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The acetaminophen-derived bioactive N-acylphenolamine AM404 inhibits NFAT by targeting nuclear regulatory events

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ABSTRACT

AM404 is a synthetic TRPV1/CB_1 hybrid ligand with inhibitory activity on the anandamide transporter and is used for the pharmacological manipulation of the endocannabinoid system. It has been recently described that acetaminophen is metabolised in the brain to form the bioactive N-acylphenolamine AM404 and therefore, we have evaluated the effect of this metabolite in human T cells, discovering that AM404 is a potent inhibitor of TCR-mediated T-cell activation. Moreover, we found that AM404 specifically inhibited both IL-2 and TNF-α gene transcription and TNF-α synthesis in CD3/CD28-stimulated Jurkat T cells in a FAAH independent way. To further characterize the biochemical inhibitory mechanisms of AM404, we examined the signaling pathways that regulate the activation of the transcription factors NF-κB, NFAT and AP-1 in Jurkat cells. We found that AM404 inhibited both the binding to DNA and the transcriptional activity of endogenous NFAT and the transcriptional activity driven by the over expressed fusion protein Gal4–NFAT (1–415). However, AM404 did not affect early steps in NFAT signaling such as CD3-induced calcium mobilization and NFAT1 dephosphorylation. The NFAT inhibitory activity of AM404 seems to be quite specific since this compound did not interfere with the signaling pathways leading to AP-1 or NF-κB activation. These findings provide new mechanistic insights into the immunological effects of AM404 which in part could explain some of the activities ascribed to the widely used acetaminophen.

1. Introduction

Acetaminophen (paracetamol) is a widely prescribed analgesic and antipyretic agent with a weak anti-inflammatory activity and a low incidence of adverse effect compared to other non-steroideal anti-inflammatory drugs (NSAIDs). For many years, the action mechanism of acetaminophen in pain and fever remained a mystery. It reduces the secretion of prostaglandin (PG) metabolites when administered to humans *in vivo* [1], but it is a very weak inhibitor of PG synthesis mediated by either

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Abbreviations: AEA, anandamide; CB, cannabinoid receptor; COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; JNK, Jun N terminal kinase; NADA, *N*-arachydonoildopamine; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor of *kappa* B; PPAR, peroxisome proliferator-activated receptor; TRPV-1, transient release potential vanilloid receptor type 1

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cyclooxygenase (COX)-1 or COX-2 *in vitro* [2]. However, paracetamol inhibits COX-2 activity in rat microglial cells at therapeutic relevant concentrations [3]. Chandrasekharan et al. have identified the existence of a COX-1 variant named COX-3 that is selectively inhibited by acetaminophen [4]. However, the expression of COX-3 seems to be restricted to the brain and the spinal cord and does not explain many of the peripheral effects of acetaminophen [5]. For instance, the *in vivo* immunosuppressive effects of this atypical NSAID were described a number of years ago [6]. Nevertheless the molecular mechanism underlying these effects is still a matter of intense debate.

It has been recently described that acetaminophen undergoes two-step metabolic transformation in the brain, generating the bioactive *N*-acylphenolamine AM404 [7]. This metabolite interferes with several important molecular targets that are present in the pain and thermoregulatory pathways and provides an interesting link between acetaminophen and the endocannabinoid system [7,8]. This system comprises cannabinoid (CB) and vanilloid receptors (TRPV1), their endogenous ligands (named endocannabinoids), and the enzymes responsible for the biosynthesis and metabolism of these compounds [9]. The manipulation of the endocannabinoid system has therapeutic potential for several conditions including neuropathic pain and neurogenic inflammation [10,11], and research activity has been mainly focused on the development of CB agonists [11], TRPV1 agonists/antagonists [12–14], inhibitors of endocannabinoid inactivation [15,16], and compounds endowed with dual CB1 and TRPV1 activity.

Anandamide (*N*-arachidonoyl ethanolamine) (AEA), the first identified endocannabinoid, acts mainly by signaling through the CB_1/CB_2 receptors and type 1 vanilloid receptors (VR1 or TRPV1) [17,18]. Termination of AEA signalling involves a two-step process which implies transport across the plasma membrane followed by enzymatic hydrolysis by the fatty acid amide hydrolase (FAAH) [15]. The AEA membrane transporter (AMT) has not been identified at the molecular level but chemical inhibitors have been developed and show to have potential therapeutic value [19–21]. AM404 (*N*-[4-hydrophenyl)-eicosa-5,8,11,14-tetraenamide] was designed as a TRPV1/CB_1 hybrid ligand with inhibitory activity on the AMT [19] and different groups have demonstrated that AM404 induces hypothermia and analgesia in animal models probably by increasing the endogenous levels of AEA [22,23]. Over the past few years, there has been a growing awareness that endocannabinoids and certain synthetic analogs exert CB_1- and VR1-independent biological activities [24–26]. For instance, we have previously shown that an endogenous amide, *N*-archidonoyldopamine (NADA) and the synthetic TRPV1/CB_1 hybrid ligand, Arvanil, which show high homology to AM404, are endowed with potent immunosuppressive activities trough TRPV1/CB-independent pathways [27,28].

