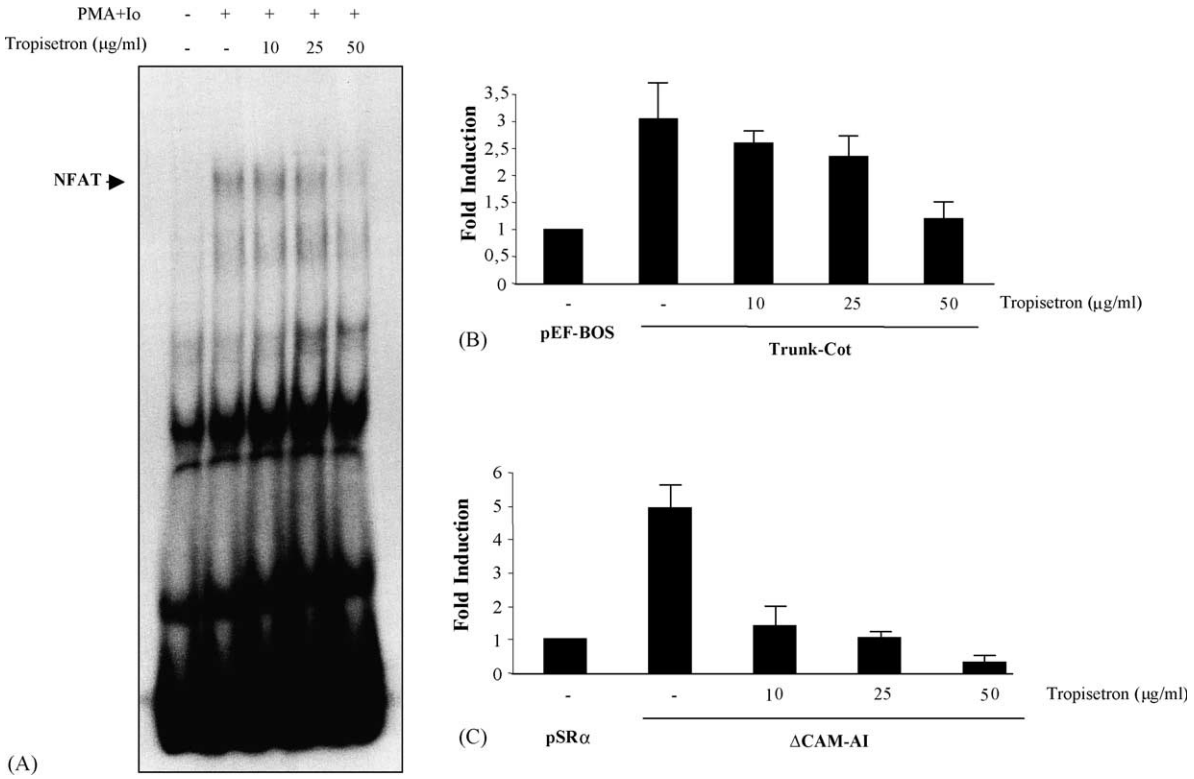

Fig. 3. Effects of tropisetron on the NFAT activation pathway. (A) NFAT DNA-binding activity was analysed for nuclear extracts from Jurkat T cells stimulated for 2 h with PMA plus ionomicyin (Io) (1 μM) in the absence or the presence of increasing concentrations of tropisetron. (B) Jurkat T cells were cotransfected with the plasmid pEF-BOS trunk-Cot containing the truncated active form of the Cot kinase together with the expression vector Gal4-hNFAT1 and the GAL4-Luc reporter plasmid. (C) Jurkat T cells were transiently cotransfected with an expression vector encoding for the catalytic subunit of calcineurin (ΔCAM-AI) together with the NFAT-Luc reporter plasmid. In all the cases and 24 h after transfection the cells were incubated with the indicated concentration of tropsisetron for 12 h and the luciferase activity measured as described above. Results are the means of three determinations expressed as fold induction.

