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Arginine salt and amide inhibitors of NO synthase and cyclooxygenase

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TITLE

Arginine Salt and Amide Inhibitors of NO Synthase and Cyclooxygenase

DESCRIPTION

The invention relates to compounds having dual biological activity, as inhibitors of both the L-Arginine/nitric oxide (NO) pathway and the cyclooxygenase pathway, to a process for their preparation and to pharmaceutical compositions containing them.

Considering the potential role of NO synthase and cyclooxygenase in physiopathology, the compounds may provide effective and favourable benefits in the treatment of:
- cardio and cerebrovascular disorders, including, for example, migraine, stroke, infarcts, ischemia, septic, endotoxinic and hemorrhagic shocks, pain;
- the various forms of inflammation, including, for example, acute rheumatic fever, rheumatoid arthritis or other types of arthritis, osteoarthrosis, asthma;
- immune disorders, including viral or non viral infections, auto-immune diseases, drug abuse, cancer and any pathologies where an excessive production of nitric oxide and/or arachidonic acid metabolites is involved in humans or animals.

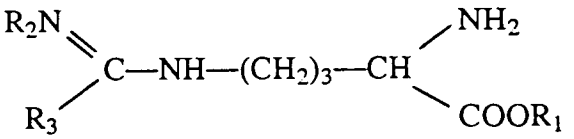
Inhibitors of cyclooxygenase or aspirin like drugs, i.e. acetylsalicylic and salicylic acid, methylated indole derivatives, such as indomethacin (DCI of 1-(4-chlorobenzoyl)--5-methoxy-2-methyl-1H-indole-3-acetic acid) and sulindac (DCI of 5-fluoro-2-methyl--1-[(4-methyl-sulphinyl-phenyl)-methylene]-1H-indene-3-acetic acid), derivatives of N-phenyl-anthranilic acids (meclofenamate, fenamates), propionic acid derivatives such as ibuprofen (DCI of p-isobutylhydratropic acid), naproxen and fenoprofen, are largely employed and have been largely demonstrated, with however some undesirable side effects at high doses, as efficient drug therapy of inflammation (R. Flower, S. Moncada and J. Vane, Mechanism of action of aspirin-like drugs - In the pharmacological basis of therapeutics Goodman and Gilman 1985, 29, 674-715). Moreover, these compounds have been used in both acute and prophylactic treatment of migraine. The value of these drugs is without question although their therapeutic responses are often incomplete and they are not considered to be an adequate treatment by some patients. Considering their anti-inflammatory and platelet anti-aggregant properties, these compounds have also been used in thrombosis and with evidence of a reduction of oedema, in brain ischemia models and hence proposed in the treatment and prevention of infarcts, stroke and cerebrovascular diseases (W. Armstrong Recent trends in research and treatment of stroke. SCRIP, PJB publications. 1991).

Biological activity of inhibitors of nitric oxide synthase has been discovered only recently and their potential therapeutic use is just being considered. These substances, whose structures are represented by L-Arginine analogues and are disclosed in British Patent Application 2240041, are inhibitors of nitric oxide (NO) generation. The current knowledge on NO has been exhaustively reviewed in 1991 by Moncada et al, (S. Moncada, R.M.J. Palmer, E.A. Higgs. Nitric oxide : Physiology, Pathophysiology, and Pharmacology. Pharmacological reviews 43, 2, 109-142). Briefly, it appears that NO serves as a transduction mechanism for the soluble guanylate cyclase in vasculature, platelets, nervous system and as an effector molecule in immunological reactions in many cells and tissues, including macrophages or neutrophils. NO is enzymatically generated from L-Arginine by an enzyme called NO synthase. This enzyme exists in two forms: a constitutive one and an inducible one, which are both inhibited by L-Arginine analogues as thereunder defined. In some pathologies, an excessive production of NO may occur, as it has been already demonstrated in shock, as described in the previously cited Patent Application. In this context, the inhibitors of NO synthase are effective drugs to prevent the vascular consequence and mortality due to the disease, especially when they are combined with inhibitors of cyclooxygenase like aspirin, indomethacin or meclofenamate. Such beneficial effects of the combination of two active principles in the same molecule, is likely to be found in human patients suffering from migraine, stroke, infarcts, cerebral ischemia, pain inflammations and various immunological diseases. The association of inhibitor of nitric oxide synthase and inhibitor of cyclooxygenase have been disclosed in the above mentioned Patent Application for the treatment of shock states. However, it has been found that the combination of such compounds provided a better synergic effect than the association.

