

## RESEARCH ARTICLE

# Three Arachidonoylamide Derivatives Inhibit Pro-Inflammatory Genes Expression by Modulating NF- $\kappa$ B and AP1 Activities

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**Abstract: Background:** Since the anti-inflammatory activity of arachidonic acid derivatives was previously reported, we synthesized three new amide derivatives of arachidonic acid (AA-Ds) and tested their anti-inflammatory effects on an *in vitro* skin inflammation model. Aim of our study was to find derivatives of natural compounds able to down regulate inflammatory signal transduction pathway.

**Methods:** Human keratinocytes cell line (HaCaT) was cultured and induced by cytokines in the presence of AA-Ds. Cytokines administration elicited an inflammatory response mediated by NF- $\kappa$ B and STAT-1 activation that induced pro-inflammatory genes expression.

**Results:** By real time PCR we found that 24 hours after induction all AA-Ds significantly inhibit inducible Nitric Oxide Synthase (iNOS), TNF $\alpha$ , Inhibitor  $\alpha$  of NF- $\kappa$ B, chemokine (C-X-C motif) ligand 9 and 10 genes expression. We analyzed their molecular effects in particular on the iNOS gene expression. Since iNOS transcript half-life did not change with AA-Ds treatment, we excluded a prominent role of post-transcriptional regulation for this gene and focused our attention on its transcriptional regulation. Starting three-five hours after cytokines induction, HaCaT cells, pre-treated with each compound, showed inhibition of both NF- $\kappa$ B DNA-binding and NF- $\kappa$ B p65-Ser536 phosphorylation. STAT1 activation was inhibited only by AA-D4 derivative. To explain why the inhibition of iNOS expression began late after induction we analyzed activities of others key transcription factors. AA-Ds treatment elicited early increases of AP1 DNA binding as well as c-Jun, c-Fos and Fra-1 mRNA levels. Our data agree with the repressing effects of AP1 on human iNOS promoter previously described in others cell systems (Kleinert *et al.*).

**Conclusion:** AA-Ds shown to be good candidates as inhibitors of several pro-inflammatory genes induction and our study provides indications for their possible use as new anti-inflammatory drugs.



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## INTRODUCTION

Arachidonic acid is a polyunsaturated omega-6 fatty acid (20:4  $\omega$ 6, AA). It is the most abundant fatty acid found in the phospholipids of cell membranes where it is stored [1]. When phospholipase A2 is activated by different inflammatory stimuli, including lipopolysaccharides (LPS), cytokines

and allergens, AA is released into the cytosol and then metabolized by cyclooxygenases and converted into prostaglandins that can activate inflammation [2]. Although AA is the precursor of pro-inflammatory mediators, the exogenous administration of AA may have pro-inflammatory as well as anti-inflammatory effects in a dose dependent manner [3]. Moreover, some arachidonic acid derivatives have various anti-inflammatory effects; in fact, Lopez *et al.* designed and synthesized 2-hydroxy-arachidonic acid (2OAA), which contains a hydroxyl group at the  $\alpha$ -carbon of arachidonic acid (AA) and inhibits the pro-inflammatory pathway [4]. 2OAA inhibits inducible Nitric Oxide Synthase (iNOS) expression and Nitric Oxide (NO) production in microglia cells, decreasing also the production of tumor necrosis factor  $\alpha$

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(TNF $\alpha$ ) [4]. Moreover, anandamide (AEA), the amide of arachidonic acid with ethanolamine, exhibits anti-inflammatory and pro-apoptotic activity [5-6]. Sancho Rocio *et al.* showed that AEA inhibits Nuclear Factor-kappaB (NF- $\kappa$ B) activation [7]. Furthermore, arvanil (*N*-[3-methoxy-4-hydroxy-benzyl]-arachidonoylamide) has a pharmacological profile suitable for the development of potent analgesic agent [8].

The pathological and physiological bases of skin inflammation consist of local cellular and vascular responses which are triggered when the body is injured by exogenous or endogenous insults. Unfortunately, inflammation may be potentially harmful to cells viability, and the goal would be to lead the cells out from the inflammatory process. Our aim is to discover new pharmacologic tools that might be able to switch off the inflammation process by interrupting its signal transduction without the detrimental side effects of the well-known anti-inflammatory drugs.

In skin inflammation, signals derive from the cross-talk between keratinocytes, nervous and the immune cells that leads to the release of mediators, such as interleukins (IL), TNF $\alpha$ , interferons (IFN) and chemokines, which can amplify the inflammatory response and influence its evolution or progression. These inflammatory mediators are able to induce the expression of several target genes, in particular, iNOS. The high amounts of NO produced by iNOS can have beneficial effects if these are time and spatially restricted [9]; in contrast, a persistent iNOS induction may have detrimental consequences and seems to be involved in the pathophysiology of multiple human diseases [10-13]. Therefore, pharmacological suppression of iNOS-dependent NO production may be helpful to shut down the inflammatory process. Regulation of iNOS gene expression is the main regulatory step in controlling iNOS activity [11-12]. The human iNOS promoter shows important regulation by the transcription factors NF- $\kappa$ B, Signal Transducer and Activator of Transcription 1 (STAT1), Activator Protein1 (AP1), and others [11, 14-16]. iNOS gene expression is finely up-regulated by a number of pro-inflammatory cytokines such as IFN $\gamma$ , TNF $\alpha$  and IL1 $\beta$ , together with gram-negative bacterial membrane-derived LPS [17]. NF- $\kappa$ B mediates the signaling of TNF $\alpha$ , IL 1 $\beta$  and LPS, while STAT1 mainly mediates the action of IFN $\gamma$  [17]. Therefore, the modulation of these nuclear factors may be considered as a new strategy in the treatment of several inflammatory diseases.

Our preliminary results show that AA is unstable when used in *in vitro* cell system. Starting from the observation of the anti-inflammatory effects of cellular administration of exogenous AA derivatives [4-8], our purpose was to find new compounds, more stable than AA and easy to use, able to down-modulate inflammation through the inhibition of pro-inflammatory gene expression, in particular iNOS. Therefore, we focused on the synthesis of three amide derivatives of arachidonic acid to see whether these molecules could specifically suppress the induction of several pro-inflammatory genes. Their molecular mechanisms of action were also investigated.

## MATERIALS AND METHODS

### Chemistry

All reagents and anhydrous dichloromethane were obtained from commercial sources and used without further purification. Anhydrous conditions were performed under N<sub>2</sub> atmosphere and all glassware were flame dried. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker AM 250 (250.13 MHz for <sup>1</sup>H, 62.89 MHz for <sup>13</sup>C) and Bruker DRX 300 (300 MHz for <sup>1</sup>H; 75 MHz for <sup>13</sup>C). J values are given in Hz. The <sup>1</sup>H chemical shifts were referenced to the solvent peak, CDCl<sub>3</sub> (7.26 ppm), and the <sup>13</sup>C chemical shifts were referenced to the solvent peak, CDCl<sub>3</sub> (77.0 ppm). ESI(+)-MS measurements were performed on a quadrupole mass spectrometer equipped with electrospray ion source. Elemental analyses were performed on the FlashEA 1112 Series with Thermal Conductivity Detector (Thermo Electron Corporation). Thin-layer chromatography was performed on Macherey-Nagel pre-coated aluminium sheets (0.20 mm, silica gel 60 with fluorescent indicator UV<sub>254</sub>) in appropriate solvent. Column chromatography was carried out using silica gel 60 (70–230 mesh ASTM, Merck).

### Synthesis of AA-D2, AA-D4, AA-D6 Compounds. General Method

A solution of arachidonic acid **1** (100 mg, 0.33 mmol) and DCC (75 mg, 0.36 mmol) in anhydrous dichloromethane (4.2 mL) was stirred at room temperature for 30 min. The appropriate amine **2** (1.0 equivalents, 0.50 mmol) in anhydrous dichloromethane (0.5 mL) was then added. The reaction mixture was further stirred at room temperature for 5h. After that, the solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (8/2 petroleum ether/ ethyl acetate as eluent) to afford the desired products in good yields.

