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New Amides of Arachidonic-Acid as Potential Antiinflammatory Drugs: A Preliminary Study.

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Abstract

The arachidonic acid is a precursor in the synthesis of eicosanoids: through the action of cyclooxygenase and peroxidase, it gives rise to prostaglandins then is converted into different substances involved in inflammation and in many physiological reactions as protectors of gastric mucosa. The aim of this research was to verify the pharmacological activity in a new series of amides of arachidonic acid. To this end, we have been synthesized new derivatives and performed cytotoxicity studies and preliminary studies of anti-inflammatory activity. Our data indicate that some compounds are able to control the NO and PGs biosynthesis on inflammatory process through the *in vitro* inhibition of iNOS activity in J744.A1 macrophage cell line.

Key words: Arachidonic acid, eicosanoids, prostaglandin, inflammation, cytotoxicity

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Introduction

The arachidonic acid is a polyunsaturated fatty acid present in human body; it's introduced trough the diet or it may arise from linoleic acid (essential fatty acid). In the cells, the arachidonic acid is linked to membrane phospholipids (phosphatidylcholine, phosphatidylinositole, phosphatidyletanolamine) and it is the precursor in the synthesis of eicosanoid; it's metabolized to several groups of lipid mediators, including prostaglandins, leukotrienes, and lipoxins [1].

Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid into prostaglandins (PGs), which play a significant role in health and disease in the gastrointestinal tract (GI) and in the renal, skeletal, and ocular systems. COX-1 is constitutively expressed and found in most normal tissues, whereas COX-2 can be expressed at low levels in normal tissues and is highly induced by pro-inflammatory mediators [2].

Also the leukotrienes comprise a family of products of the 5-lipoxigenase pathway of arachidonic acid metabolism. They are the chemical mediators of inflammatory reaction and anaphilaxis, and also have a powerful effect vasodilator and in the bronchoconstriction. Furthermore, arachidonic acid is also used in anandamide's biosynthe
sis [3].

The anandamide, or arachidonoiletanolammide (AEA), is part of a new class of lipid mediators, acting predominantly olocrina and paracrine, collectively known as endocannabinoids.

During inflammatory setting, both COX-2 and the inducible isoform of nitric oxide synthase (iNOS) are detected in a variety of cells, resulting in the production of large amounts of pro-inflammatory PGs and cytotoxic nitric oxide (NO) molecules. Increasing evidences suggest that intracellular concentrations of NO and PGs may be relevant in switching on/off inflammatory cells by modulating their own biosynthesis and NOS/COX enzymes [4].

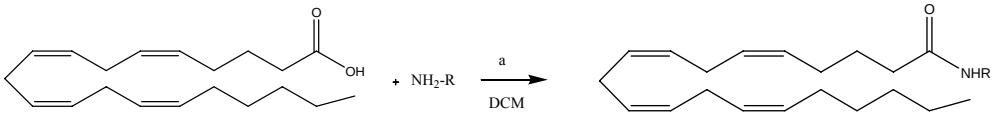
The goal of this work was to produce new compounds (1-9) obtained by adding a series of amines to the arachidonic acid molecule and to evaluate their anti-inflammatory activity, preliminarily on NO production by LPS-induce macrophages.

Experimental section

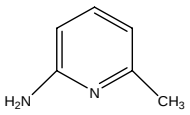
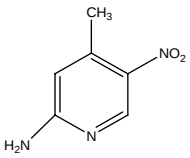
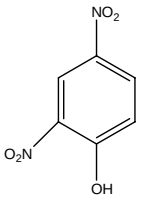
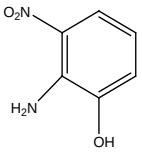
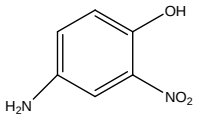
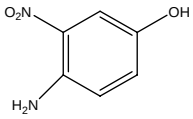
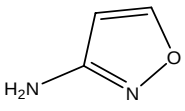
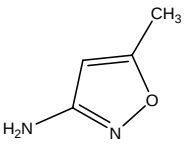
Melting points were taken on a Gallenkamp melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded with a Bruker Avance 300 MHz spectrometer. Chromatographic separations were performed on silica gel column (Kiesalgel 40, 0040-0,063mm, Merck). Reactions and product mixtures were routinely monitored thin

layer chromatography (TLC) on silica gel precoated F254 Merck plates.

Scheme



a= TEA; PPAA

N° COMPOUND	R
1	
2	
3	
4	
5	
6	
7	
8	

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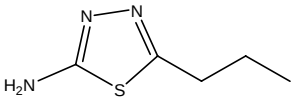

***General method of synthesis of products** 1-9*

Arachidonic acid (1eq) was added to a series of amines (1,5eq) in dichloromethane (2-3 ml) in the presence of TEA (4eq) and PPAA (2eq), at room temperature[5]. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (silica gel) using petroleum ether/ ethylacetate as eluent.