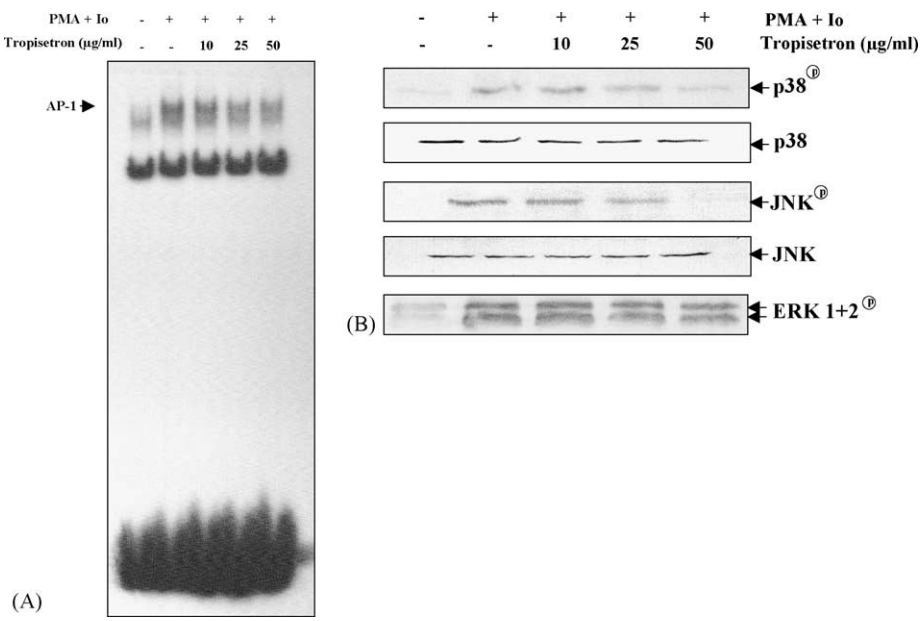

Fig. 4. Effects of tropisetron on AP-1 binding to DNA and MAPKs activation. (A) Jurkat T cells were preincubated with tropisetron at the indicated concentrations for 30 min followed by stimulation with PMA + Ionomycin (Io) for 1 h. The nuclear binding activity from total cell extracts was then assayed by EMSA using a γ-^{32}P-labelled AP-1 oligonucleotide. (B) Tropisetron inhibits the phosphorylation of the p38 and JNK but not the phosphorylation of ERK 1 and 2 kinases. Jurkat T cells were incubated with increasing concentrations of tropisetron and further stimulated with PMA plus Io for 30 min and the phosphorylation status for the MAPKs analysed by immunoblots using specific anti-phospho mAbs.

the phosphorylated and activated forms of the three major MAPKs, we observed that tropisetron, at the same AP-1 inhibitory concentrations, was able to inhibit the phosphorylation of JNK and p38, but not mitogen-induced activation of both ERK isoforms (ERK-1 and -2). Tropisetron pretreatment did not affect the steady state levels of the non-phosphorylated form of the MAPKs p38 and JNK (Fig. 4B).

3.5. Tropisetron selectively inhibits the NF-κB activation pathway induced by PMA plus ionomycin in Jurkat cells

Previous reports have suggested that calcineurin represents a critical link between Ca^{2+} signalling and NF-κB activation [28,29]. Therefore we analysed the effect of tropisetron on the NF-κB signalling pathways activated by PMA plus Ionomycin or TNFα in Jurkat cells. Firstly, the NF-κB DNA binding activity was studied by gel retardation assays using nuclear proteins isolated from cells stimulated with PMA and ionomycin in the presence or the absence of tropisetron. As shown in Fig. 5, Jurkat cells pretreated with tropisetron led to a dose-related inhibition of PMA plus ionomycin-induced NF-κB binding activity. The DNA-binding specificity was studied by supershift experiments using a specific anti-p65 (RelA) antibody and by cold competition experiments with unlabelled competitors and we identified the heterodimer p50/p65 as the main complex activated by PMA plus ionomycin in Jurkat cells (Fig. 5). Next, to investigate the level at which tropisetron exerted its inhibitory effect on NF-κB activation, we stimulated Jurkat cells with PMA and ionomycin for different times in the presence or absence of tropisetron (25 μg/ml), and proteins from total cell extracts were used for studying the steady state levels of IκBα by western blot. The kinetic experiments revealed that PMA plus ionomycin treatment led to a clear phosphorylation and degradation of IκBα, which were completely abrogated in the presence of tropisetron (Fig. 6A). Interestingly, tropisetron also inhibited PMA plus ionomycin-dependent p65-phosphorylation (serine 536) (Fig. 6A). To further analyze whether tropisetron inhibits directly p65-transcriptional