The invention provides a salt of a cyclooxygenase inhibitor and the L-form of an arginine analogue,

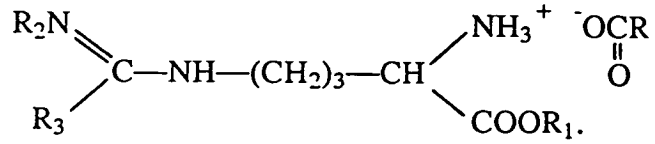
the cyclooxygenase inhibitor having the general formula RCOOH wherein the carboxy group COOH is an accessible acidic function and R stands for the appropriate radical of the cyclooxygenase inhibitor,

the L-form of an arginine analogue having the general formula

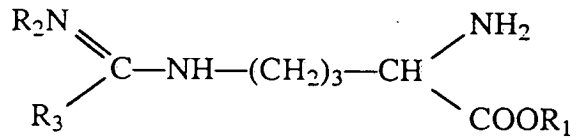


wherein R_1 represents a hydrogen atom or a methyl or ethyl group, R_2 represents a hydrogen atom or a nitro group and R_3 represents an amino, methylamino, ethylamino, hydrazino, methyl or ethyl group, with the proviso that if R_2 represents a hydrogen atom

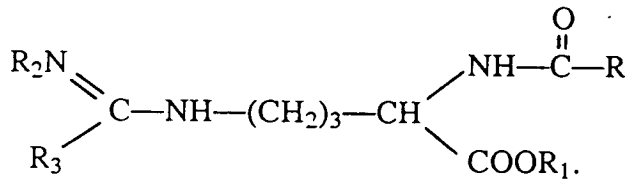
then R_3 does not represent an amino group,
the salt having the general formula



The invention also provides an amide of a cyclooxygenase inhibitor and the L-form of an arginine analogue, the cyclooxygenase inhibitor having the general formula RCOOH wherein the carboxy group COOH is an accessible acidic function and R stands for the appropriate radical of the cyclooxygenase inhibitor, the arginine analogue having the general formula



wherein R_1 represents a hydrogen atom or a methyl or ethyl group, R_2 represents a hydrogen atom or a nitro group and R_3 represents an amino, methylamino, ethylamino, hydrazino, methyl or ethyl group,
the amide having the general formula



In a preferred embodiment, the cyclooxygenase inhibitor is salicylic acid, acetylsalicylic acid, mefenamic acid, ibuprofen, indomethacin or sulindac. In a preferred embodiment, the arginine analogue is N-ω-monomethyl-L-arginine, N-ω-nitro-L-arginine or N-ω-nitro-L-arginine methyl ester.

The invention provides a process for the preparation of a salt according to the invention, the process comprising reacting, in substantially equimolar proportions in water or in a mixture of water and alcohol, at a temperature of from ambient temperature to the boiling point of the reaction mixture, a cyclooxygenase inhibitor as herein defined with an arginine analogue as herein defined. The cyclooxygenase inhibitor may be used in this process as such or in the form of a salt, such as its sodium salt. The alcohol used in admixture with water may be methanol or ethanol.

The invention provides a process for the preparation of an amide according to the invention, the process comprising reacting, in substantially equimolar proportions in

acetonitrile in the presence of a base, at a temperature of from 0°C to ambient temperature, an arginine analogue as herein defined and a compound RCOX wherein R is as herein defined and X represents a halogen atom. The arginine analogue may be used in this process as such or in the form of, for instance, its hydrochloride. The base is preferably triethylamine.

The invention also provides a pharmaceutical composition comprising a salt or amide according to the invention in admixture with a pharmaceutically acceptable diluent or carrier.

The following Examples illustrate the invention.

Example 1

The salt of acetylsalicylic acid and N-ω-monomethyl-L-Arginine (L-NMMA)

99 mg (0.52 mmol) of L-NMMA and 95 mg (0.52 mmol) of acetylsalicylic acid were dissolved in 10 ml of ethanol (95%) at room temperature. Stirring was maintained for three hours at room temperature. The solution was concentrated to dryness and the obtained residue was treated with 25 ml of water, then lyophilised to yield 190 mg of the required compound (white solid; m.p. = 170°C).
^{1}H-NMR (100 MHz, D_2O):
7.80-6.60 (m, 4H, aromatic); 3.50 (t, 1H, CHCOOH); 3.10 (m, 2H, CH_2-NH); 2.60 (s, 3H, CH_3-NH); 2.15 (s, 3H, CH_3-CO); 1.90-1.30 (m, 4H, CH-C$\underline{H}_2$-C$\underline{H}_2$).