### (5Z, 8Z, 11Z, 14Z)-N-(4-methoxybenzyl)icosa-5,8,11,14-tetraenamide (AA-D2)

Colorless oil (75% yield) <sup>1</sup>H-NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.18 (d, J = 8.5 Hz, 2H), 6.83 (d, J = 8.5 Hz, 2H), 5.81 (bs, 1H), 5.37- 5.33 (m, 8H), 4.33 (d, J = 5.5 Hz, 2H), 3.77 (s, 3H), 2.81- 2.77 (m, 6H), 2.18 (t, J = 7.5 Hz, 2H), 2.15- 2.00 (m, 4 H), 1.77- 1.67 (q, J = 7.5 Hz, 2H), 1.34- 1.24 (m, 6H), 0.87 (t, J = 7.0 Hz, 3H); <sup>13</sup>C-NMR ( 75 MHz, CDCl<sub>3</sub>):  $\delta$  172.5, 158.9, 130.4, 129.1, 128.6, 128.5, 128.1, 127.7, 127.4, 113.9, 55.2, 42.9, 35.9, 31.4, 29.2, 27.1, 26.6, 25.5, 25.4, 22.5, 14.0. MS: m/z 423 (M<sup>+</sup>). Anal. calcd. for C<sub>28</sub>H<sub>41</sub>NO<sub>2</sub>: C, 79.39; H, 9.76; N, 3.31%; found: C, 79.37; H, 9.73; N, 3.29%.

### (5Z, 8Z, 11Z, 14Z)-N-(3,4-dimethoxybenzyl)icosa-5,8,11,14-tetraenamide (AA-D4)

Pale yellow oil (60% yield) <sup>1</sup>H-NMR (250 MHz, CDCl<sub>3</sub>):  $\delta$  6.75 (m, 3H), 5.93 (bs, 1H), 5.40- 5.23 (m, 8H), 4.30 (d, J = 5.8 Hz, 2H), 3.80 (s, 6H), 2.80- 2.74 (m, 6H), 2.17 (t, J = 7.2 Hz, 2H), 2.11- 1.96 (m, 4H), 1.75- 1.63 (q, J = 7.2 Hz, 2H), 1.34- 1.18 (m, 6H), 0.87 (t, J = 6.5 Hz, 3H); <sup>13</sup>C-NMR ( 62.89 MHz, CDCl<sub>3</sub>):  $\delta$  172.6, 149.0, 148.3, 130.9, 130.4, 129.0, 128.7, 128.5, 128.1, 128.0, 127.7, 127.4, 120.0, 111.0, 110.9, 55.7, 43.3, 36.0, 31.4, 29.2, 27.1, 26.6, 25.5, 22.5, 14.0. MS: m/z 453 (M<sup>+</sup>). Anal. calcd. for C<sub>29</sub>H<sub>43</sub>NO<sub>3</sub>: C,

76.78; H, 9.55; N, 3.09%; found: C, 76.76; H, 9.53; N, 3.12%.

**(5Z, 8Z, 11Z, 14Z)-N-(2,4-dimethoxybenzyl)icoso-5,8,11,14-tetraenamide (AA-D6)**

Pale yellow oil (70% yield) <sup>1</sup>H-NMR (250 MHz, CDCl<sub>3</sub>): δ 7.13 (d, J = 7.8 Hz, 1H), 6.40- 6.37 (m, 2H), 6.00- 5.96 (bs, 1H), 5.41- 5.24 (m, 8H), 4.31(d, J = 5.8 Hz, 2H), 3.77 (s, 3H), 3.74 (s, 3H), 2.80- 2.74 (m, 6H), 2.31-1.98 (m, 6H), 1.73- 1.61 (q, J = 7.7 Hz, 2H), 1.34- 1.20 (m, 6H), 0.88 (t, J = 6.5 Hz, 3H); <sup>13</sup>C-NMR ( 62.89 MHz, CDCl<sub>3</sub>): δ 172.2, 160.3, 158.4, 130.4, 130.3, 129.1, 128.5, 128.4, 128.1, 128.0, 127.7, 127.4, 118.8, 103.8, 98.4, 60.2, 55.2, 38.7, 36.0, 31.4, 29.2, 27.1, 26.5, 25.5, 25.4, 22.5, 20.9, 14.0. MS: m/z 453 (M+). Anal. calcd. for C<sub>29</sub>H<sub>43</sub>NO<sub>3</sub>: C, 76.78; H, 9.55; N, 3.09%; found: C, 76.81; H, 9.53; N, 3.07%.

**Human Keratinocytes (HaCaT) Cell Culture and Treatment with AA-Ds**

The immortalized human keratinocytes cell lines (HaCaT) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-invitrogen #61695), containing antibiotic (20 U/ml penicillin G, 20µg/ml streptomycin) and antimycotic (2.5µg/ml Amphotericin B) (Gibco-invitrogen #15240), and supplemented with 10% fetal bovine serum (FBS) (Gibco-invitrogen ). HaCat cells were incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Cells were plated onto 6 cm diameter dishes for real time PCR, or onto 10 cm diameter dishes for Electrophoretic Mobility Shift Assay

centrifuged at 4°C for 10 minutes at 2300×g and the supernatant containing membranes and cytosolic fraction were discarded. Pellets, consisting of cell nuclei, were suspended for 15 minutes at 4°C in 10-15 µl of hypertonic solution (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 25% Glycerol, 0.2 mM EDTA, 0.5 mM DTT) added with protease inhibitor mixture (Roche, Mannheim, Germany). Samples were centrifuged at 25000×g for 30 minutes at 4°C and, finally, the supernatants containing nuclear proteins were kept at -80°C until use.

**Quantification of Protein Concentration**

Protein concentration in the nuclear or whole cell extracts was determined using the Bradford method [19]. The quantity of proteins can be estimated by determining the amount of dye in the blue form and this is achieved by measuring the absorbance of the solution at 595 nm with the double-beam spectrophotometer (Jasco V-650). The absorbance of the sample is then compared to a standard curve generated by the reaction of known amounts of a standard protein Bovine Serum Albumin (BSA).

**Electrophoretic Mobility Shift Assay (EMSA)**

<sup>32</sup>P end-labelled DNA fragment, when binds specifically to a protein, retards its electrophoretic mobility in a non-denaturing polyacrylamide gel electrophoresis, showing discrete bands corresponding to the individual protein-DNA complexes. Double stranded oligonucleotides were purchased from MWG Biotech AG (Germany):

Gene	Sequence	Complementary
STAT1	AGCTTCATTCCCGTAAATCCCTA	TAGGGATTTACGGGAAATGAAGCT
NF-kB	CCTACTGGGGACTCTCCCTTG	CAAAGGGAGAGTCCCCAGTAGG
AP-1	CGCTTGATGAGTCAGCCGGAA	TTCCGGCTGACTCATCAAGCG
Oct-1	GGGAACAGTTATGCAAAATAG	CTATTTTGCATAACTGTTCCC

(EMSA) as well as for Western Blot analysis. At 70% confluence cell medium was replaced with serum free DMEM and cells were cultured under these condition for 24 hrs. Two hours before the induction, different concentrations of Arachidonic acid amide derivatives (AA-Ds) were added to the cell culture. The pre-treated cells were induced with pro-inflammatory cytokines TNF-α (10ng/ml) and IFN-γ (10ng/ml) (PeproTech). At the end of treatments, HaCaT cells were washed in phosphate saline buffer (PBS, BioWhittaker, Lonza, Bergamo, Italy) at 4°C, harvested by scraping, centrifuged for 5 minutes at 1500×g, and the pellets were stored at -80°C until use.