In T cells, the signal transduction pathways triggered by the activation of the T-cell receptor (TCR)/CD3 complex in combination with a second signal, provided by costimulatory molecules such as CD28, lead to the immediate activation of transcription factors that coordinately regulate a great variety of activation-associated genes [29]. NFAT, NF-κB and AP-1 are probably the three most important transcription factor families in T cells, all of them being activated downstream from TCR engagement. The signal transduction pathways involved in T-cell activation are initiated by the activation of phospholipase C-γ by specific tyrosine kinases at the immunological synapse resulting in the hydrolysis of the phosphatidylinositol 4,5 bisphosphate and the generation of inositol (1,4,5) triphosphate (InsP3) and diacylglycerol (DAG). $InsP_3$ binds to the $InsP_3$ receptor in the membrane of the endoplasmic reticulum (ER), which is the main intracellular Ca^{2+} store, and initiates release of the stored Ca^{2+}. As a consequence of an increase of intracellular Ca^{2+} levels, several signaling pathways are activated [30]. For instance, 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 [31]. NFAT is a family of transcription factors present in cells and tissues both inside and outside of the immune system and is composed of at least four structurally related members: NFAT1, NFAT2, NFAT3, and NFAT4, that are expressed in the cytoplasm of the resting cells as well as the constitutively nuclear NFAT5 member [32]. In the nucleus, NFAT binds to the DNA either alone or in conjunction with AP-1 proteins [33]. In addition to NFAT, TCR activation orchestrates intracellular protein tyrosine phosphorylation events in the immunological synapse leading to the activation of the so called canonical NF-κB activation pathway [34].

In this report we show that AM404, but not paracetamol, inhibits T-cell activation by targeting the NFAT pathway. The inhibitory activity is independent of FAAH activity and cannabinoid receptors.

2. Material and methods

2.1. Cell lines and reagents

Jurkat cells (ATCC, Rockville, MD) were maintained in exponential growth in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 1 mM Hepes and Penicillin/Streptamicin (Invitrogen). The anti-IκBα mAb 10B was a gift from R.T. Hay (St. Andrews, Scotland), the mAb anti-tubulin was purchased from Sigma (St. Louis, MO), and the rabbit polyclonal anti-NFAT1 (672) was a gift from J.M. Redondo (CBM, Madrid, Spain). The anti phospho-ERK 1 + 2 (sc-7383) was from Santa Cruz Biotechnology (Santa Cruz, CA), the mouse monoclonal anti-JNK from BD-Biosciences (Erembodegem, Belgium), the anti-ERK 1 + 2 from Sigma, the anti-phospho-JNK (9255S) and the anti-phospho-p65 (3031S) were from Cell Signaling (Hitchin, UK). Anti-CD3 and anti-CD28 mAbs were purified from ATCC hybridomas (clone OKT3 and clone 15E8). Acetaminophen and ρ-aminophenol were from Sigma. AM404 (*N*-(4-hydroxyphenyl)-arachidonamide), AM1172 (*N*-(5Z, 8Z, 11Z, 14Z eicosatetraenyl)-4-hydroxybenzamide) and URB597 (3′-carbamoyl-biphenyl-3-y-cyclohexylcarbamate) were from Alexis (Lausen, Switzerland). [γ-^{32}P] ATP (3000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). All other reagents not cited above or later were from Sigma.

2.2. Plasmids

The IL-2-Luc (−326 to +45 of the IL-2 promoter) and the TNF-Luc (−1185 pTNFα-Luc) plasmids were previously described

[35,36]. The KBF-Luc contains three copies of the MHC enhancer κB site upstream of the conalbumin promoter followed by the luciferase gene [37]. 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), the NFAT-Luc plasmid contains three copies of the NFAT binding site of the IL-2 promoter fused to the luciferase gene [35]. The plasmids pGal4–NFAT1 (1–415) and pGal4–NFAT1 (1–171) have already been described [38]. The Gal4-Luc reporter plasmid includes five Gal4 DNA-binding sites fused to the luciferase gene.

2.3. Isolation of human peripheral mononuclear cells and T-cell proliferation assays

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) [40]. T cells from PBMC were purified by nylon wool column. Purified T cells were labelled with CFSE (Molecular Probes Cell trace CFSE Cell Proliferation Kit (Invitrogen)) following the manufacturers instructions. Cells were seeded at 10^6 ml^{-1} in RPMI and were stimulated with anti-CD3 (2.5 μg/ml) plus anti-CD28 (1 μg/ml) in the absence or the presence of different AM404 concentrations. After 5 days the cells were analyzed by flow cytometry in an EPIC XL flow cytometer (Coulter, Hialeah, FL). Ten thousand gated events were collected per sample and the fluorescence pattern was determined.

2.4. Ca^{2+} mobilization assay in Jurkat cells

Jurkat cells were incubated in Tyrode's salt solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12.0 mM $NaHCO_3$, 5.6 mM D-glucose) containing 5 μM Indo1-AM (Invitrogen) for 30 min at 37 °C in the dark. Cells were then harvested, washed three times with buffer to remove extracellular Indo1 dye, readjusted to 10^6 cells/ml in Tyrode's salt solution and analyzed in a spectrofluorimeter operated in the ratio mode (Hitachi F-2500 model, Hitachi Ltd.) under continuous stirring and at a constant temperature of 37 °C using a water jacketed device. After 5-min accommodation to equilibrate temperatures, samples were excited at 338 nm and emission was collected at 405 and 485 nm, corresponding to the fluorescence emitted by Ca^{2+}-bound and free Indo-1, respectively. Cells were then treated or not with different AM404 concentrations and then stimulated with α-CD3. $[Ca^{2+}]_i$ was calculated using the ratio values between bound- and free-Indo-1 fluorescence, and assuming an Indo-1 Kd for Ca^{2+} of 0.23 μM. $[Ca^{2+}]_i$ changes are represented as changes in the ratio of bound-to-free calcium (405 nm/485 nm).

2.5. Transient transfections and luciferase assays

Jurkat cells (3×10^6 ml^{-1}) were transiently transfected with the indicated plasmids. The transfections were performed using Lipofectamine™ reagent (Invitrogen) according to the manufacturer's recommendations for 24 h. After incubation with the compounds (AM404, AM1172) for 30 min, transfected cells were stimulated for 6 h as indicated. In experiments using FAAH inhibitor (URB597), this was added 30 min before the compounds addition. 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 (EG&G Berthold, USA) following the instructions of the luciferase assay kit (Promega, Madison, WI). The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation expressed as % induction over α-CD3/α-CD28 was determined. All the experiments were repeated at least four times.