Example 2

The salt of salicylic acid and N-ω-monomethyl-L-Arginine (L-NMMA)

0.52 mmol of N-ω-monomethyl-L-Arginine acetate and 0.52 mmol of sodium salt of salicylic acid were dissolved in water at room temperature. Stirring was maintained at room temperature until the solution became limpid. The sodium acetate formed was eliminated and the solution was lyophilised to yield 160 mg of the required compound (white solid; m.p. = 172-175°C).
^{1}H-NMR (100 MHz, D_2O):
7.75-6.70 (m, 4H, aromatic); 3.55 (t, 1H, C$\underline{H}$-COOH); 3.00 (m, 2H, CH_2-NH); 2.60 (s, 3H, NH-C$\underline{H}_3$); 1.90-1.30 (m, 4H, CH_2-CH_2).

Example 3 :

The salt of acetylsalicylic acid and N-ω-nitro-L-Arginine (L-NO)

500 mg (2.28 mmol) of N-ω-nitro-L-Arginine and 411 mg (2.28 mmol) of acetylsalicylic acid were dissolved in a mixture of ethanol / H_2O (100 ml/70 ml) in the heat. The stirring was maintained for one hour under reflux. The solution was concentrated to dryness and the obtained residue was treated with 100 ml of water, then lyophilised to yield 900 mg of the required compound (white solid ; m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.85-6.70 (m, 4H, aromatic) ; 3.70 (t, 1H, C<u>H</u>-COOH) ; 3.10 (m, 2H, C<u>H_2</u>-NH) ; 2.16 (s, 3H, CH_3) ; 1.90-1.35 (m, 4H, CH_2-CH_2).

Example 4 :

The salt of salicylic acid and N-ω-nitro-L-Arginine (L-NO)

500 mg (2.28 mmol) of N-ω-nitro-L-Arginine and 315 mg (2.28 mmol) of salicylic acid were dissolved in a mixture of ethanol / H_2O (100 ml / 70 ml) in the heat. The stirring was maintained for one hour under reflux. The solution was concentrated to dryness and the obtained residue was treated with 100 ml of water, then lyophilised to yield 810 mg of the required compound (white solid ; m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.78-6.71 (m, 4H, aromatic), 3.53 (t, 1H, C<u>H</u>-COOH), 3.11 (m, 2H, C<u>H_2</u>-NH), 2.00-1.30 (m, 4H, CH_2-CH_2).

Example 5 :

The salt of acetylsalicylic acid and N-ω-nitro-L-Arginine methyl ester (L-NAME)

This compound has been prepared by mixing, in equimolar proportions, acetyl salicylic acid and N-ω-nitro-L-Arginine methyl ester, according to the method as described in example 3 (yield 98.7 %) ; (white solid ; m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.8-6.70 (m, 4H, Ph) ; 3.80 (t, 1H, C<u>H</u>-CO_2H) ; 3.65 (s, 3H, CO_2C<u>H_3</u>) ; 3.10 (m, 2H, C<u>H_2</u>NH) ; 2.11 (s, 3H, C<u>H_3</u>CO) ; 1.90-1.35 (m, 4H, CH_2-CH_2)

Example 6 :

The salt of indomethacin and N-ω-nitro-L-Arginine (L-NO)

This compound has been prepared by mixing, in equimolar proportions, indomethacin and N-ω-nitro-L-Arginine according to the method as described in example 3 (yield 97.8 %) ; (white solid ; m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.70-6.35 (m, 7H, aromatic) ; 3.95 (m, 1H, C<u>H</u>-COOH) ; 3.62 (s, 3H, CH_3O) ; 3.35 (s, 2H, C<u>H</u>$_2$-COOH) ; 3.08 (m, 2H, C<u>H</u>$_2$-NH) ; 2.10 (2s, 3H, CH_3-C=) ; 1.72-1.30 (m, 4H, CH_2-CH_2).

Example 7 :

The salt of sulindac and N-ω-monomethyl-L-Arginine (L-NMMA)

This compound has been prepared by mixing, in equimolar proportions, sodium salt of sulindac and N-ω-methyl-L-Arginine acetate, according to the method as described in example 2 (yield 98 %) ; (orange solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.45 (m, 4H, Ph-SO) ; 6.95-6.60 (m, 3H, Ph-F) ; 6.29 (m, 1H, =C-H) ; 3.15 (m, 5H, C<u>H</u>-COOH, C<u>H</u>$_2$-COOH, C<u>H</u>$_2$NH) ; 2.72 (s, 3H, C<u>H</u>$_3$-NH) ; 2.60 (s, 3H, CH_3-SO) ; 1.83 (2s, 3H, CH_3-C=) ; 1.40 (m, 4H, CH_2-CH_2).