**Total and Nuclear Protein Extracts**

Nuclear extracts were prepared from cell cultures as previously described [18]. Briefly, cellular pellets were kept for 10 minutes at 4°C in 40 µl of hypotonic solution (10 mM HEPES pH 7.9, 0.1% Nonidet-P40, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT) containing a detergent in order to break down the membrane phospholipids. The buffer solution was added with complete protease inhibitor mixture EDTA-free (Roche, Mannheim, Germany). Lysed cells were

The lyophilized oligonucleotides were solubilised in Tris-EDTA (100 pmol/µl) and 5 pmol of forward and reverse oligonucleotides were annealed at 95°C in 10 mM Tris HCl pH 8.0, 1 mM EDTA and 150 mM NaCl. Then they were radio-labelled with [γ-<sup>32</sup>P]ATP (PerkinElmer, Monza, Italy) by using T4 Polynucleotide Kinase (PNK, USB Corporation, Ohio, USA) at 37°C for 30 minutes The <sup>32</sup>P end-labelled oligonucleotides were purified from the precursors <sup>32</sup>P ATP by Sephadex G-50 column (GE Healthcare, Milan, Italy).

4 µg of nuclear extract were incubated at room temperature for 20 minutes with 5 x 10<sup>4</sup> cpm of one <sup>32</sup>P-labelled double stranded oligonucleotide, in 15 µl of a reaction mixture containing 20 mM HEPES pH 7.9, 50 mM KCl, 10% glycerol, 0.5 mM DTT, 0.1 mM EDTA and 1 µl of 1 µg/µl poly(dI-dC). Samples were fractioned by electrophoresis on a non-denaturing 5% polyacrylamide gel in 0.5X TBE (50 mM Tris base, 50 mM boric acid, 1 mM EDTA). The gel was subjected to a previous run for 30 minutes at 100 V and electrophoresis was carried out for 2.5 hours at 140 V. At the end of the electrophoresis the gel was dried by gel dryer (BioRad, Hercules, CA, USA) for 40 minutes and then ex-

posed to an auto radiographic film (Hyperfilm MP, GE Healthcare, Milan, Italy).

### Western Blotting

Nuclear extracts (20-30 µg/sample) were added with reducing buffer (Sample Buffer, 1 M Tris HCl pH 6.8, 20% SDS, 5% β-mercaptoethanol), boiled for 3 minutes and the immediately put and kept on ice for 3 minutes.

The samples were fractionated by SDS-PAGE in a 7.5-10-12% gel and a marker of known molecular weight (SharpMass VI, EuroClone SpA) was used. The electrophoresis was performed in running buffer (25 mM Tris HCl pH 8.3, 192 mM glycine, 0.1% SDS) at 15 mA until the stacking gel was passed, and at 25 mA in the running gel.

Protein were electro-blotted onto PVDF membrane (Immobilion P, Millipore, Milan, Italy) for 1 hour at 350 mA on ice in the transfer solution (25 mM Tris Base pH 8.3, 192 mM glycine, 10% methanol) using a Bio-Rad Mini Protean II™ system (Bio-Rad, Segrate, Italy).

The membrane was incubated with a blocking solution (5% BSA or 4% non-fat dried milk, 10 mM Tris HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature under stirring and overnight at 4°C with the addition of a primary antibody. We used the following primary antibodies: anti-STAT1 phospho-Tyr701 (#9177; Cell Signaling Technology, Milan, Italy) diluted 1:1000 in blocking 5% BSA solution, anti-STAT1 phospho-Ser727 (#06-802, Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:1000 in blocking 5% BSA solution, anti-STAT1 p84/p91 (sc-346X; Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:1000 in blocking 4% milk solution, anti-p65-NF-κB phospho-Ser536 (#3033; Cell Signaling Technology, Milan, Italy) diluted 1:1000 in blocking 5% BSA solution, anti-p65-NF-κB (#3987; Cell Signaling Technology, Milan, Italy) diluted 1:1000 in blocking 4% milk solution, anti βactin antibody diluted 1:1000 in blocking 4% milk solution (Santa Cruz Biotechnology, Heidelberg, Germany)

The membrane was washed three times for 5 minutes with washing solution (10 mM Tris HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) and incubated with a secondary antibody anti-rabbit IgG-peroxidase conjugated (Cell Signaling Technology, Milan, Italy) diluted 1:2000 in blocking solution or anti-mouse IgG horseradish peroxidase linked (GE Healthcare, Milan, Italy) diluted 1:1000, for 1 hour at room temperature under agitation. Membrane was washed again three times for 10 minutes to wash-out any excess of the antibody.

The immunoreactive proteins on the blot were detected using Immun-Star™ WesternC™ Chemiluminescent Kit (Bio-Rad, Segrate, Italy), an enhanced chemiluminescence detection system, by ChemiDoc XRS (Bio-Rad, Segrate, Italy).

### RNA Extraction

For total RNA extraction, HaCaT cells were cultured in 6 cm diameter plates and then processed using the Pure-Link® RNA Mini Kit (Ambion Life technologies, Milan, It-

aly). Cells were first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate and 1% 2-mercaptoethanol containing buffer. Ethanol was added and the sample was applied to an RNeasy Mini spin column, where the total RNA binds to the membrane. High quality RNA was then eluted in RNase-free water. Then, we used the Turbo DNA-free™ kit (Ambion Life technologies, Milan, Italy) to remove contaminating DNA: samples were added with Buffer DNase and DNase I and incubated at 37°C for 30 minutes, then centrifuged at 10.000g for 2 minutes. The RNA was then transferred into a fresh tube for the concentration and purification step. Precipitation was performed with ethanol/Na Acetate solution. After -20°C storage and centrifugation, the pellet, containing total RNA, were re-suspended in RNase-free water. Quantification of purified RNA was determined by measuring the absorbance at 260 nm with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Milan, Italy).

To perform real-time PCR, RNA was first reverse transcribed into cDNA in a reverse transcription reaction. For generating single-strand cDNA, SuperScript® VILO cDNA Synthesis Kit (Invitrogen, Milan, Italy) was used as previously reported [20]. The reverse transcription was performed with Rotor-Gene™ 6000 thermocycler (Corbett Research, Cambridge, UK), using the following program: 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 85°C. We diluted the cDNA in RNase-free water to have 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> solution samples.

### Real-time PCR

Real-time PCR was performed with a QuantiTect SYBR Green PCR Kit (Qiagen, Milan, Italy), SensiFAST SYBER No-ROX Kit (Bioline, Milan, Italy), a Rotor-Gene™ Q thermocycler (Qiagen, Milan, Italy) and PowerSYBR Green PCR Master Mix (Applied biosystems, Warrington, UK), and a 7900HT Fast Real-Time PCR System (Applied biosystems, Warrington, UK) **using listed in Table 1 human primers:**

The expression data of GAPDH and 18S were used as internal control for data normalization.

### Statistical Analysis

Data are expressed as mean ± SD of at least three independent experiments. Statistical analysis was performed by one-way analysis of variance. (ANOVA). If statistical significance was reached for ANOVA (P < 0,05), a Turkey-Kramer post hoc test was applied.

## RESULTS

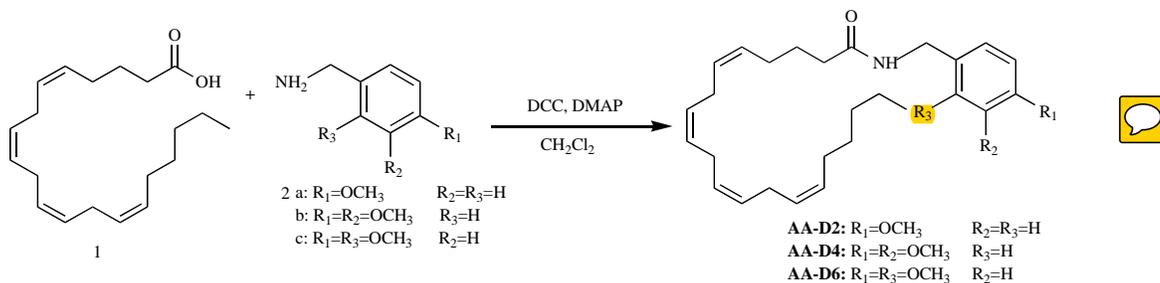
### Synthesis of AA-derivatives

The synthesis of amides AA-D2, AA-D4 and AA-D6 is reported in Scheme 1. The title compounds were prepared from commercially available arachidonic acid **1** by direct condensation between **1** and the corresponding amine **2** in the presence of DCC. The coupling reactions were carried out in dichloromethane to give the title compounds in 60-75% yield after column chromatography. The structure of the compounds was deduced on the basis of their spectral data.