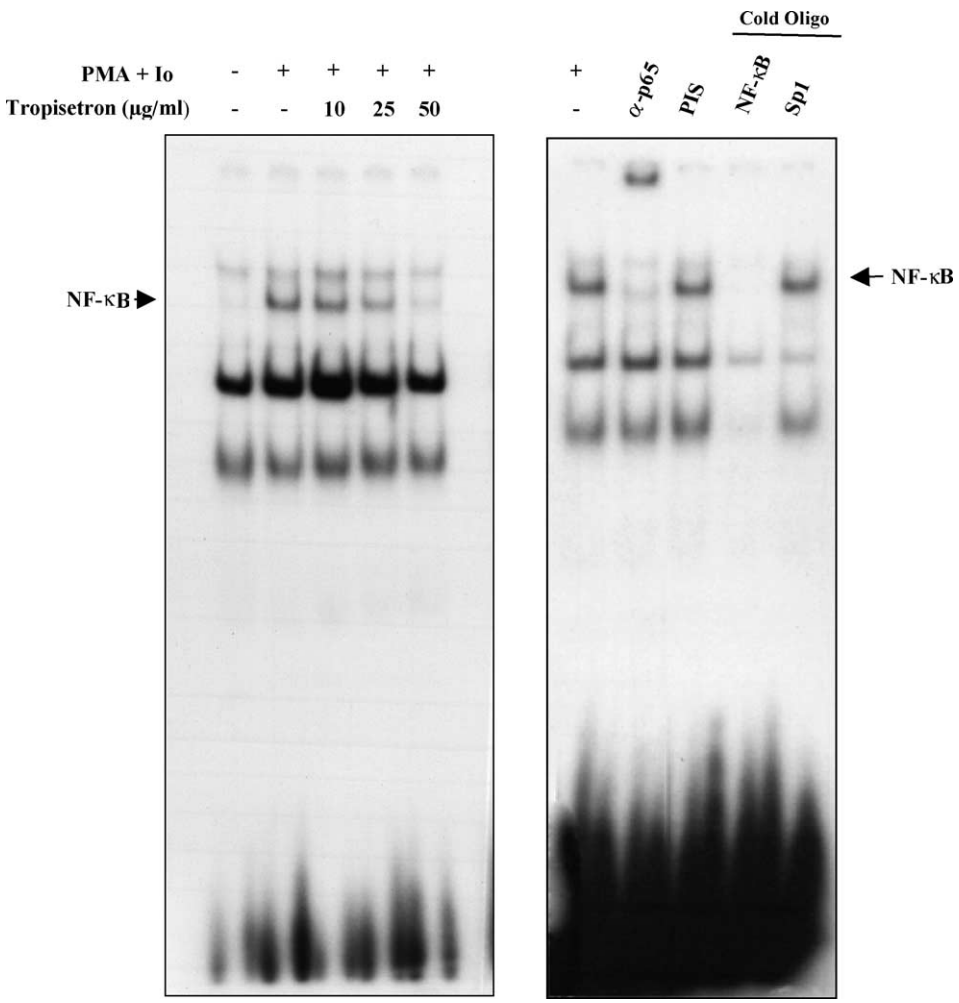

Fig. 5. Effects of tropisetron in NF-κB/DNA binding. Jurkat cells, either untreated or pre-treated with tropisetron at the indicated doses, were incubated with PMA plus Io for 30 min. The nuclear binding activity from total cell extracts was then assayed by EMSA using a γ-^{32}P-labelled NF-κB oligonucleotide (left panel). Cold competition and supershift experiments were assayed using proteins extracted from PMA plus Io treated cells (right panel).