2.6. Western blots

For kinetic studies, Jurkat cells (1×10^6 cells/100 μl) were stimulated with anti-CD3/anti-CD28/Protein-A mixture (4 μg/2 μg/10 μg) for different times in the absence or presence of AM404 at the indicated concentration. For dose–response studies cells (1×10^6 cells/100 μl) were stimulated with anti-CD3/anti-CD28/Protein-A mixture (4 μg/2 μg/10 μg) for 15 min in the absence or presence of AM404/AM1172 at different doses. In both cases cells were then washed with PBS and whole cell extracts (WCE) were obtained by adding 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 NaF, 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 (or 6% SDS/polyacrylamide for NFAT detection). Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 °C) for 30 min. 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, HRP-conjugated secondary antibodies and the ECL system (Amersham Biosciences, Piscataway, NJ).

2.7. Isolation of nuclear extracts and mobility shift assay

Jurkat cells (5×10^6 ml^{-1}) were treated with plastic coated α-CD3 (10 μg/ml) and α-CD28 (2 μg/ml) mAbs for 3 h in the presence or absence of AM404 at different concentrations. Cells were then washed twice with cold PBS and proteins from nuclear extracts were isolated as previously described [39]. 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 site for NFAT or AP-1 were end-labeled with [γ-^{32}P] ATP. The sequences of the oligonucleotides (5′ to 3′) used in EMSAs were gatcGGAGGAAAAACTGTTTCA-TACAGAAGGCGT (distal NFAT site of human IL-2 promoter) and CGCTTGATGAGTCAGCCGGAA (AP-1). The binding reaction mixture contained 5 μg of nuclear extract, 0.5 μg poly(dI-dC) (Amersham Biosciences, Piscataway, NJ), 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. Where indicated, 0.5 μl of rabbit anti-NFAT1 or pre-immune 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 4% gel containing 89 mM Tris-base, 89 mM boric acid and 1 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. Inhibition of CD3/CD28-induced IL-2 transcription by AM404

We have recently shown that the endocannabinoid *N*-arachidonoyl dopamine (NADA) inhibits antigen induced T-cell activation events trough TRPV1/CB-independent pathway [27,39]. Due to the high structural similarity between NADA and AM404, we studied the effects of AM404 and other acethaminophen metabolites on IL-2 gene transcription. Jurkat cells were transfected with a luciferase reporter construct under the control of the human IL-2 gene promoter and 24 h later the cells were stimulated for 6 h with the agonistic α-CD3/α-CD28 mAbs in the absence or in the presence of AM404, arachidonic acid (AA), acetaminophen or *p*-aminophenol at the indicated concentrations. In Fig. 1 it is shown that CD3/CD28-induced IL-2 gene transcription was markedly inhibited in a concentration dependent manner by AM404. However, neither AA, nor *p*-aminophenol nor acetaminophen was found to affect CD3/CD28-induced IL-2 promoter activation at the concentrations tested.

3.2. AM404 inhibitory effect is FAAH independent

AM404 is a potential inhibitor of the putative anandamide transporter (AMT) [19] and it has also been described to be hydrolyzed by the FAAH [41] that is expressed in T cells [25]. Thus, to investigate the potential involvement of FAAH and AMT on the AM404 inhibitory mechanism, we transfected Jurkat cells with either the IL-2-Luc or the TNF-α-Luc plasmids and 24 h later the cells were incubated with AM1172, an AM404 analogue resistant to FAAH degradation [41], prior to CD3/CD28 stimulation. We found that, at the doses tested, AM1172 was as potent as AM404 in inhibiting both IL-2 (Fig. 2A) and TNF-α (Fig. 2B) gene transcription ruling out the possibility that FAAH-generated AM404 metabolites were responsible for the AM404 inhibitory activity. Moreover, preincubation with URB597, a specific FAAH inhibitor [20], clearly enhanced the inhibitory activity of AM404 on CD3/CD28 mediated IL-2 gene transcription (Fig. 2A). URB597 alone slightly increased the luciferase activity driven by the IL-2 promoter in CD3/CD28-stimulated Jurkat cells.

3.3. AM404 inhibits T-cell proliferation and cytokine release in Jurkat T

To correlate the IL-2 inhibitory activity of AM404 with T-cell activation events, we studied the effects of AM404 on T-cell proliferation induced by stimulation with the agonistic α-CD3/α-CD28 mAbs in purified human peripheral T cells. CFSE-loaded primary T cells were stimulated with solid-bound α-CD3 (2.5 μg/ml) and soluble α-CD28 mAb (1 μg/ml) in the presence or the absence of increasing concentration of AM404 and 5 days later the percentages of dividing cells were identified by flow cytometry. The fluorescence intensity of cells stained with CFSE decreases upon mitogenesis and