Example 8 :

The salt of ibuprofen and N-ω-nitro-L-Arginine (L-NO)

This compound has been prepared by mixing, in equimolar proportions, ibuprofen and N-ω-nitro-L-Arginine, according to the method as described in example 3 (yield 99 %) ; (white solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7 (m, 4H, aromatic) ; 3.6-3.3 (m, 2H, 2$CHCO_2H$) ; 3.05 (m, 2H, CH_2N) ; 2.35 (d, 2H, CH_2 Ph) ; 1.8-1.3 (m, 5H, CH_2-CH_2 and C<u>H</u>$(CH_3)_2$) ; 1.2 (d, 3H, CH-C<u>H</u>$_3$) ; 0.9 (d, 6H, 2CH_3).

Example 9 :

The salt of mefanamic acid and N-ω-nitro-L-Arginine (L-NO)

This compound has been prepared by mixing, in equimolar proportions, mefenamic acid and N-ω-nitro-L-Arginine, according to the method as described in example 3 (yield 98 %) ; (white solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, CD_3OD) :

8 (m, 1H, H arom. on o. de CO_2H) ; 7.3-6.5 (m, 6H, aromatic) ; 3.6 (t, 1H, C<u>H</u>CO_2H) ; 3.3 (m, 2H, CH_2NH) ; 2.2 and 2.3 (2s, 6H, $2CH_3$) ; 1.85-1.6 (m, 4H, CH_2-CH_2).

Example 10 :

The salt of indomethacin and N-ω-monomethyl-L-Arginine (L-NMMA)

This compound has been prepared by mixing, in equimolar proportions, sodium salt of indomethacin and N-ω-methyl-L-Arginine acetate, according to the method as described in example 2 (yield 99 %) ; (yellow solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.70-6.35 (m, 7H, aromatic) ; 3.67 (2s, 3H, CH_3O) ; 3.47 (m, 1H, C<u>H</u>-COOH) ; 3.35 (s, 2H, C<u>H</u>$_2$-COOH) ; 2.97 (m, 2H, C<u>H</u>$_2$-NH) ; 2.57 (s, 3H, C<u>H</u>$_3$-NH) ; 2.05 (2s, 3H, CH_3-C=) ; 1.72-1.30 (m, 4H, CH_2-CH_2).

Example 11 :

The salt of sulindac and N-ω-nitro-L-Arginine methyl ester (L-NAME)

This compound has been prepared by mixing, in equimolar proportions, sodium salt of sulindac and N-ω-nitro-L-Arginine methyl ester hydrochloride, according to the method as described in example 2 (yield 98.6 %) ; (orange solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.30 (m, 4H, Ph-SO) ; 6.70 (m, 3H, Ph-F) ; 6.11 (m, 1H, =C-H) ; 3.78 (m, 1H, C<u>H</u>-COOH) ; 3.62 (s, 3H, O-CH_3) ; 3.18 (bs, 2H, C<u>H</u>$_2$-COOH) ; 3.00 (m, 2H, C<u>H</u>$_2$-NH) ; 2.61 (s, 3H, CH_3-SO) ; 1.83 (bs, 3H, CH_3-C=) ; 1.90-1.30 (m, 4H, CH_2-CH_2).

Example 12 :

The salt of mefanamic acid and N-ω-monomethyl-L-Arginine (L-NMMA)

This compound has been prepared by mixing, in equimolar proportions, mefenamic acid and N-ω-methyl-L-Arginine, according to the method as described in example 3 (yield 99 %) ; (white solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, CD_3OD) :

8 (m, 1H, H arom. on o. de CO_2H) ; 7.3-6.5 (m, 6H, Ph) ; 3.56 (t, 1H, C<u>H</u>CO_2H) ; 3.2 (m, 2H, C<u>H$_2$</u>NH) ; 2.85 (s, 3H, C<u>H$_3$</u>NH) ; 2.2 and 2.3 (2s, 6H, $2CH_3$) ; 1.8-1.6 (m, 4H, CH_2-CH_2).

Example 13 :

The salt of sulindac and N-ω-nitro-L-Arginine (L-NO)

This compound has been prepared by mixing, in equimolar proportions, sulindac and N-ω-nitro-L-Arginine, according to the method as described in example 3 (yield 98 %) ; (orange solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.30 (m, 4H, Ph-SO) ; 6.70 (m, 3H, Ph-F) ; 6.11 (m, 1H, =C-H) ; 3.78 (m, 1H, C<u>H</u>-COOH) ; 3.18 (bs, 2H, C<u>H$_2$</u>-COOH) ; 3.00 (m, 2H, C<u>H$_2$</u>-NH) ; 2.61 (s, 3H, CH_3-SO) ; 1.83 (bs, 3H, CH_3-C=) ; 1.90-1.30 (m, 4H, CH_2-CH_2).