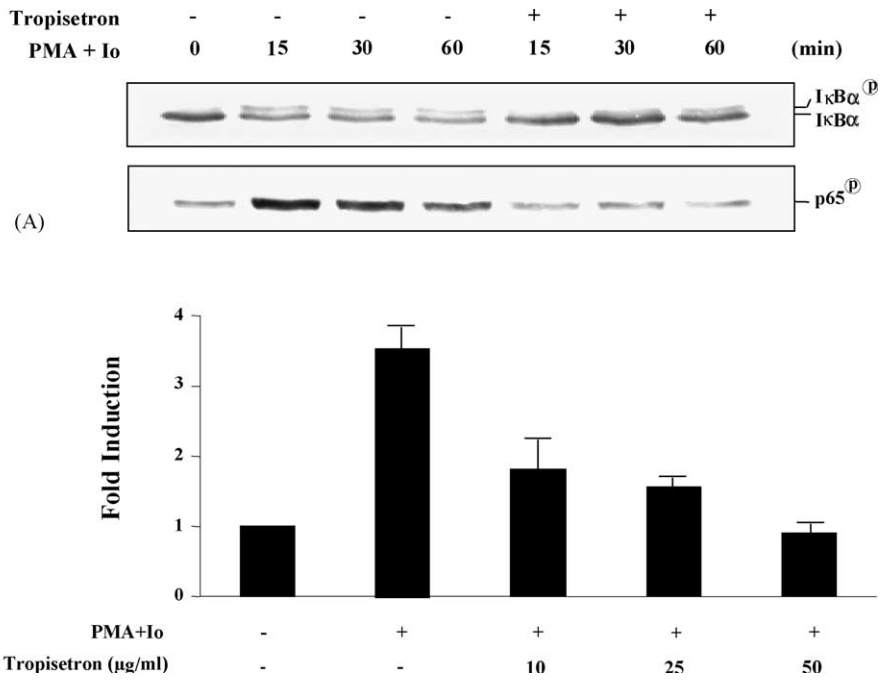

Fig. 6. Effects of tropisetron on NF-κB activation pathway. Jurkat T cells were incubated with tropisetron for 30 min and then treated with PMA and Ion for the indicated times and then tested for IκBα phosphorylation and degradation and p65 phosphorylation (A). Tropisetron inhibits p65 transcriptional activity. Jurkat T cells were transiently cotransfected with the plasmids Gal4-p65 and pGal4-Luc. Twenty-four hours after transfection, the cells were incubated for 30 with increasing concentration of tropisetron, stimulated with PMA plus Io for 6 h and the luciferase activity measured. Results are the means ± S.E. of three different experiments and represented in RLU.