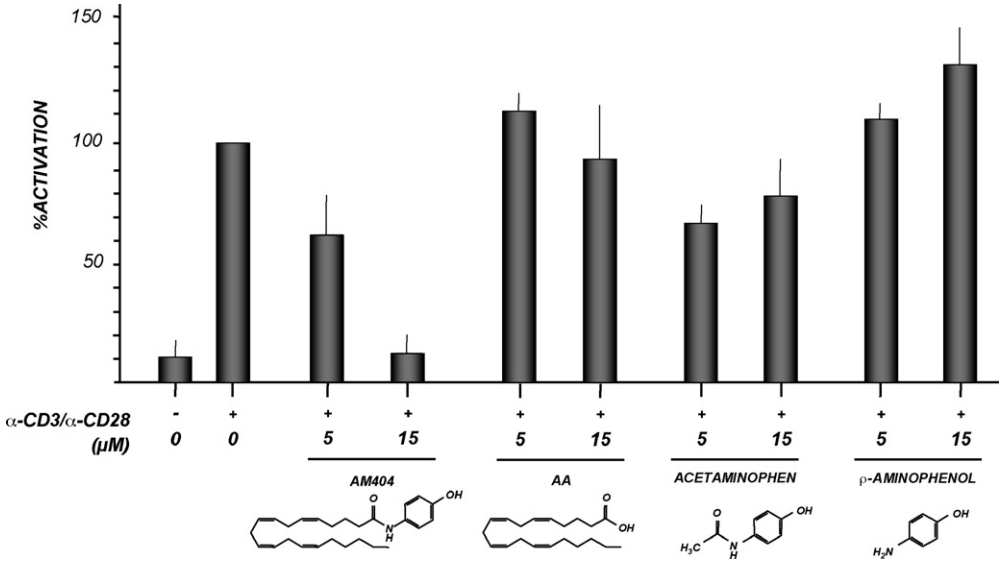

Fig. 1 – Effect of AM404, arachidonic acid, acetaminophen and *p*-aminopenol on IL-2 promoter dependent transcription. Jurkat T cells were transfected with the IL-2 promoter luciferase reporter plasmid as described in Section 2. Twenty-four-hour post-transfection the cells were treated with AM404, arachidonic acid, acetaminophen or *p*-aminophenol and 30 min later α-CD3/α-CD28 was added for additional 6 h. Luciferase activity in the cell lysates was measured and represented as the % of activation (considering α-CD3/α-CD28 as 100% activation).

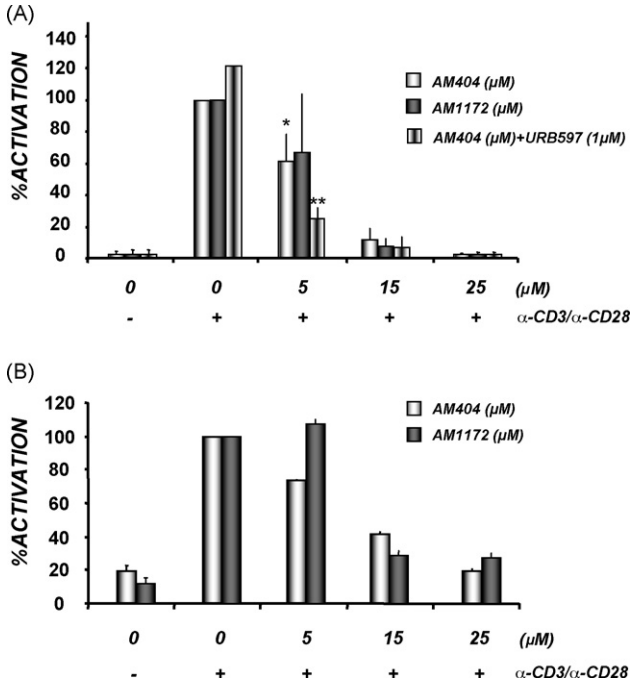

Fig. 2 – The effect of AM404 on IL-2 and TNF-α is FAAH independent. Jurkat T cells transfected with IL-2 (A) or TNF-α (B) promoter luciferase reporter plasmids were treated for 30 min with increasing concentrations of AM404 or AM1172, and then stimulated with α-CD3/α-CD28 for 6 h and luciferase activity measured in the cell lysates. Where indicated, FAAH inhibitor URB597 was added 30 min prior AM404 addition. Results are the mean ± S.D. of three determinations expressed as % activation (considering α-CD3/α-CD28 as 100% activation) ($^{*}P < 0.05$, $^{**}P < 0.001$).

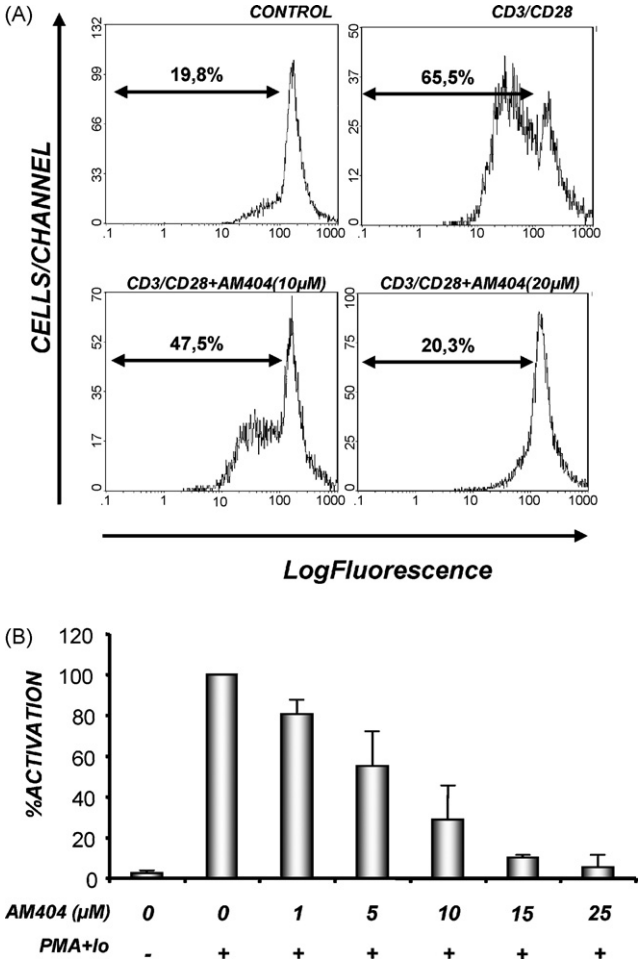

Fig. 3 – AM404 inhibits α-CD3/α-CD28 induced T-cell proliferation and TNF-α release by Jurkat cells. (A) Nylon purified human T cells labelled with CFSE were stimulated with anti-CD3 (2.5 μg/ml) plus anti-CD28 (1 μg/ml) in the absence or the presence of AM404 at different concentrations. After 5 days the cells were analyzed by flow cytometry in an EPIC XL flow cytometer. The percentage of proliferating cells is indicated. (B) Jurkat cells were treated with AM404 at the indicated doses and stimulated with PMA (50 ng/ml) plus ionomycin (1 μM). After the stimulation time, TNF-α in the supernatant was measured by ELISA. The results are the mean ± S.D. of three determinations.