Table 1. Primer sequences used in real-time PCR.

Name Gene	Forward	Reverse
IKBalpha	GCTATTCTCCCTACCAGCTC	CAGTCATCATAGGGCAGCTC
TNFalpha	GCCCTGGTATGAGCCCATCT	TAGTCGGGCCGATTGATCTC
CIITA	CCGACACAGACACCATCAAC	CTTTTCTGCCAACTTCTGC
iNOS	CAGCGGGATGACTTTCCAA	AGGCAAGATTTGGACCTGCA
ICAM1	GCTCTGCAACCCTGGAGGT	GGGCCATACAGGACACGAAG
CXCL9	GTGAGAAAGGGTCGCTGTTC	AATTTTCTCGCAGGAAGGGC
CXCL10	GCAAGCCAATTTTGTCCACG	TGATGGCCTTCGATTCTGGA
c-Jun	AGGAAGCTGGAGAGAATCGC	CTGTTCCCTGAGCATGTTGG
c-Fos	AGTGGAACCTGTCAAGAGCA	ATCAAAGGGCTCGGTCTTCA
Fra-1	GACCTACCCTCAGTACAGCC	CGGGCTGATCTGTTCACAAG
Fra-2	CTGTCATCAAGCCCATCAGC	CCAGGACGCTAGGATAGGTG
ATF-2	CAGTACAGTCAGAAGCAAAAATGAG	CGCTACCATGACCTTTGACA
FOSB	TTTCCCCGGAGACTACGAC	CCACCGAAGACAGATATTGAGAC
18S	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
GAPDH	AACGGATTTGGTCGTATTGGGC	TCGCTCCTGGAAGATGGTGATG



Scheme 1. General synthetic scheme for title compounds.

### Inhibition of iNOS Gene Expression

In HaCaT cells, we analyzed the inhibitory effects on iNOS gene expression by different concentrations (30, 60 and 100  $\mu$ M) of AA and AA-Ds. HaCaT cells were pre-treated with AA or AA-Ds for 2 hours and stimulated with the indicated pro-inflammatory cytokines for 24 hours. At the end of treatment, the amounts of iNOS mRNA were determined by real time PCR. Data demonstrated a dose-dependent inhibition of iNOS gene induction after 24 hours treatments with all compounds (Fig. 1). It is worth noting that AA activity was similar to that of AA derivatives only if AA was used immediately after opening the vial. If instead it was used after several hours, its inhibitory activity decreased considerably (data not shown).

### Half-life of iNOS Transcript

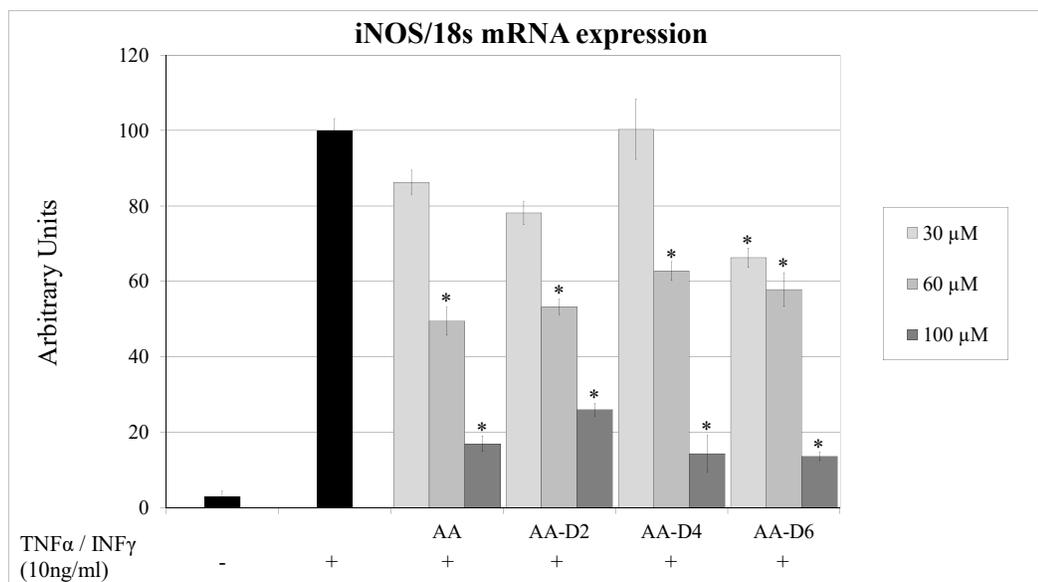
To verify whether the decrease of iNOS mRNA levels elicited by AA-Ds could depend on post-transcriptional regulation, we performed experiments utilizing the transcriptional inhibitor actinomycin D. To measure iNOS transcript half-life, HaCaT cells were grown to 70% confluence and treated with or without 100  $\mu$ M AA-D4 for 2 hours. Then, cell samples were induced with pro-inflammatory cytokines

for 6 hours. At this time, actinomycin D (5  $\mu$ g/ml) was added to both treated with AA-Ds and non-treated cells to stop *de novo* RNA synthesis. Total RNA was extracted from cell samples at 0, 1, 3, 4, 5, 6 hours after actinomycin D addition. iNOS and GAPDH mRNA levels were measured by real time-PCR. As shown in (Fig. 2), the iNOS mRNA half-life was not modified by AA-D4 treatment. Similar results were obtained using AA-D2 or 6 (data not shown).

### DNA Binding Activity of NF- $\kappa$ B and STAT1

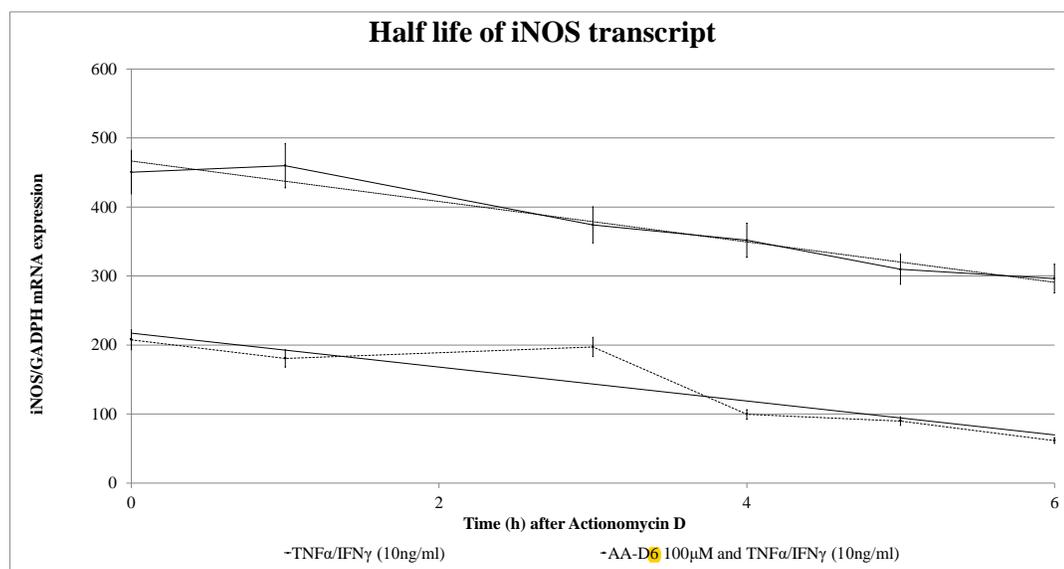
Having previously excluded the possibility that iNOS gene expression was regulated at the post-transcriptional level, we focused our attention on iNOS transcriptional gene regulation. As reported in the literature [21], several transcription factors, such as NF- $\kappa$ B, STAT1, AP-1, Oct-1, have been shown to be important in iNOS gene induction. First, we decided to test by EMSA, in a time course experiment, whether AA-Ds could inhibit NF- $\kappa$ B and/or STAT1 transcription factors DNA binding activity.