Example 14 :

The salt of salicylic acid and N-ω-nitro-L-Arginine methyl ester (L-NAME)

This compound has been prepared by mixing, in equimolar proportions, sodium salt of salicylic acid and N-ω-nitro-L-Arginine methyl ester hydrochloride, according to the method as described in example 2 (yield 97.8 %) ; (white solid, m.p. > 260° C).

^{1}H-NMR (100 Mz, D_2O) :

7.70-6.68 (m, 4H, Ph) ; 4.00 (t, 1H, C<u>H</u>-COOH) ; 3.65 (s, 3H, $COOCH_3$) ; 3.11 (m, 2H, C<u>H$_2$</u>-NH) ; 2.00-1.30 (m, 4H, CH_2-CH_2).

Example 15 :

The salt of indomethacin and N-ω-nitro-L-Arginine methyl ester (L-NAME)

This compound has been prepared by mixing, in equimolar proportions, sodium salt of indomethacin and N-ω-nitro-L-Arginine methyl ester hydrochloride, according to the method as described in example 2 (yield 98.5 %) ; (yellow solid, m.p. > 260° C).

^{1}H-NMR (100 MHz, D_2O) :

7.70-6.35 (m, 7H, aromatic) ; 3.95 (m, 1H, C<u>H</u>-COOH) ; 3.67 (bs, 6H, CH_3O, $COOCH_3$) ; 3.35 (s, 2H, C<u>H$_2$</u>-COOH) ; 3.08 (m, 2H, C<u>H$_2$</u>-NH) ; 2.10 (2*s*, 3H, CH_3-C=) ; 1.72-1.30 (m, 4H, CH_2-CH_2).

Example 16 :

The amide of acetylsalicylic acid and N-ω-nitro-L-Arginine methyl ester (L-NAME)

N-ω-nitro-L-Arginine methyl ester hydrochloride (675 mg, 2.5 mmol) was suspended in anhydrous acetonitrile (15 ml), then 0.7 ml of triethylamine (5 mmol) was added under stirring. The resultant limpid solution was cooled to 0° C then acetyl salicoyl chloride (0.5 g, 2.5 mmol) in acetonitrile (8 ml) was added and a precipitate formed. The stirring was maintained for two hours at room temperature. Thereafter the precipitate was filtered off and the filtrate concentrated until dryness ; the obtained residue was chromatographed on silica column ($CHCl_3$/MeOH 95/5 as eluent), to yield the required compound (73 %) ; (white solid, m.p.=180° C).

^{1}H-NMR (100 MHz, $CDCl_3/D_2O$) :

8.10-6.90 (m, 4H, Ph) ; 4.85 (m, 1H, C<u>H</u>-COOH) ; 3.82 (s, 3H, OCH_3) ; 3.40 (m, 2H, C<u>H$_2$</u>-NH) ; 2.40 (s, 3H, CH_3-CO) ; 2.20-1.50 (m, 4H, CH_2-CH_2).

Example 17 :

The amide of sulindac and N-ω-nitro-L-Arginine methyl ester (L-NAME)

This compound has been prepared by using chloride of sulindac and N-ω-nitro-L-Arginine methyl ester, according to the method as described in example 16 (yield 70 %) ; (yellow solid, m.p. = 154-156° C).

^{1}H-NMR (100 MHz, $CDCl_3/D_2O$) :
7.30 (m, 4H, Ph-SO) ; 6.70 (m, 3H, Ph-F) ; 6.11 (m, 1H, =C-H) ; 3.60 (s, 3H, OCH_3) ; 3.15-3.05 (m, 5H, CH_2CON, $CHCO_2CH_3$, CH_2NH) ; 2.61 (s, 3H, CH_3SO) ; 2.05 (2s, 3H, CH_3-C=) ; 1.8-1.3 (m, 4H, CH_2-CH_2).

Example 18 :

The amide of ibuprofen and N-ω-nitro-L-Arginine methyl ester (L-NAME)

This compound has been prepared by using bromide of ibuprofen and N-ω-nitro-L-Arginine methyl ester according to the method as described in example 16 (yield 78 %) ; (white solid, m.p. = 213° C).
^{1}H-NMR (100 MHz, $CDCl_3/D_2O$) :
7 (m, 4H, Ph) ; 3.6 (s, 3H, $0CH_3$) ; 3.5-3.3 (m, 2H, C<u>H</u>CON, C<u>H</u>CO_2CH_3) ; 3.10 (t, 2H, $C\underline{H}_2N$) ; 2.35 (d, 2H, CH_2, Ph) ; 1.8-1.3 (m, 5H, CH_2-CH_2, C<u>H</u>$(CH_3)_2$) ; 1.2 (d, 3H, CH-C<u>H</u>$_3$) ; 0.8 (d, 6H, $2CH_3$).