therefore, we found that CD3/CD28-mediated proliferation resulted in a decrease in the percentage of CFSE bright cells (up to 65.5%) that was clearly inhibited by AM404 (Fig. 3A). Next, we were interested in studying the effects of AM404 on cytokine release, and since we found that AM404 inhibits the TNF-α promoter in Jurkat T cells (Fig. 2B), we studied whether or not the inhibition in the transcriptional activity correlated with an inhibition in TNF-α protein release. Jurkat cells were pre-incubated with increasing concentrations of AM404, and then treated with PMA (PKC agonist) and ionomycin (calcium ionophore), a combination that mimics CD3/CD28 stimulation in T cells, for 6 h and the concentration of TNF-α in the supernatants was determined by ELISA. AM404 was found to be a potent inhibitor of TNF-α release that paralleled with its inhibitory activity at the TNF-α promoter (Fig. 3B).

3.4. *AM404 does not affect CD3/CD28-induced NF-κB or AP-1 activation*

Transcriptional activity of both IL-2 and TNFα genes depends on the coordinated activation of several transcription factors including NFAT, NF-κB and AP-1 families. Thus, we evaluated the effect of AM404 on the signaling pathways that activates these transcription factors. The signaling pathways that activate NF-κB and AP-1 include a complex activation of regulatory proteins [33,34]. To investigate the effect of AM404 in these NF-κB and AP-1 regulatory steps we stimulated Jurkat cells with α-CD3/α-CD28 mAbs for different times in the presence or the absence of AM404 (10 μM), and proteins from total cell extracts were analyzed for IκBα levels and p65 phosphorylation status (ser536) by Western blot. The kinetic experiments revealed a clear increase in IκBα phosphorylation and degradation and p65 phosphorylation after 5 min of stimulation that was maintained after 30 min. Neither IκBα phosphorylation and degradation nor p65 phosphorylation was affected by AM404 in CD3/CD28-stimulated Jurkat cells

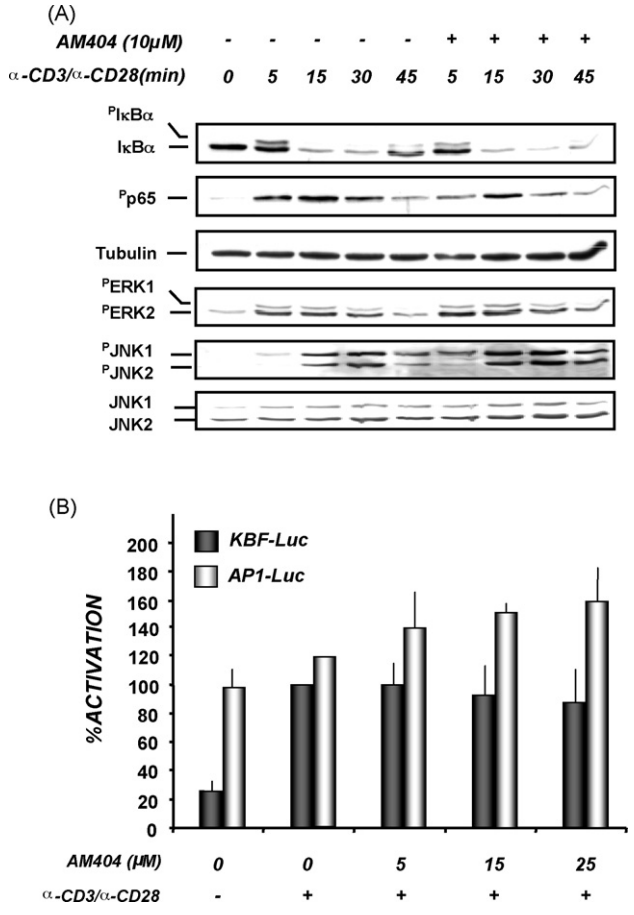

Fig. 4 – Effect of AM404 on α-CD3/α-CD28 induced NF-κB and AP-1 activation. (A) Jurkat cells were pre-treated with AM404 (10 μM) before the addition of α-CD3/α-CD28 for the indicated times and the level of IκBα, phosho-p65 (Ser536), phospho-JNK and phospho-ERK was analyzed by Western blot using specific mAbs. (B) Jurkat T cells were transiently transfected with the luciferase reporter plasmid KBF-Luc or AP-1-Luc, as described in Section 2. Cells were preincubated for 30 min with AM404 at the concentrations indicated, before stimulation with α-CD3/α-CD28 for 6 h. Luciferase activity was measured and the results are the means ± S.D. of three determinations expressed as the percentage of activation (considering CD3/CD28 activation as 100%).