EMSA data showed a significant decrease of NF- $\kappa$ B retarded bands in cells treated with 100  $\mu$ M of AA-D2, AA-D4, AA-D6 in respect of not treated cells (Fig. 3). It is worth noting that the inhibition of NF- $\kappa$ B activity with AA-D4 and AA-D6 treatments was shown to occur at later times, start-



**Fig. (1).** *iNOS* mRNA levels after treatment with AA-Ds.

Total cellular RNA was isolated and analysed by real time PCR. HaCaT cells were pre-treated with AA or AA-Ds (30, 60 and 100  $\mu$ M) for 2 hours. After that, cells induction was obtained with the pro-inflammatory cytokines TNF $\alpha$  & IFN $\gamma$ . The level of 18S ribosomal RNA was used for data normalization. \*Significant difference for pre-treated and induced vs induced cells;  $p \leq 0.05$ . Black columns show not pre-treated cells.



**Fig. (2).** *Half-life of iNOS* transcript.

Total RNA was extracted and analysed for iNOS mRNA expression by real time PCR. The level of GAPDH mRNA was used to normalize the data. 6 hours after induction by cytokines to both induced and pre-treated cells the transcriptional inhibitor Actinomycin D was added. The decrease of iNOS mRNA relative levels for induced and pre-treated cells are reported as fold mRNA expression over the basal level of not induced cells. Values are the means ( $\pm$ SD) of three independent experiments.

ing, respectively, 3 hours and 5 hours after cell induction (Fig. 3).

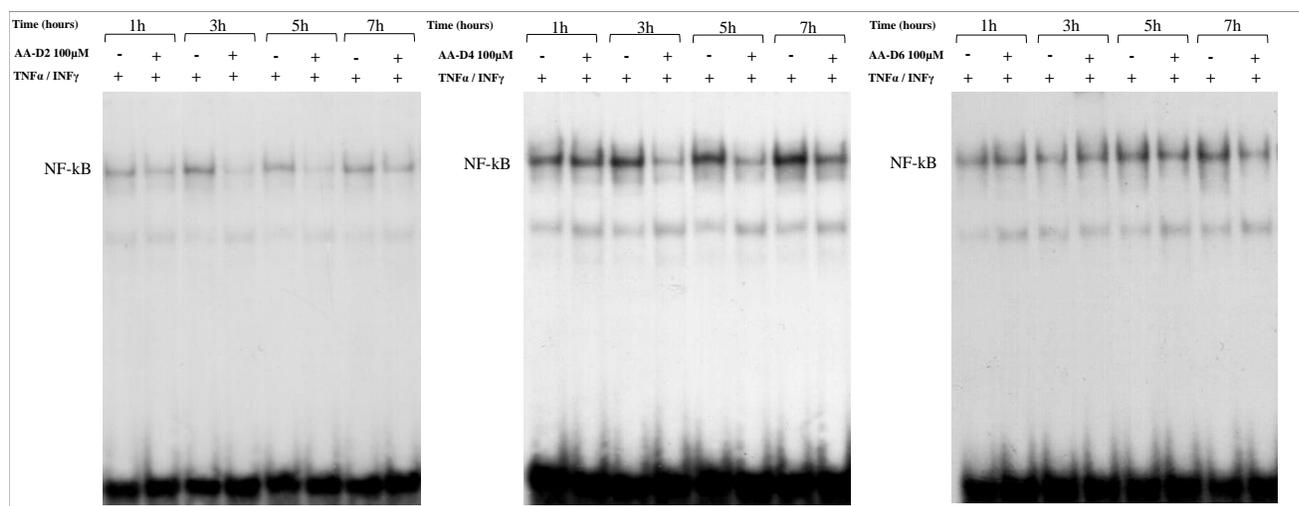
EMSA data did not show a significant decrease of STAT1 retarded bands in cells treated with 100  $\mu$ M of AA-D2 and AA-D6 in respect of not treated cells, whereas, a faint inhibition of DNA binding activity could be seen starting three hours after AA-D4 treatment (Fig. 4).

#### STAT1 and NF- $\kappa$ B Phosphorylation

The association of the NF- $\kappa$ B with its inhibitor I $\kappa$ B $\alpha$  plays a pivotal role in regulating nuclear translocation and

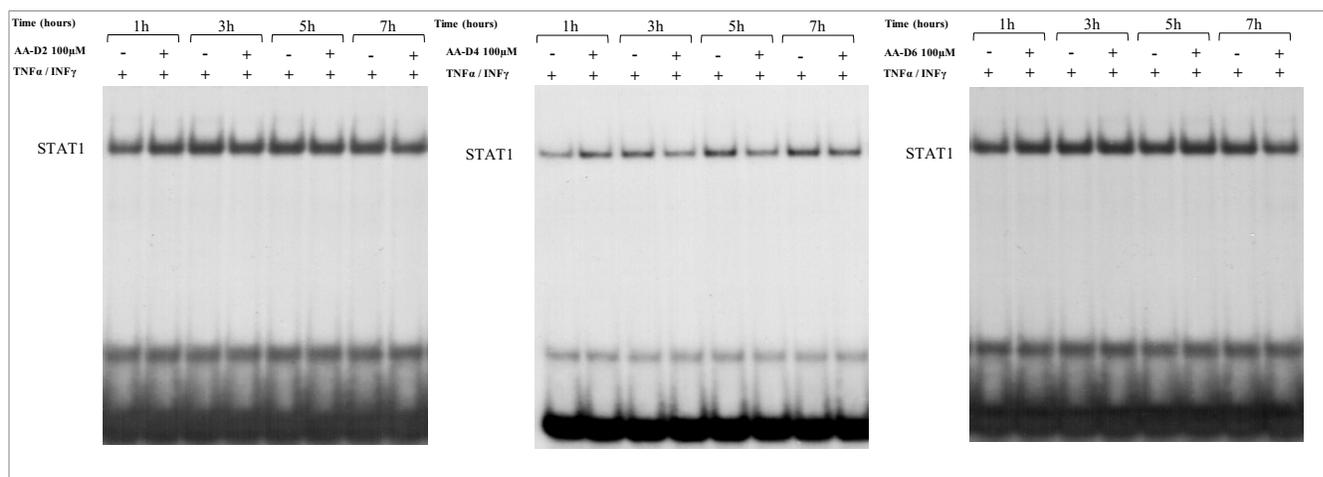
gene transcription; moreover, serine phosphorylation at various sites of the p65 subunit of NF- $\kappa$ B has been shown to be important for initiating transcription [22]. The transcriptional activity of p65, *i.e.*, phosphorylation at serine 536, has a distinct set of target genes in respect of non-phosphorylated protein. It may represent a non-canonical NF- $\kappa$ B activation pathway independent of I $\kappa$ B $\alpha$  regulation [22].

Thus, the level of p65 phosphorylation at serine 536 residue can help us to understand whether or not a non-canonical pathway of NF- $\kappa$ B is also involved in it.



**Fig. (3).** DNA binding of NF- $\kappa$ B transcription factor on consensus motifs of iNOS promoter.

4  $\mu$ g of nuclear protein extracts of the different treated-cells were incubated with a  $^{32}$ P-labeled double-stranded oligonucleotide containing binding sequence for NF- $\kappa$ B, isolated by non-denaturing polyacrylamide gel electrophoresis and exposed to an auto radiographic film. Each of these gels represents three independent experiments.



**Fig. (4).** DNA binding of STAT1 transcription factor.

4  $\mu$ g of nuclear protein extracts of the various treated-cells were incubated with a  $^{32}$ P-labeled double stranded oligonucleotide containing binding sequence GAS, which was isolated by non-denaturing polyacrylamide gel electrophoresis and exposed to an auto radiographic film. Each of these gels represents three independent experiments.

The results showed that a significant inhibition of p65 phosphorylation at Ser536 occurs with AA-D4 treatment at all times analyzed, while it can be observed only at later times with AA-D6 and AA-D2 treatments (Fig. 5 and data not shown). It is worth noting that only in the case of the AA-D4 treatment the total amount of p65 also decreases. Nevertheless, densitometric analysis of the bands and the subsequent results obtained from normalization of the phosphorylated bands versus those of total p65 maintain statistical significance (data not shown). These results confirm the EMSA data and suggest a broad inhibition of NF- $\kappa$ B transcriptional activity with all AA-Ds studied.