The compounds of the invention have been subjected to some biological tests in vitro and in vivo, to prove their activity to block the nitric oxide synthase (constitutive and inducible) and the cyclooxygenase ; moreover, the combination is biologically more active than the simple association of the two active principles. Their activity has been also assessed in pathological models in animals ; they have been compared with reference substance such as aspirin, indomethacin and L-N^G-mono-methyl arginine (L-NMMA), and the simple association of these compounds.

1- *In vitro* effect on constitutive NO synthase in the isolated rat aorta :

Preparations of isolated rat aorta with endothelium were prepared as previously described (M. Auguet, S. Delaflotte, P.E. Chabrier and P. Braquet - Comparative effects of endothelium and phorbol 12-13 dibutyrate in rat aorta, Life Sciences, 1989, 45, 2051-2059).

Male Sprague Dawley rats (270-360 g, Charles River, Paris) were sacrified by cervical dislocation and the thoracic aorta removed and cleaned of the surrounding tissue. Rings 2 mm wide were suspended in organ baths containing 10 ml of physiological solution (for composition, see below) under a tension of 2 g at 37° C and gassed with O_2 / CO_2 (95 % / 5 %). Contractile responses were measured, using force displacement transducers (Statham UC_2) coupled to a Gould 8000 S polygraph. An equilibration period of one hour

was allowed before experimentation. Normal physiological solution was composed of (mM) : NaCl, 118 ; KCl, 4.7 ; $CaCl_2$, 2.5 ; KH_2PO_4, 1.2 ; $MgSO_4$, 0.6 ; $NaHCO_3$, 25 ; glucose, 11. After equilibration in normal medium, the preparation was subjected to a near maximal dose (about 95 %) of phenylephrine (PE, 1 µM). When the contraction was stable, carbachol (10 µM) was tested in order to evaluate the presence or absence of endothelium.

After washing the preparation and a further reequilibration period of 45 minutes, the preparation was subjected to PE (1 µM) and carbachol (10 µM) was administered to accomplish maximal relaxation. The antagonists were then tested in cumulative-dose-fashion and the IC_{50} (Inhibitory Concentration 50 %) to reverse the relaxation of carbachol was calculated. The results are summarised in the following table, paragraph 2-, on the first column of results, entitled "Test on constitutive NO synthase".

2- *In vitro* effect on inducible NO synthase in the isolated rat aorta :

As previously published (M. Auguet, J.M. Guillon, S. Delaflotte, E. Etiemble, P.E. Chabrier and P. Braquet - Endothelium independent protective effect of N^G-monomethyl-L-Arginine on endotoxin-induced alterations of vascular reactivity, Life Sciences, 1991, 48, 189-193), the compounds were tested on isolated rat aorta from shocked animal.

Male Sprague Dawley rats (240-320 g) were intrapertioneally injected with endotoxin (10 mg/kg) or with solvent (saline, 1 mg/kg). Three hours later, endotoxin-treated animals displayed the signs of endotoxemia including piloerection, diarrhea and lethargy. The rats were sacrified by cervical dislocation and the thoracic aorta removed and cleaned of the surrounding tissue. Rings 2 mm wide were suspended under a tension of 2 g in organ baths containing 10 ml of Krebs-Henseleit solution (mM) : NaCl, 118 ; KCl, 4.7 ; $CaCl_2$, 2.5 ; KH_2PO_4, 1.2 ; $MgSO_4$, 1.2 ; $NaHCO_3$, 25 ; glucose, 11. This solution was continuously gassed with O_2 / CO_2 (95 % / 5 %). The endothelium was mechanically disrupted by gently rolling a small forceps on the luminal surface of the rings. After an equilibration period of 90 minutes, contraction was induced by application of a maximal concentration of phenylephrine (PE, 1 µM). When contraction studies were accomplished, carbachol (10 µM) was tested in order to verify the integrity of endothelium (11). Antagonists were introduced into the bath 45 minutes before application of PE and the IC_{50} was calculated. The results are summarised in the following table, on the second column of results, entitled "Test on inducible NO synthase".