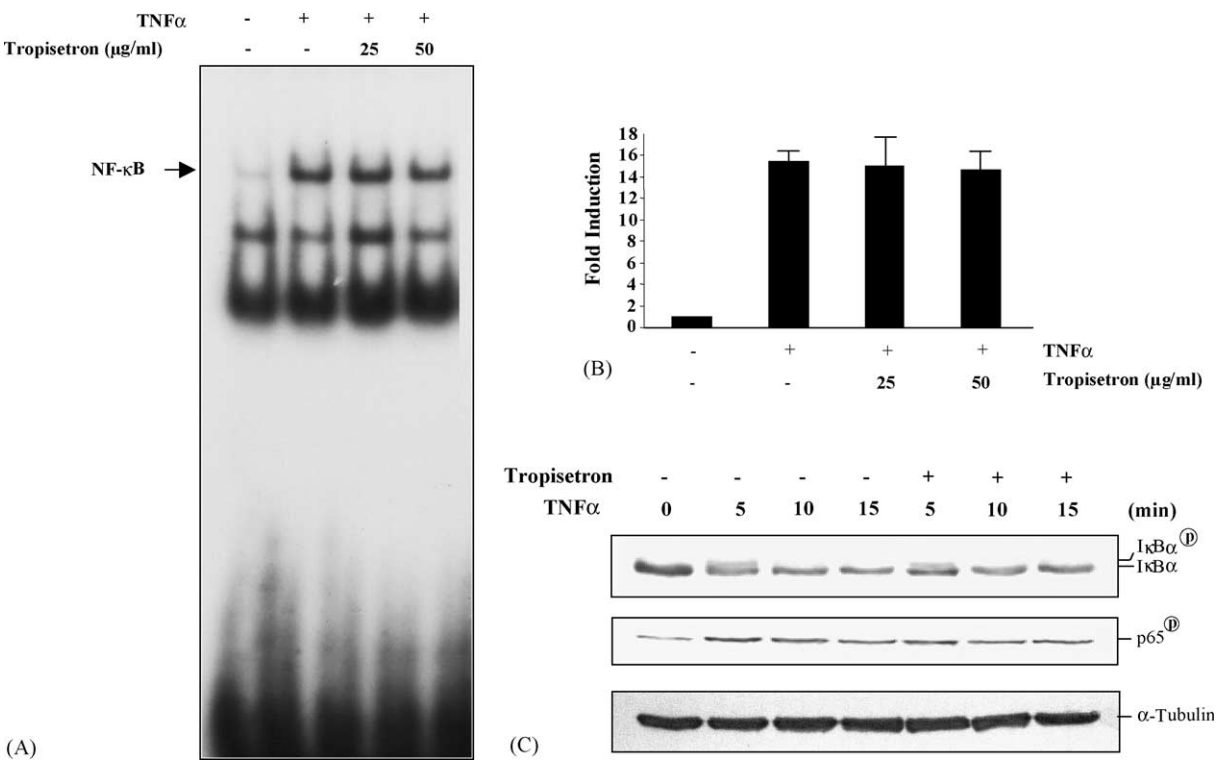

Fig. 7. Tropisetron does not affect the TNFα-induced NF-κB activation pathway. 5.1 cells, either untreated or pre-treated with tropisetron at the indicated doses, were incubated with TNFα for 30 min and the NF-κB/DNA binding activity studied by gel retardation (A). 5.1 cells were pre-treated with different doses of tropisetron and treated with TNFα for 6 h, after which luciferase activity was measured. The results show fold induction ± S.D. over the control ($n = 3$). The relative light units in control cells were 296 ± 83/μg of protein (B). Western blot analysis of IκBα degradation and p65 phosphorylation performed in 5.1 cells pretreated with tropisetron (25 μg/ml) and stimulated with TNFα for the indicated period of time. As a control, the housekeeping protein α-tubulin was analysed (C).

activity, we performed cotransfection experiments using Gal4-p65, a fusion protein between the transactivation domain of p65 (amino acids 286–551) and the DNA binding domain of the yeast Gal4 transactivator, together with a reporter plasmid containing the luciferase gene under the control of a Gal4-responsive element (Gal4-Luc). One of the advantages of this system is that the Gal4 transactivator fusion protein is exclusively nuclear, and thus, is regulated irrespective of IκBs. The results presented in Fig. 6B revealed that transcriptional activity of Gal4-p65 was increased (≅3.5-fold) upon treatment of the cells with PMA plus ionomycin, and this induction was inhibited by the presence of tropisetron in a concentration-dependent manner.

Although the NF-κB activation pathways induced by TCR engaging in T cells and mimicked by PMA plus ionomycin stimulation, are not well understood, the components of the NF-κB activation cascade triggered by TNFα are relatively well characterized and it is known as the canonical pathway of NF-κB activation. To investigate the effect of tropisetron on this NF-κB activation pathway we used the cloned 5.1 cell line, a Jurkat derived clone stably transfected with a plasmid encoding the luciferase gene driven by HIV-1-LTR promoter, which is responsive to TNFα through the NF-κB pathway. In Fig. 7A, it is shown that tropisetron did not affect the NF-κB DNA binding activity in TNFα-stimulated cells. In addition, when the cells were pre-incubated with tropisetron prior to TNFα stimulation we found that this 5-HT_3R antagonist was not able to inhibit the luciferase activity induced by TNFα (up to 16-fold induction over the non-stimulated control cells) (Fig. 7B). To further confirm the lack of effects of tropisetron on the canonical pathway of NF-κB activation in 5.1 cells, we pre-incubated these cells with tropisetron (25 μg/ml) for 15 min and then stimulated with TNFα for the indicated times. Total cell extracts were analysed in parallel for IκBα degradation and p65 phosphorylation and in Fig. 7C, it is shown that TNFα-treatment induced a rapid phosphorylation and degradation of the IκBα as well as the phosphorylation (ser536) of the NF-κB p65 subunit, which were not affected by the presence of tropisetron.

4. Discussion

5-HT is a neurotransmitter widely distributed in the central nervous system. Moreover, 5-HT is released in peripheral tissues by platelets, mast cells and noradrenergic nerve terminals that are in close contact with immune cells in lymphoid organs [30]. Compared to the immense amount of data elucidating the role of 5-HT and 5-HT receptors in the brain, the biological role of 5-HT in cells form the immune system is far to be understood and the pharmacological manipulation of the serotoninegic system should help to better understand its role in the immune response. In this sense, both 5-HT_{1A} and 5HT_3 receptor antagonists have been shown to inhibit 5-HT-induced T cell co-stimulation [6,31]. In this report, we show for the first time that tropisetron, a 5-HT_3R antagonist, is an effective in vitro inhibitor of the signalling pathway leading to the activation of the NFAT, AP-1 and NF-κB transcription factors. As a consequence, tropisetron inhibits IL-2 gene transcription and protein release, IL-2Rα expression and proliferation in antigen-stimulated human T cells.