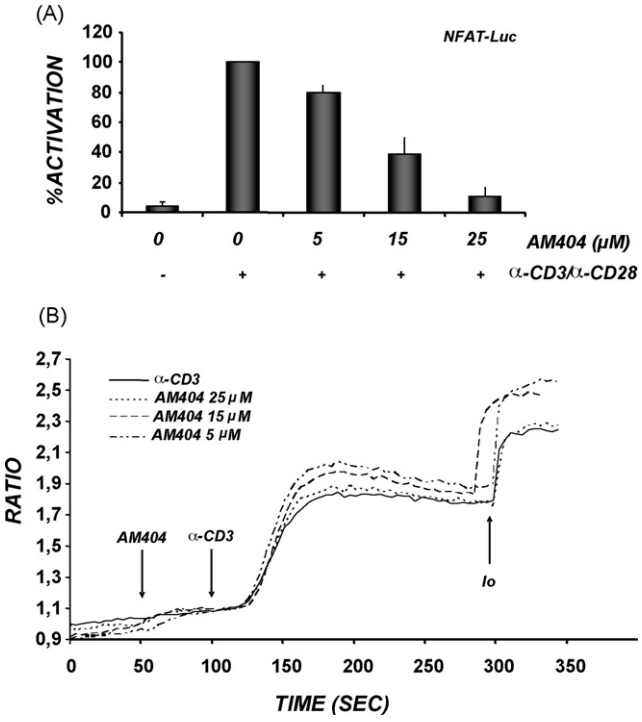

Fig. 5 – AM404 inhibits NFAT dependent transcription in a Ca^{2+} mobilization independent way. (A) Jurkat T cells were transfected with the NFAT-Luc reporter plasmid as described in Section 2. Twenty-four-hour post-transfection the cells were treated with AM404 at the indicated concentrations and after 30 min α-CD3/α-CD28 mAbs were added for additional 6 h. Luciferase activity in the cell lysates was measured and represented as the percentage of activation. (B) Jurkat cells were loaded with Indo1-AM, treated with AM404 at different doses, stimulated with protein A crosslinked OKT3 and the calcium mobilization measured by ratiometric fluorescence as indicated in Section 2. After 4–5 min recording, ionomycin (1 μg/ml) was added to standardize results. $[Ca^{2+}]_i$ changes are presented as changes in the ratio of bound to free calcium (405 nm/485 nm). The calcium traces are representative of at least three independent experiments.

(Fig. 4A). We also found that AM404 did not interfere with the activation of the MAPKs ERK (1 + 2) and JNK that were activated in CD3/CD28-stimulated cells (Fig. 4A). To further confirm that AM404 did not interfere with the NF-κB and AP-1 pathways we transfected Jurkat cells with KBF-Luc and AP-1-Luc plasmids that contain the luciferase gene under the control of minimal promoter containing binding sites for each of each transcription factor. In agreement with previous report [42], CD3/CD28-stimulation of Jurkat cells resulted in activation of luciferase gene expression driven by the NF-κB minimal promoter (nearly five-fold) but not by the AP-1 promoter, which is strongly activated by PMA plus ionomycin (data not shown), and we found that AM404 did not have any effect in CD3/CD28-induced NF-κB-dependent transcriptional activity (Fig. 4B) or in PMA plus ionomycin-induced AP-1-dependent transcription (data not shown).

3.5. *AM404 inhibits NFAT dependent transcription without affecting CD3-induced calcium mobilization*

In addition to NF-κB, the transcription factor NFAT plays a key role in the transcriptional regulation of IL-2 and TNF-α genes among others [43]. Since AM404 did not interfere with the signaling pathways that regulate the activation of both NF-κB and AP-1 transcription factors, we studied the effect of this acetaminophen-derived metabolite on NFAT activation. Firstly, NFAT-luc transfected Jurkat T cells were pretreated with different doses of AM404 and then stimulated with α-CD3/α-CD28 mAbs for 6 h. As shown in Fig. 5A, AM404

efficiently inhibited CD3/CD28-induced NFAT dependent luciferase activity with an IC50 between 5 μM and 15 μM. The distinguishing feature of NFAT is its regulation by calcium and calcium/calmodulin-dependent serine phosphatase calcineurin. Therefore, to investigate the effects of AM404 on TCR-induced Ca^{2+} mobilization, Jurkat T cells were loaded with the indicator dye Indo-1 and treated with increasing concentrations of AM404 for 50 s at 37 °C before CD3 stimulation with the mAb α-CD3 as described in Section 2. As shown in Fig. 5B, CD3 stimulation induced a rapid increase in calcium mobilization that was maintained during the time and was not prevented by the preincubation with AM404 demonstrating that the NFAT inhibitory activity of AM404 is not due to calcium signaling inhibition.

3.6. AM404 inhibits the binding of NFAT to DNA without affecting its de-phosphorylation

In unstimulated cells, phosphorylated NFAT proteins localize in the cytoplasm and as a consequence of an increase of $[Ca^{2+}]_i$ levels calcineurin is activated and subsequently NFAT is dephosphorylated allowing its nuclear shuttling. Therefore, to dissect the mechanism responsible for NFAT inhibition by AM404, we first assessed the kinetics of NFAT dephosphorylation upon treatment with agonistics α-CD3/α-CD28 mAbs in the presence or the absence of AM404. Western blot analysis of cytoplasmic and whole cell extracts from CD3/CD28-stimulated Jurkat cells showed that AM404 partially prevented NFAT1 dephosphorylation in a concentration dependent