***N-(6-methylpyridin-2-yl)icosa-5,8,11,14-tetraenamide* (1).**
Arachidonic acid (70mg, 0,23mmol, 1eq) is dissolved in dry DCM (2ml); to this solution are added: TEA (130µl, 0,92mmol, 4eq), PPAA (136µl, 0,46mmol, 2eq) and 2-amino-6-methylpyridine (38 mg, 0,35mmol, 1,5eq).
The reaction mixture is kept under reflux for about 7 hours, then the solvent is evaporated and the resulting residue was purified by silica gel column chromatography (eluents: petroleum ether/ethyl acetate 85:15) to get an oily compound with a yield of 50%.

^{1}H NMR (CDCl3) :8.00 (s, 1H); 7.80 (d, 1H); 7.50 (t, 1H); 7.25 (d, 1H); 5.45-5.25 (m, 8H); 2.63-2.58 (m, 6H); 2.50 (s, 3H); 2.00 (m, 4H); 2.20 (t, 2H); 1.62 (m, 2H); 1.36-1.30 (m, 6H); 0.98 (t, 3H).

N-(4-methyl-5-nitropyridin-2-yl) icosa-5,8,11,14- tetraenamide (2).
Arachidonic acid (70mg, 0.23mmol, 1eq) is dissolved in dry DCM (2ml); to this solution are added TEA (130µl, 0.92mmol, 4eq), PPAA (136µl, 0,46mmol, 2eq) and then is added 2-amino-4-methyl-5-nitropyridine (54mg, 0,35mmol, 1,5eq).

The reaction mixture is kept under reflux for about 5 hours, then the solvent is evaporeted and the resulting residue was purified by silica gel column (eluents: petroleum ether/ethyl acetate 85:15) to get an oily compound with a yeld of 60%.

^{1}H NMR (CDCl3): 9.40 (s, 1H); 8.45 (s, 1H); 5.45-5.26 (m, 8H); 2.65 (m, 6H); 2.50 (s, 3H); 2.20 (t, 2H); 1.98 (m, 6H); 1.58 (m, 1H); 1.55-1.25 (m, 6H); 0.98 (t, 3H).

***N-(2-hydroxy-5-nitrophenyl)icosa-5,8,11,14-tetraenamide* (3).**
Arachidonic acid (70mg,0.23mmol, 1eq) is dissolved in dry DCM (2ml); to this solution are added TEA (130µl, 0.92mmol, 4eq), PPAA (136µl, 0,46mmol, 2eq) and then is added 2-amino-4-methyl-5-nitropyridine (54mg, 0,35mmol, 1,5eq).

The reaction mixture is kept at room temperature for 5 hours; then the solvent is evaporeted and the resulting residue was purified by silica gel column (eluents: petroleum ether/ethyl acetate 9:1) to get an oily compound with a yeld of 55%.

^{1}H NMR (CDCl3) : 8.28 (s, 1H); 7.90 (s, 1H); 7.86 (s, 1H); 7.00 (s, 1H); 5.45-5.35 (m, 8H); 4.90 (s, 1H); 2.60 (m, 6H); 2.25 (t, 2H); 2.00 (m, 4H); 1.72 (m, 2H); 1.38-1.28 (m, 6H); 0.99(t, 3H).

N-(2-hydroxy-5-nitrophenyl)icosa-5,8,11,14-tetraenamide (4).
Arachidonic acid (90mg, 0.30mmol, 1eq) is dissolved in dry DCM (3ml); to this solution are added TEA (165µl, 1.18mmol, 4eq), PPAA (177µl, 0.60mmol, 2eq) and then is added 2-amino-3-nitrophenol (70mg, 0.45mmol, 1.5eq).

The reaction mixture is stirred at room temperature, for 3 hours ; then the solvent is evaporeted and the resulting residue was purified by silica gel column chromatography (eluents: petroleum ether/ethyl acetate 7:3) to get an oily compaund with a yeld of 68%.

^{1}H NMR (CDCl3): 8.80 (s, 1H); 7.80 (d, 1H); 7.20 (d, 1H); 7.18 (d, 1H); 5.48-5.37 (m, 8H); 4.20 (s,
1H); 2.62 (m, 6H); 2.25 (t, 2H); 2.00 (m, 4H); 1.72 (m, 2H); 1.30-1.27 (m, 6H); 0.98 (t, 3H).