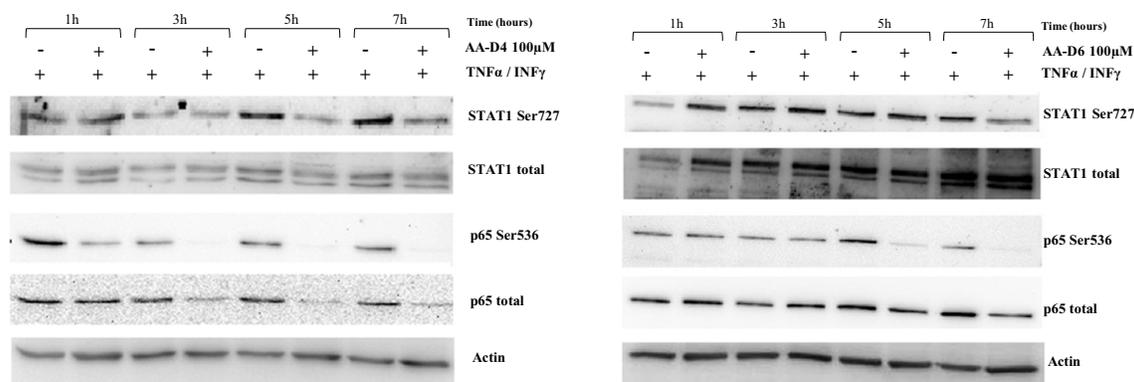
In the IFN $\gamma$ /STAT1 pathway, STAT1 activation occurs after the phosphorylation of STAT1 proteins by Janus Kinases (JAKs). Phosphorylation in Tyr701 of the two STAT1 monomers is the necessary and sufficient condition for dimerization, nuclear translocation and DNA binding [23]; [24]. Although phosphorylation in Ser727 of STAT1 is

not necessary to DNA binding, it is very important for maximal transcriptional activity [25]. Thus, western blot analysis was performed with nuclear extracts, using rabbit polyclonal antibody that recognizes STAT1 phosphorylated Ser727.

As far as the modulation of STAT1 transcription factor activity is concerned, the inhibition of STAT1 Ser 727 phosphorylation as well as STAT1 binding appear to be peculiar characteristics of the AA-D4 treatment, although a faint inhibition of Ser727 STAT1 phosphorylation occurring 7 hours after induction was also observed with the AA-D6 compound (Fig. 5 and data not shown).

#### *The Inhibitory Effect of AA-Ds on the Expression of Several Pro-inflammatory Genes*

In order to verify whether the inhibitory effect of AA-Ds is restricted to iNOS gene expression or might concern other pro-inflammatory targets, we investigated the effect of 100



**Fig. (5).** Effect of AA-Ds on Ser727 phosphorylation of STAT1 and Ser536 phosphorylation of p65-NF- $\kappa$ B in HaCaT cells induced with TNF $\alpha$  and IFN $\gamma$ .

Nuclear extracts of pre-treated cells were isolated by denaturing SDS-PAGE. 12  $\mu$ g of them were electroblotted onto PVDF membrane and probed with antibodies for phosphorylated STAT1 Ser727, STAT1 total, and antibodies for phosphorylated p65 NF- $\kappa$ B Ser536, p65 total. Data are shown in a time course manner until 7 hours induction. As for AA-D4 and AA-D6 treatments, the gels are representative of three independent experiments each (Data do not shown for AA-D2).

$\mu$ M of AA-Ds on iNOS expression and compared it with the expression levels of TNF $\alpha$ , class II major histocompatibility complex transactivator (CIITA), I $\kappa$ B $\alpha$ , chemokine (C-X-C motif) ligand 9 (CXCL9), chemokine (C-X-C motif) ligand 10 (CXCL10) genes in a time dependent manner, from 1 to 24 hours after the induction of cells with cytokines. Total RNA was purified from HaCaT cells and the amount of specific mRNA was quantified by Real-Time PCR, as described in the Materials and Methods section.

Our results have shown that pre-treatment with AA-Ds decreased the mRNA levels of several pro-inflammatory genes (Fig. 6, a-f). While real time PCR data showed that 100  $\mu$ M AA-D4 was able to decrease all the pro-inflammatory genes mRNA levels studied by us, AA-D2 and AA-D6 tested at the same dose were not able to inhibit CIITA gene (data not shown). The time course experiment demonstrated that all these inhibitions are delayed since they do not start before 4 hours after the induction of cytokines.

#### Potential Mechanisms of the AA-Ds Inhibitory Effect on iNOS Gene Expression

Kleinert H. *et al.* [12,15] showed that the human iNOS promoter holds more than 15 transcription factors regulating its activity, the most important of which are: STAT1, NF- $\kappa$ B, Oct-1, AP-1.

The results obtained suggest that there were no differences of *in vitro* DNA-binding activity of Oct-1 transcription factor between cells treated or non-treated with AA-Ds (data not shown).

On the contrary, preliminary experiments showed that pre-treatments with AA-Ds increased the DNA-binding activity of transcription factor AP-1 (data not shown). Thus, we performed time course experiments until 7 hours. EMSA results of AA-D4 treated cells showed that the retarded bands increased at all times, but in particular 1 hour after cytokines induction (Fig. 7). Similar results were obtained for AA-D2 and AA-D6 treatments (data not shown).

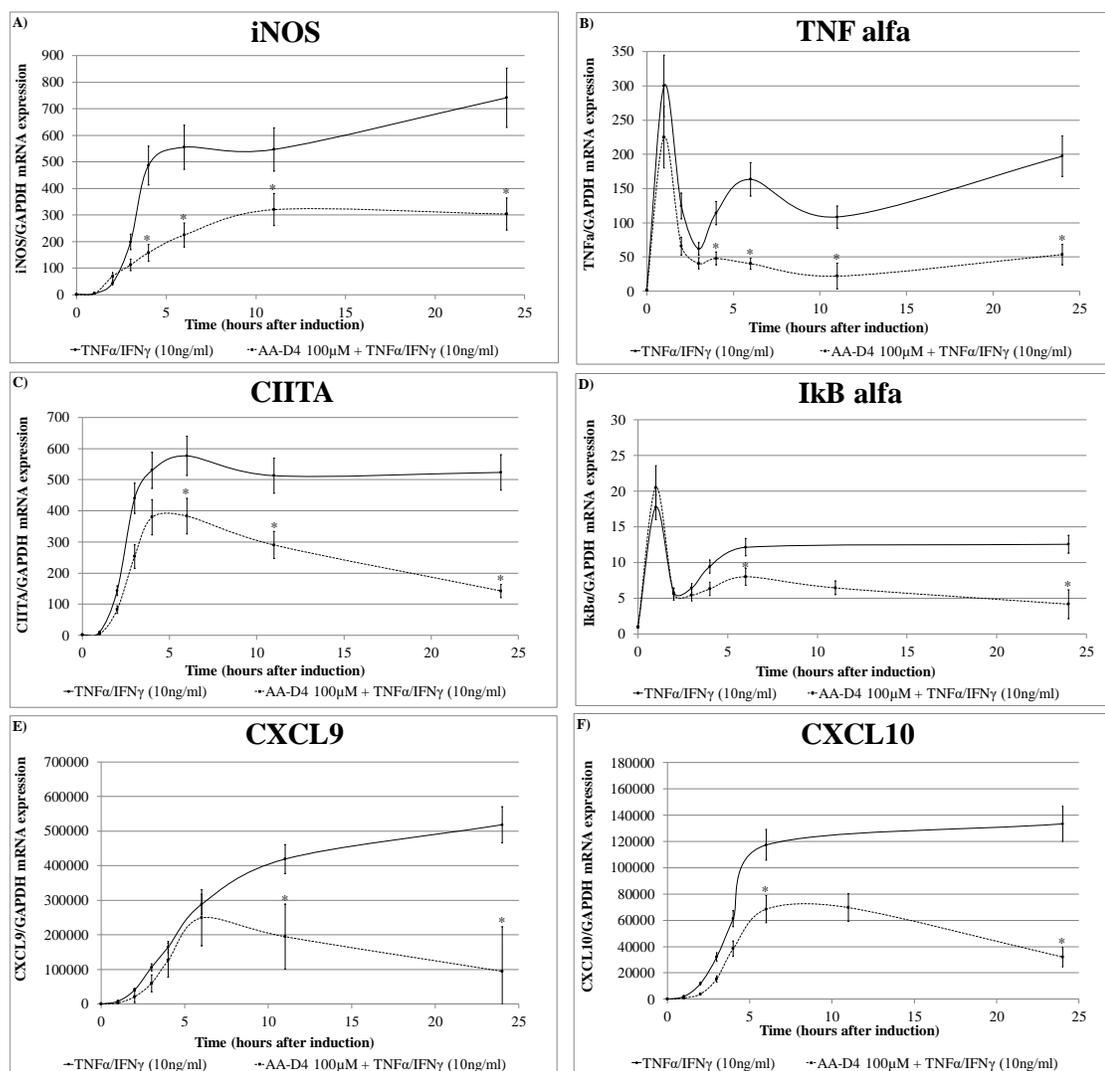
AP-1 transcription factor consists of a homodimer or a heterodimer of the Jun/Fos family proteins. For this reason the expression of several genes involved in AP-1 complex was investigated. Real Time PCR data showed a significant increase of mRNA transcript levels of c-Jun and Fra-1 at all times, from 1 to 24 hours, after induction, in cells pre-treated with AA-D4 and simulated with cytokines (Fig. 8, A and B). In addition, our results showed a higher c-Fos expression level with AA-D4 pre-treatment, in particular at 1, 4, 6 and 11 hours after induction, even though its expression returned to control levels after 24 hours (Fig. 8, C). Although no significant differences were found for JunB mRNA levels, FosB and ATF2 showed a higher level of gene expression at short and long time after induction, respectively (Fig. 8, D, E and F). Similar results were obtained in the case of AA-D2 and AA-D6 treatments (data not shown).