Although it is not clear that human primary T cells can produce endogenous 5-HT, lymphocytes carrying a serotonine transporter (SERT) may transport 5-HT [32]. Therefore, it is possible that SEB stimulation in primary T cells could induce the release of 5-HT from internal stores that in turn may bind at the 5-HT receptors expressed at the cell surface and then activate the cells in an autocrine manner. In this sense, it has been shown that SEB can induce 5-HT release in rodent mast cells by an unknown mechanism [33]. Another source of 5-HT in our cellular system is provided by the fetal calf serum since it has been shown that fetal serum contains unusually high concentration of 5-HT compared to adult or newborn serum [34]. Serum 5-HT could be co-mitogenic in both antigen-stimulated primary T cells and in PMA plus ionomycin-stimulated Jurkat cells, especially if the 5-HT_3R is previously sensitized. Interestingly, the functionality of this receptor can be modulated by PKC and probably by tyrosine kinases [35,36] and it is possible that antigen or PMA plus ionomycin stimulation can phosphorylate this receptor in T cells rendering them more susceptible to exogenous 5-HT. However, this will imply an exclusive role for the 5-HT_3R in T cell stimulation, a situation that is difficult to reconcile with data showing a key role for 5-HT_{1A} receptor on T cell stimulation [31,37] and with the fact that tropisetron does not affect the functionality of the 5-HT_{1A} receptor.

We have found that tropisetron inhibits early and late events on T cell activation with an IC-50 of approximately 50 μM, which are at least five orders of magnitude higher than would be required to block 5-HT_3R-mediated response. Moreover, both ondasetron and granisetron that antagonized the 5-HT_3R with similar potency than tropisetron showed a different profile on SEB-induced T cell activation (cell proliferation and IL-2 production). Thus, while ondasetron partially inhibited T cell proliferation granisetron was with no effect at the concentrations tested. Due to the structural similarity of the three 5-HT_3R antagonists and because the differential effects on T cell function we believe that the molecular target for the immunosuppressive activity of tropisetron at these concentrations may be independent of its binding to the 5-HT_3receptor.

Thus, our results show that tropisetron strongly inhibits NFAT activation induced by the overexpression of an active form of the phosphatase calcineurin and also inhibits the signalling pathways that activate NF-κB in response to

PMA plus Ionomycin but not to TNFα. This is in agreement with previous reports showing that calcineurin plays a crucial role in the calcium-mediated induction of the NF-κB pathway in T cells [28,29]. Moreover, tropisetron also inhibits the activation of JNK and AP-1 signalling pathways and a cross talking between the calcineurin and the JNK pathways has been also demonstrated [38]. Therefore, our data strongly suggest that the phosphatase calcineurin and/or associated proteins may represent a new molecular target for tropisetron and perhaps for other related analogues such as ondasetron, which also inhibits IL-2 production in SEB-stimulated primary T cells (data not shown). Interestingly, 5-HT3R-independent effects have been described for ondasetron, which blocks potassium channel voltage-gated potassium channels in TE671 human neuroblastoma cells [39].

Our data showing that 5-HT_3R antagonists inhibit IL-2 expression suggest that these compounds may be used as immunomodulatory agents. The well established immunosuppressant compound cyclosporine A has a similar inhibitory profile to tropisetron as demonstrated here. Both compounds target the Ca(2+)/calmodulin-dependent protein phosphatase 2B/calcineurin resulting in the inhibition of NFAT and AP-1 activation and IL-2 production. In contrast to tropisetron, which is well tolerated, the therapy with cyclosporine A is associated with serious toxic side effects. Therefore, tropisetron, as lead compounds, might serve to for development of new compounds with fewer side effects than cyclosporin A. To the best of our knowledge no immunosuppressant activity has been reported in patients using tropisetron for anti-emesis indications. However, we must take into account that tropisetron is commonly used in combination with anti-tumoral chemotherapy that it is immunosuppressive by itself in most of the cases. In addition, the anti-emetic clinical doses of tropisetron did not shown immunomodulatory activity in our ''in vitro'' assays. The relatively high concentrations of tropisetron required for the ''in vitro'' inhibition of T cell activation should not be seen as evidence against its potential use in clinical since this type of compounds are well tolerated at least in local injections at the inflammatory sites and in intravenous bolus. However, further studies are required to validate the anti-inflammatory effects of tropisetron through 5-HT_3 receptor-independent pathways.

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