***N-(4-hydroxy-3-nitrophenyl)icosa-5,8,11,14-tetraenamide* (5).**
Arachidonic acid (90mg, 0.30mmol, 1eq) is dissolved in dry DCM (3ml); to this solution are added TEA (165µl, 1.18mmol, 4eq), PPAA (177µl, 0.60mmol, 2eq) and then is added 4-amino-2-nitrophenol (70mg, 0.45mmol, 1.5eq).

The reaction mixture is stirred at room temperature for 5 hours; then the solvent is evaporeted and the resulting residue was purified by silica gel column chromatography (eluents: petroleum ether/ethyl acetate 7:3) to get an oily compaund with a yeld of 65%.

^{1}H NMR (CDCl3): 8.30 (s, 1H); 7.85 (t, 1H); 7.20 (s, 1H); 7.00 (d, 1H); 5.55-5.35 (m, 8H); 4.50 (s, 1H); 2.63

(m, 6H); 2.25 (t, 2H); 1.98 (m, 4H); 1.70 (m, 2H); 1.35-1.31 (m, 6H); 0.98 (t, 3H).

***N-(4-hydroxy-2-nitrophenyl)icosa-5,8,11,14-tetraenamide* (6).**
Arachidonic acid (90mg, 0.30mmol, 1eq) is dissolved in dry DCM (3ml); to this solution are added TEA (165µl, 1.20mmol, 4eq), PPAA (177µl, 0.60mmol, 2eq) and then is added 4-amino-3-nitrophenol (55mg, 0.36mmol, 1.2eq).

The reaction mixture is stirred at room temperature for about 6 hours; then the solvent is evaporeted and the resulting residue was purified by silica gel column chromatography (eluents: petroleum ether/ethyl acetate 7:3) to get an oily compaund with a yeld of 60%.

^{1}H NMR (CDCl3): 8.00 (s, 1H); 7.75 (d, 1H); 7.65 (s, 1H); 6.90 (s, 1H); 5.45-5.35 (m, 8H); 4.50 (s, 1H); 2.64-2.60 (m, 6H); 2.25 (t, 2H); 1.98 (m, 4H); 1.73 (m, 2H); 1.32-1.28 (m, 6H); 0.99 (t, 3H).

N-(isoxazol-3-yl)icosa-5,8,11,14-tetraaenamide (7).
Arachidonic acid (90mg, 0.30mmol, 1eq) is dissolved in dry DCM (3ml); to this solution are added TEA (160µl, 1.20mmol, 4eq), PPAA (177µl, 0.60mmol, 2eq) and then is added 5-amino-isoxazol (33µl, 0.45mmol, 1.5eq).
The reaction mixture is stirred at room temperature for about 6 hours; then the solvent is evaporeted and the resulting residue was purified by silica gel column chromatography (eluents: petroleum ether/ethyl acetate 7:3) to get an oily compaund with a yeld of 63%.

^{1}H NMR (CDCl3): 8.50 (s, 1H); 8.00 (s, 1H); 7.40 (s, 1H); 5.48-5.39 (m, 8H); 2.68 (m, 6H); 2.19 (t, 2H); 1.98 (m, 4H); 1.58 (m, 2H); 1.30-1.27 (m, 6H); 1.00 (t, 3H).

***N-(5-methylisoxazol-3-yl)icosa-5,8,11,14-tetraenamide* (8).**
Arachidonic acid (90mg, 0.30mmol, 1eq) is dissolved in dry DCM (3ml); to this solution are added TEA (165µl, 1.20mmol, 4eq), PPAA (177µl, 0.60mmol, 2eq) and then is added 3-amino-5-methylisoxazol (44.14mg, 0.45mmol, 1.5eq).

The reaction mixture is stirred at room temperature for about 6 hours; then the solvent is evaporeted and the resulting residue was purified by silica gel column chromatography (eluents: petroleum ether/ethyl acetate 7:3) to get an oily compaund with a yeld of 48%.

^{1}H NMR (CDCl3): 8.50 (s, 1H); 7.40 (s, 1H); 5.46-5.37 (m, 8H); 2.62 (m,6H); 2.38 (m, 3H); 2.20 (t, 2H); 2.00 (m, 4H); 1.73 (m, 2H); 1.35-1.30 (m, 6H); 0.97 (t, 3H).

***N-(5-propyl-1,3,4-thiadiazol-2-yl)icosa-5,8,11,14-tetraenamide* (9).**
Arachidonic acid (90mg, 0.30mmol, 1eq) is dissolved in dry DCM (3ml); to this solution are added TEA (165µl, 1.20mmol, 4eq), PPAA (177µl, 0.60mmol, 2eq) and then is added 2-amino-5-propylthiadiazol (58.13mg, 0.45 mmol, 1.5eq).