#### DISCUSSION

Inflammation consists of local cellular and vascular responses, which are triggered when the body is injured or invaded by antigens, which can lead to the necrosis of cells and tissues. Aberrant iNOS induction during inflammation may have detrimental consequences and seems to be involved in the pathophysiology of many human diseases [10-13]. The discovery of new compounds able to keep low iNOS and other pro-inflammatory gene expression might be useful to control the inflammatory state.

Human keratinocyte cell lines activated with cytokines were used as an *in vitro* model for studying acute inflammation. In this cell system, we showed that three amide derivatives of arachidonic acid inhibited, in a dose dependent manner, iNOS gene expression (Fig. 1).

Concerning the molecular mechanism by which AA-Ds decrease iNOS gene expression, we first excluded the possibility that the half-life of iNOS transcript might be modified in the presence of AA-Ds (Fig. 2 and data not shown). The results obtained suggested us when new transcription is blocked by Actinomycin D that AA-Ds could inhibit iNOS gene expression by down-modulating its transcription.



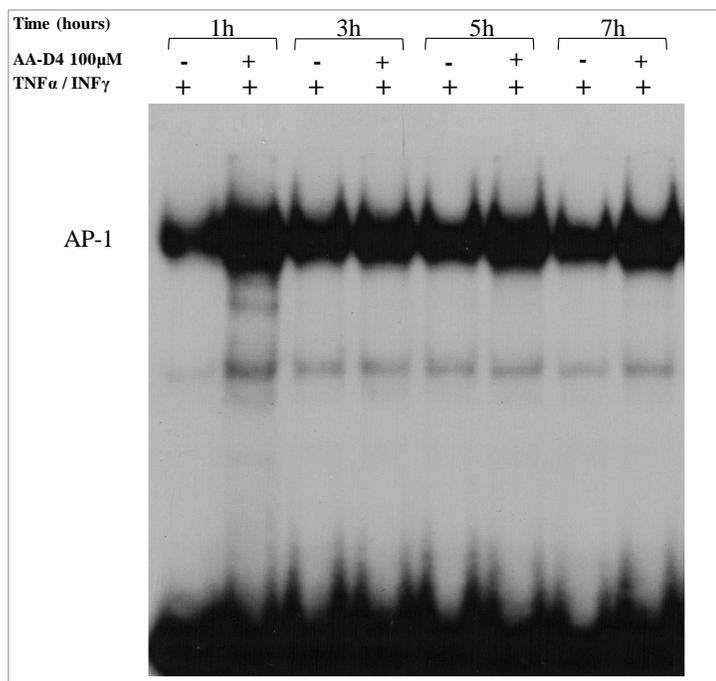
**Fig. (6).** Inhibition of pro-inflammatory gene expression.

HaCaT cells were pre-treated with 100 $\mu$ M of AA-D4 for 2 hour and induced with TNF $\alpha$  (10ng/ml) and IFN $\gamma$  (10ng/ml) until 24 hours; total RNA was extracted, purified and quantified as described in the Materials and Methods section. The data show that compound AA-D4 is able to inhibit gene expression levels of iNOS, TNF- $\alpha$ , CIITA, I $\kappa$ B- $\alpha$ , CXCL9 and CXCL10. GAPDH mRNA was used as an internal control for data normalization. Values are the average of three independent experiments  $\pm$  S. D. \*Significant differences for pre-treated and induced vs induced cells: p < 0.05.

Several reports agree on the primary regulation of human iNOS gene at the transcriptional level [12, 21]. Since NF- $\kappa$ B and STAT1 transcription factors activation, induced by pro-inflammatory cytokines, is essential for the iNOS gene induction, we think that these factors could be the targets of the inhibitory action of AA-Ds. Therefore, we analyzed, by EMSA, the DNA binding of NF- $\kappa$ B and STAT1 in cells treated or not with AA-Ds. Data concerning NF- $\kappa$ B DNA binding showed less intensity retardation bands in sample cells treated with AA-D2, AA-D4 and AA-D6 in respect of the control ones (Fig. 3). On the other hand, EMSA assay for STAT1 did not show any difference in DNA binding for AA-D2 and AA-D6 treatments, although a slight inhibition could be seen in EMSA in the case of AA-D4 treatment (Fig. 4). In order to study the possible occurrence of important phosphorylation of these nuclear transcription factors, a western blot analysis was carried out in a time course experiment with antibodies against NF- $\kappa$ B p65 phospho-

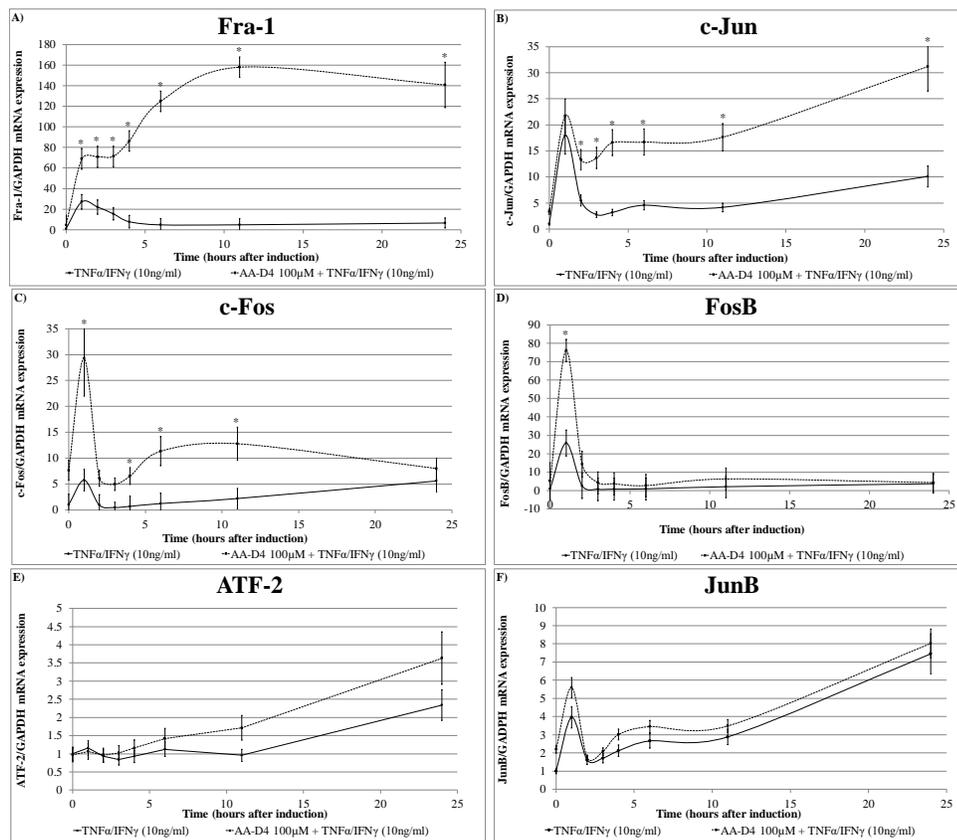
Ser536 and STAT1 phospho-Ser727. The results obtained showed that the pre-treatment with all AA-Ds inhibits Ser536 phosphorylation of p65 in a time-dependent manner (Fig. 5). Thus, the inhibition of both canonical and not canonical pathways of NF- $\kappa$ B could be responsible for the lower transcriptional activity of the iNOS promoter. It should also be mentioned that apart from the decrease of the phosphorylated p65 protein, the total p65 protein level also decreased with AA-D4 treatment. In this case, the decrease in nuclear translocation of the p65 NF- $\kappa$ B subunit might be responsible for the results obtained, since western blot was performed with only nuclear protein extracts. On the other hand, the decreases in p65 phosphorylation remain statistically significant also if data are normalized versus p65 total protein amount.