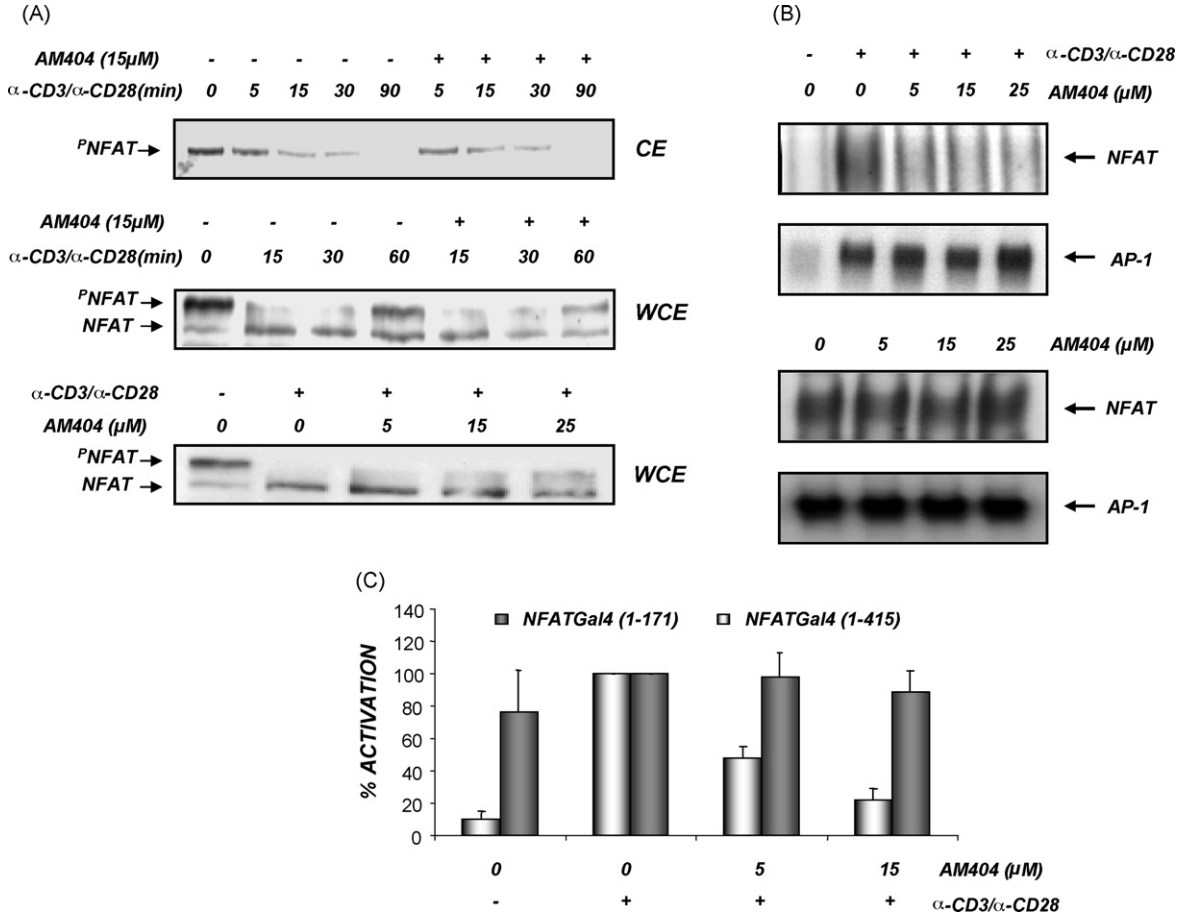

Fig. 6 – Effect of AM404 on NFAT dephosphorylation and NFAT-DNA binding. (A, upper panel) Jurkat cells were pre-treated with AM404 (15 μM) prior stimulation with α-CD3/α-CD28 for the indicated times. Cytoplasmic protein fraction (CE) was extracted and NFAT level was detected by Western blot. (A, middle panel) NFAT phosphorylation state was analysed by Western blot in whole cell extract (WCE) from cells stimulated with α-CD3/α-CD28 mAbs at different times in the absence or the presence of AM404 (15 μM). (A, lower panel) Jurkat cells were pretreated with increasing doses of AM404 and then stimulated with α-CD3/α-CD28 for 15 min. WCE were extracted and Western blot analysis of NFAT phosphorylation was performed. (B) NFAT and AP-1 DNA-binding activities were analysed in nuclear extracts from Jurkat T cells stimulated for 3 h with α-CD3/α-CD28 mAbs in the absence or the presence of increasing concentration of AM404 (upper panels). Nuclear extracts from α-CD3/α-CD28-stimulated cells were incubated in the binding buffer with increasing concentrations of AM404 and then subjected to EMSA for NFAT and AP-1 detection (lower panels). (C) Jurkat cells were cotransfected by lipofection with the Gal4-Luc reporter plasmid together with the expression vectors coding for either the fusion protein pGal4–NFAT1 (1–415) or pGal4–NFAT1 (1–171). After 24 h, cells were pretreated or not with increasing doses of AM404 and further stimulated with α-CD3/α-CD28 for 6 h. Results are represented as the mean ± S.D. of three determinations expressed as the percentage of inhibition.

manner (Fig. 6A). Similar results were found with the stable analog AM1172 (data not shown). Taken together, these results suggest that AM404 target could be located downstream NFAT dephosphorylation and nuclear translocation. To get insights into the mechanism of NFAT inhibition by AM404, we investigated the next step in the activation pathway of this transcription factor by analyzing its binding to the distal NFAT sequence present in the IL-2 promoter. Thus, we performed electrophoretic mobility shift assays with nuclear extracts of Jurkat cells stimulated with α-CD3/α-CD28 mAbs for 6 h in the presence or absence of increasing concentrations of AM404 and we found a major complex that was retarded in α-CD3/α-CD28 treated cells and the binding to DNA of this complex was clearly inhibited in the presence of increasing concentrations of AM404 (Fig. 6B). This complex was characterized as NFAT1 by supershift experiments with an anti-NFAT1 antiserum and by cold competition experiments (data not shown). As expected, we found that AM404 did not interfere with the binding of AP-1 to DNA. To study the possibility that AM404 could interfere with the binding of NFAT to DNA nuclear extracts from α-CD3/α-CD28 stimulated cells were incubated with increasing concentrations of AM404 in the binding buffer and then subjected to gel retardation assays. As depicted in Fig. 6B (lower panels) AM404 did not interfered with the DNA-binding activities of both NFAT and AP1 transcription factors. Next, to explore the inhibitory mechanisms of AM404 on NFAT activation, a Gal4-derived reporter system was employed. Jurkat cells were co-transfected with the chimeric vector pGal4–NFAT1 (1–415), encoding the Gal4 DBD fused to amino acids 1–415 of human NFAT1 along with the reporter plasmid Gal4-Luc. The fusion protein pGal4–NFAT1 (1–415) contains both the calcineurin-binding regulatory and the transactivation domains. As shown in Fig. 6C, AM404 prevented the transactivation function of NFAT1 induced by α-CD3/α-CD28 mAbs in a concentration-dependent manner. As expected, AM404 did not affect the transcriptional activity of the construct pGal4–NFAT1 (1–171) that contains only the transactivation domain of NFAT and confirm our data showing that AM404 did not interfere with the DNA-binding activity of this factor.