The reaction mixture is stirred at room temperature for about 6 hours; then the solvent is evaporeted and the resulting residue was purified by silica gel column chromatography (eluents: petroleum ether/ethyl acetate 7:3) to get an oily compaund with a yeld of 70%.

^{1}H NMR (CDCl3): 8.00 (s, 1H); 5.45-5.30 (m, 8H); 2.63 (m, 6H); 2.58 (m, 2H); 2.20 (t, 2H); 1.95 (m, 4H); 1.68 (m, 2H); 1.66 (m,2H); 1.35-1.30 (m, 6H); 0.98 (t, 6H).

Toxicological and Pharmacological Methods

Materials
The murine macrophage cell line (J774A.1) the murine fibrosarcoma cells (WEHI-164) and human epithelial kidney cells (HEK-293) was obtained from American Tissue Culture Collection (ATCC). *E. coli* lipopolysaccharide (LPS) was obtained from Fluka (Milan, Italy). 3-(4,5-Dimethylthiazolyl-2-yl) 2,5-diphenyltetrazolium bromide (MTT), phosphate buffer solution (PBS), bovine serum albumin (BSA), were obtained from Sigma Chemical Co. (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, HEPES, glutamine, fetal calf serum (FCS), and horse serum were from Hy Clone (Euroclone-Cellbio, Pero, Milan, Italy).

Cells
J774.A1, murine monocyte/macrophage cells, were grown in adhesion on Petri dishes and maintained at 37 °C as previously described [6]. WEHI-164 and HEK-293 were maintained in adhesion on Petri dishes with DMEM supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 100 u/mL penicillin, and 100 µg/mL streptomycin.

Cytotoxic Activity
J774A.1, WEHI-164 and HEK-293 (3.5 x 10^4 cells/well) were plated on 96-well microtiter plates and allowed to adhere at 37 °C in a 5 % CO_2 atmosphere for 2 h. Thereafter, the medium was replaced with of fresh medium and serial dilution of each test compound was added and then the cells incubated for 72 h. Serial dilutions of 6-mercaptopurine, as reference drug, were also added to cells. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] (MTT) to formazan and cells viabil-

ity was assessed accordingly to the method of Mosmann. Briefly 5 μL of MTT (5 mg/mL) were added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 μL of a solution containing 50% (v:v) N, N-dimethylformamide, 20 % (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-mercaptopurine was calculated as: % dead cells=100-(OD treated/OD control)x 100 [7].

Analysis of Nitrite

J774A.1 (5.0 x 10^4 cells/well) were plated on 96-well microtiter plates and allowed to adhere at 37°C in a 5% CO_2 atmosphere for 2 h. Examined compounds (0.01-1 mM) was added 1h before and simultaneously to LPS (6x10^3 u/ml), used to induce inducible nitric oxide synthase (iNOS). Nitric oxide release (NO), evaluated as nitrite (NO_2^-) accumulation in the cell culture medium, were performed 24 h after LPS stimulation by Griess reagent [8]. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium. Results are expressed as percentages of inhibition calculated versus cells treated with LPS alone.

Results and Conclusions

To establish the effects on cell viability of new compounds (**1-9)** obtained by adding a series of amines to the arachidonic acid *in vitro*, (0.01-1 mM), were each tested on J774.A1, WEHI-164, and HEK-293 cells using MTT test. Among tested compounds only 3, 5 and 6 didn't show cytotoxic effect on cells (data not shown).

The involvement of NO on inflammatory process and its relation with PGs biosynthesis led us to investigate the *in vitro* activities of iNOS, evaluating NO biosynthesis, in J744.A1 macrophage cell line. NO release in the cellular medium of LPS-stimulated J774.A1 macrophages, incubated with new compounds which didn't resulted cytotoxic (0.01−1 mM) was evaluated 24 h after LPS (6 × 10^3 u/mL) challenge. Results were expressed as % of inhibition calculated *versus* macrophages treated with LPS alone. The results of the present study indicate that compounds **5** and **6** added 1 h before and simultaneously with LPS, significantly inhibited at the concentrations 0.1 and (54.0%) 1m M (67.0%), NO release ($P<0.001$). Results on NO release also showed that compound 3 was the most active inhibiting at all tested concentrations, and in a related- manner, NO release ($P<0.001$ vs LPS alone) (62.0% and 82,6%, respectively).

Therefore, our data indicate that compounds 3, 5 and 6 are able to control the NO and PGs biosynthesis on inflammatory process through the *in vitro* inhibition of iNOS activity in J744.A1 macrophage cell line.

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