Although the phosphorylation of serine 727 usually does not affect STAT1-DNA binding [25], the inhibition of



**Fig. (7).** EMSA time course until 7 hours after cytokines induction.

DNA binding of AP-1 transcription factor on consensus motifs of iNOS promoter. 4 µg of nuclear protein extracts of the various treated-cells were incubated with a <sup>32</sup>P-labeled double stranded oligonucleotide containing binding sequence for AP-1, which was isolated by non-denaturing polyacrylamide gel electrophoresis and exposed to an auto radiographic film. This gel is representative of three independent experiments.



**Fig. (8).** Modulation of the expression levels of several genes of Fos and Jun family.

HaCaT cells were pre-treated with 100µM of AA-D4 for 2 hours, and induced with TNFα (10ng/ml) and IFNγ (10ng/ml) until 24 hours; the specific RNA levels for Fra1, c-Jun, c-Fos, FosB, Atf-2 and JunB were quantified by Real Time PCR. GAPDH mRNA was used as an internal control for data normalization. \*Significant differences for pre-treated and induced vs induced cells: p ≤ 0.05.

STAT1-Ser 727 phosphorylation that was seen by western blot analysis with AA-D4 treatment is in agreement with the faint reduction of retarded bands observed in EMSA with the same compound (Figs. 4 and 5). All the results obtained show that AA-D4 treatment affects the activity of both NF- $\kappa$ B and STAT1.

In addition, we have shown that AA-Ds compounds had inhibitory effects on a large spectrum of pro-inflammatory gene targets, such as TNF $\alpha$ , I $\kappa$ B $\alpha$ , CXCL9 and CXCL10 pro-inflammatory gene expression at different times until 24 hours after induction (Fig. 6). Although AA-Ds showed anti-inflammatory activity at broad-spectrum, their inhibitory action on iNOS and the other pro-inflammatory gene induction was delayed, as it was detectable only 4 hours after cytokines administration (Fig. 6). To explain the delay in the onset of these effects we could hypothesize the involvement of newly expressed proteins, which subsequently inhibit pro-inflammatory gene transcription.

To better understand the molecular mechanisms underlying the delay of this inhibitory effect, we focused on iNOS gene regulation and studied the activation of other transcription factors besides NF- $\kappa$ B and STAT1, since the iNOS gene promoter is regulated by several factors [21] and its transcriptional activity can derive from the integration of transcriptional activators and inhibitors. We chose to measure the DNA binding activity of Oct-1 and AP-1, both involved in iNOS gene promoter regulation. We did not find any significant reduction in Oct-1 transcription factor DNA binding (data not shown), whereas EMSA data showed a significant increase in AP1-DNA binding in samples of cells pre-treated with AA-Ds (Fig. 7). Time course of EMSA experiments showed that AP-1 DNA binding increased early, in particular after 1 hour, and it is still visible 7 hours after cells induction (Fig. 7). This result could suggest an early repressor effect of AP-1 proteins and could be in agreement with previous data demonstrating an inhibitory effect of the AP-1 binding on human iNOS transcription [12]. AP-1 transcription factor consists of homodimer or heterodimer of c-Jun, c-Fos and ATF protein families. Several proteins of the AP1 complex must be *de novo* synthesized after cytokines induction. The composition of the AP1 subunits is very important to give different transcriptional property to modulate gene target expression [26]. Thus we studied by real time-PCR the presence of transcripts of different AP-1 subunits through a time course assay. Our data showed that Fra-1 c-Jun, and c-Fos gene expression significantly increases in samples pre-treated with AA-Ds in comparison with the untreated ones. Instead, FOSB gene expression increases, but only in the first hour and JunB gene expression remains unchanged (Fig. 8). It is worth noting that the induction of several genes belonging to the AP-1 complex occurs early after cytokines induction. This was seen by EMSA with the significant increase of AP-1-DNA binding, in particular in the first hour after cytokines administration.

These results precede in time those concerning the inhibitory effects on NF- $\kappa$ B activity. Our data could not demonstrate a causal relationship between the modulation of AP-1 and NF- $\kappa$ B activities and iNOS gene expression inhibition although they seem to suggest it. Other experiments using RNA interfering or dominant negative protein transfection

techniques will be necessary to prove that point. It would also be interesting to study the mechanism by which AA-Ds could improve or inhibit the AP-1 and NF- $\kappa$ B transcription factor activity.

We don't know whether the anti-inflammatory activity of AA-Ds is direct or mediated by their metabolites. However, the short duration of the pre-treatment might suggest a possible direct effect. Pharmacokinetic studies in animals could answer this question.

As mentioned above we verified by real time-PCR the general anti-inflammatory effects of AA-Ds on the expression of several pro-inflammatory genes, such as TNF $\alpha$ , I $\kappa$ B $\alpha$ , CXCL9 and CXCL10. The results obtained demonstrate wide inhibitory effects of all AA-Ds on the expression of these pro-inflammatory genes. It should be mentioned that AA-D4 results to be the most active compound because it also showed an inhibitory effect on CIITA gene expression, this result being in agreement with its ability to inhibit both STAT1 Ser727 phosphorylation and STAT1-DNA binding. On the contrary, the CIITA mRNA level was unchanged by AA-D2 or AA-D4 treatment (data not shown). This results agrees with the inability of AA-D2 and AA-D4 to affect STAT1 binding and/or phosphorylation. It is worth mentioning that the CIITA gene expression is directly activated by IFN $\gamma$ , which works through STAT1 transcription factor activation, and the CIITA gene is the only gene studied by us that is not regulated by NF- $\kappa$ B, in addition to STAT1. Thus, the different behavior regarding the inhibitory effects on CIITA by AA-D4 versus AA-D2 o 6, allow us to exclude the direct involvement of the STAT1 pathway on iNOS gene expression inhibition by AA-D2 and AA-D6 compounds. This dissimilarity of action may be the basis of further studies on the structure-activity relationship between these anti-inflammatory compounds.

## CONCLUSION

In conclusion the new arachidonic acid derivatives studied here show to be powerful inhibitors of the induction of several pro-inflammatory genes. The molecular mechanism of their activity goes through the damping of signal transduction pathways elicited by pro-inflammatory cytokines. All AA derivatives are able to inhibit NF- $\kappa$ B activation, while the inhibition of STAT1 only occurs in the case of the treatment with AA-D4. This derivative appears to be, in fact, the most active compound. However, AA-Ds are also able to increase the signal transduction pathway of AP-1 by enhancing the expression of some immediate early genes belonging to the Jun and Fos families. Consequently, our study provides indications for the use of these AA-Ds as new anti-inflammatory drugs.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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