4. Discussion

Neurogenic inflammation is a very complex process where the immune system plays a key role. For instance, invasion of lymphocytes into brain parenchyma is a common feature in different brain pathologies including viral encephalitis, multiple sclerosis, stroke and other post-traumatic processes [44]. Thus, the role of the immune system in neurodegenerative diseases is an important area of investigation, and new therapeutic strategies based on immune modulation are under consideration especially for autoimmune disorders such as multiple sclerosis [45]. In this sense, pharmacological modulation of the endocannabinoid system has been shown to alleviate symptoms in rodent models of experimental allergic encephalomyelitis (EAE) [46]. It has been recently shown that AM404 was effective to reduce the magnitude of the neurological impairment in EAE induced both in mouse and in rats [47,48], but the effect of AM404 on the immune system was never investigated. In this report, we extended our studies to analyze the immunosuppressive effects AM404, and we describe for the first time that this compound, but not acetaminophen or *p*-aminophenol, inhibits CD3-induced T-cell activation and the release of pro-inflammatory cytokines such is TNF-α in Jurkat cells.

In our experiments, we found that in CD3/CD28 activated Jurkat cells, AM404 completely suppressed both IL-2 and TNFα gene transcription with an IC50 around 10 μM. Since the brain concentrations of AM404 after paracetamol intake in mice are in the nM order [7], it is difficult to speculate about the physiological relevance of this finding in relation to the pharmacological activity of paracetamol in the brain. However, AM404 is formed in the nervous system trough a FAAH-dependent mechanism and therefore, we could expect that considerable higher amount of AM404 can be produced in brain regions expressing high levels of FAAH and specially in CNS inflammatory focus where also FAAH-expressing immune cells are recruited. In any case, it should be interesting to measure the AM404 in different fluids after paracetamol intake, especially in humans.

Since human peripheral T cells and Jurkat T cells do not express the TRPV-1 receptor (data not shown), and AM404 does not bind the CB2 receptor, the only CB receptor in Jurkat T cell [25,49], it is likely that the inhibitory activity of AM404 on T cells activation is mediated by a novel pathway, which seems to be independent of the vanilloid and cannabinoid receptors identified so far. However, a possible interaction of AM404 with the CB2 at the concentrations tested cannot be formally discarded. One possibility is that AM404 may enter into the cells by a process of simple diffusion and once inside the cells, AM404 interacts with a specific component of the signaling cascade leading to inhibition of NFAT signaling. Interestingly, Rockwell et al., have recently shown that 2-arachidonoyl glycerol (2-AG), which shows structural similarity with AM404, inhibits IL-2 production in splenocytes from CB1/CB2 null mice and NFAT activation in Jurkat cells [26], and they suggested that the inhibitory mechanism of 2-AG was mediated by its interaction with the peroxisome proliferator-activated receptor γ (PPARγ). It has been shown that PPARγ ligands, such is ciglitazone, interact with nuclear NFAT and thus prevents its binding to DNA [50,51]. However, Jurkat cells express low levels of PPARγ [50] and PPARγ ligands also repress NF-κB transcriptional activity which is not the case for AM404. Moreover, we found that AM404 acts as an inverse agonist in Jurkat cells transfected with an expression vector encoding the full cDNA for PPARγ (R. Sancho and F.J. Caballero, unpublished results) and therefore, we postulate that the NFAT inhibitory activity of AM404 may be PPARγ-independent, at least in Jurkat cells.

Stimulation of NFAT does not only involve its nuclear translocation, but also the intrinsic function of the transactivation domain, which is located at the N terminus of NFAT. Here we show that AM404 inhibits both the binding of NFAT to DNA and the transcriptional activity induced by the Gal4–NFAT fusion protein (amino acids 1–415), which contains the strong acidic transactivation domain-A and the whole regulatory domain fused to the Gal4 DNA-binding domain. The pGAL4–NFAT1 fusion protein is constitutively expressed in the nucleus because of the strong nuclear localization signal

at the N terminus of GAL-4 [52]. The N-terminal transactivation domain of NFAT has been found to contain an inducible phosphorylation site required for full transactivation [53]. In addition, this domain has been reported to be phosphorylated in vitro by protein kinase Cζ [54]. Moreover, Cot kinase [55] and PKCζ [54] have been reported to increase NFAT-mediated transactivation in a Cyclosporin A-independent manner. We are currently investigating whether some of the pharmacological properties of AM404 and other endocannabinoids involve Cot kinase or PKCζ in T cells and in other non-T cell types.

Apart from the immune system, NFAT is expressed in other organs and especially in the brain. Activation of NFAT has been proposed to have both beneficial and deleterious effects in the central nervous system depending of the model investigated [32,56,57]. For instance, NFAT activation is involved in methamphetamine-induced neuronal apoptosis by up-regulating Fas ligand [58], and this neuronal apoptotic pathway could be also activated in other neurodegenerative diseases. Recently, it has been shown that NFAT is a key player in the production of TNF-α by neuronal cells [59], and numerous evidences suggest that this cytokine plays an important role in the pathogenesis of inflammatory and neurodegenerative diseases related to memory [60]. In addition, TNF-α plays a role in neuronal apoptosis [61] and the perception of neuropathic pain [62,63]. Therefore, treatment with NFAT inhibitors able to accumulate in the CNS may be of special relevance for the development of novel therapies for certain CNS pathologic conditions. In this sense an important different between AM404 and the analogues Arvanil and NADA is the selectivity to target exclusively the NFAT pathway making this compound more attractive as a lead compound for pharmaceutical development.

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