	Test on constitutive NO synthase	Test on inducible NO synthase
compounds	IC_{50} (M)	IC_{50} (M)
L-NMMA	2.10^{-5}	2.10^{-5}
aspirin	NA	NA
indomethacin	NA	NA
example 1	10^{-5}	2.10^{-5}
example 2	3.10^{-6}	9.10^{-6}
example 3	6.10^{-6}	6.10^{-6}
example 4	2.10^{-6}	10^{-5}
example 5	10^{-5}	5.10^{-6}
example 6	8.10^{-6}	8.10^{-6}
example 8	4.10^{-6}	10^{-6}
example 10	5.10^{-6}	6.10^{-6}
example 12	10^{-5}	4.10^{-6}
example 13	3.10^{-6}	10^{-6}
example 16	9.10^{-6}	4.10^{-6}
example 17	5.10^{-6}	5.10^{-6}

NA = Non Active

3- *In vitro* effect on inducible NO synthase in the lipopolysaccharides (LPS) treated vascular smoth muscle cells :

Some of the compounds were also tested on the smooth muscle cells in culture, where the NO synthase was induced by LPS (M. Auguet, M.O. Lonchampt, S. Delaflotte, P.E. Chabrier and P. Braquet - FEBS Letters, 1992, in press).

Smoth muscle cells were isolated by enzymatic (elastase and collagenase) digestion of rat thoracic aorta, as previously described (P.E. Chabrier, P. Roubert, M.O. Lonchampt, P. Plas and P. Braquet - J.Biol.Chem., 1988, 263, 13199-13202). They were cultured for 4 days in DMEM with 10 % foetal calf serum and used between passage 3 and 7. Cells monolayers were washed and the medium was replaced with 2 ml of DMEM containing 2 mM glutamine, antibiotics, 0.1 mM isobutylxanthine (IBMX), with or without LPS (Escherichia Coli). After 24 hours, cGMP was extracted from cells, by rapid aspiration of the medium and addition of 1 ml of 0.1 n HCl to each well. The samples were frozen until cGMP determination, by

radioimmunoassay (NEN kit). To study the inhibitory effect, cells were incubated for 24 hours in RPMI 1640 (the concentration of L-arginine was 1.2 mM), with or without LPS (0.1 µg/ml). IBMX (0.1 mM) was added 30 minutes before cGMP extraction, in presence or not of the tested substances (10^{-4} M). Diminution of cGMP production was measured (% of diminution) and the obtained results are summarised as follows.

	diminution of cGMP production (%)
control	0
L-NMMA	35
example 1	30
example 2	35
example 3	25
example 4	25
example 5	35
example 6	35
example 8	25
example 13	25
example 17	30

4- *In vitro* effect on arachidonic acid induced aggregation of washed rabbit platelets :

This protocol was used to assay the effect of the compounds on the cyclooxygenase. Measurement of platelet aggregation was carried out according to Cazenave et al. (Ann. Biol. Chem. 1983, 41, 167-179). Blood was taken from the auricular artery of male New Zealand rabbit (2.5 kg mean body weight) on ACD (citric acid / sodium citrate / dextrose) as anticoagulant. The washed platelets were prepared and then transferred in the cuvet of the aggregometer (Chronolog aggregometer Coultronics). Additions of the antagonists and arachidonic acid (0.5 mM) were made and percentage of transmission corresponding to aggregation (or its inhibition) was measured, in order to determine the IC_{50}. The results are summarised as follows, with the NA abbreviation meaning "non active".

compounds	IC_{50} (M)
aspirin	2.10^{-4}
indomethacin	2.10^{-5}
L-NMMA	NA
example 1	3.10^{-4}
example 2	$>5.10^{-4}$
example 3	2.10^{-4}
example 4	$>5.10^{-4}$
example 5	5.10^{-4}
example 6	4.10^{-5}

5- *In vitro* effect on nitrite production induced by LPS + INFγ on J774 A_1 monocyte / macrophage cell line :

Macrophage type cells like J774 A_1 cell line are interesting to used since they are important cells in inflammation and express large amount of NO (due to the induction of NO synthase) and cyclooxygenase products. They are activated with lipopolysaccharide (LPS) in presence of interferon γ (INFγ). This assay was used to compare the effects of compounds of the invention with the association of their separated parent compounds.

Murine monocyte/macrophage cells were grown in Dubecco's modified Eagle's medium at 37° C. The cells were plated in 24 well culture plate (NUNC) and were used for experiments at about 2 x 10^5 cells/plate. The cells were activated with LPS (1µg/ml) from E. Coli (SO55:B5) and murine recombinant IFNγ (50 U/ml) and then incubated in the presence or absence of the compounds. After 48 h nitrite (NO_2-) levels, which corrolate with the activation of NO synthase, were assessed in the culture media by the colorimetric method according to Green et al (L. Green, D. Wagner, J. Glogowski, P. Skipper, J. Wishwok and S. Tannenbaum, Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Analytical Biochemistry 126, 131-138, 1982).

The production of NO_2- was undetectable in absence or presence of the compounds when the cells were not activated. In activated cells, the IC_{50} for L-NMMA, L-NO and L-NAME were 8 x 10^{-6} M, 1.5 x 10^{-5} M and 10^{-3} M, respectively, whereas the cyclooxygenase inhibitor counterparts salicylic acid, acetyl salicylic acid, indomethacin, meclofenamate were virtually inactive, giving a non significative inhibition between 0.5 to 15 %.

To illustrate the more potent activity of the compounds in comparison with the association, some examples are presented in the following table, which presents the percentage of inhibition of nitrite production, induced by LPS + INF on J774 cell line :

Compounds	% OF INHIBITION		
	Concentration (M)		
	10^{-6}	10^{-5}	10^{-4}
L-NMMA + acetyl salicylic acid	8 %	27 %	29 %
Example 1	43 %	48 %	67 %
L-NMMA + salicylic acid	11 %	29 %	33 %
Example 2	53 %	61 %	75.5 %
L-NO + acetyl salicylic acid	2 %	44 %	40 %
Example 3	46 %	49 %	76 %
L-NMMA + indomethacin	15 %	34 %	29 %
Example 10	51 %	63 %	66 %
L-NO + sulindac	21 %	37 %	40 %
Example 13	56 %	63 %	71 %
L-NAME + sulindac	7 %	24 %	29 %
Example 17	49 %	57 %	73 %

The results show that compounds of the invention are more active, at equivalent concentration, than the association of the separate parent compounds from which they are originated. It indicates also a potentialising effect of the combination of a NO synthase inhibitor and a cyclooxygenase inhibitor.

6- *In vitro* effect on prostaglandin production induced by LPS + IFNγ on J774A1 monocyte / macrophage cell line :

In the same experiments as above described, we compared the effects of compounds of the invention with the assocation on the production of cyclooxygenase products, in order to verify if the enhancement in activity of the compounds are also correlated on the inhibition of cyclooxygenase.

Levels of one of the main cyclooxygenase products produced by J774A1 cell line (i.e. : 6 keto $PGF_{1\alpha}$) the stable metabolite of PGI_2 were assessed in the culture media by a specific radioimmunoassay (NEN, Kit NEK 025).

Firstly, it was observed that the release of 6 keto $PGF_{1\alpha}$ in the culture media was not affected by L-NMMA, L-NAME or L-NO but abolished in a dose dependent manner with indomethacin and aspirin with an approximate IC_{50} of 10^{-6}M and 10^{-5}M.

The percentage of inhibition of 6-keto PG F1 production induced by LPS + INF in J774A1 cell line, are presented in the following table, which shows that combinations present a greater activity than the association.

Compounds	% OF INHIBITION			
	Concentration (M)			
	10^{-7}	10^{-6}	10^{-5}	10^{-4}
L-NO + Acetyl salicylic acid	-	30 %	58 %	74 %
Example 3	-	58.5 %	78.5 %	91.5 %
L-NMMA + Indomethacin	55 %	85 %	84 %	-
Example 10	94 %	97 %	100 %	-
L-NAME + Sulindac	-	37 %	48 %	63 %
Example 17	-	74 %	83 %	92 %

7- *In vitro* effect on nitrite production from murine activated macrophages :

To confirm the results obtained in J774 cell line showing a more potent activity of the synthetized compounds when compared with the association of an inhibitor of cyclooxygenase with an inhibitor of NO synthase, similar experiments were performed on murine activated macrophages.

Peritoneal macrophages were obtained from the peritoneal cavity of femal BBA/2 mice at 7-8 week of age, 3 days after injection of thioglycollate (3 % 1.5 ml/mouse). Macrophages (2.10^5 cells/well) were activated at 37° C with LPS (E. Coli : 0111B4) (0.1 µg/ml) and murine recombinant INFγ (100 U/ml) in wells of a 96 well-microplate for 20 h on RPMI 1640, 10 % FBS, then washed and further incubated for 24 h. Cells were incubated in absence or presence of the different compounds and nitrite levels were measured in the culture media as already described.

The percentage of the variation compared to the control, is of from 56 % for the combination of indomethacin and L-NMMA (i.e. example 10), 32 % for the association of indomethacin and L-NMMA, and less than 30 % for each separate compound.

Moreover, the percentage is of from 48 % for the combination of indomethacin and L-NAME (i.e. example 15), 25 % for indomethacin, and less than 15 % for L-NAME and the association.

8- *In vitro* effect on lethality induced by NMDA (N-Methyl-D-Aspartate) :

Glutamate and aspartate are important neuroexcitotix mediators which are involved in cerebral ischemia. Their actions are notably mediated through the activation of NMDA receptor. IV injection of a high dose of NMDA induced the mortality in nice in less than 35 sec. Any compounds which can delay the time of lethality in this test are considered as potential antiischemic compounds. Since the effects of glutamate or aspartate are possibly mediated by an exaggerated release of NO, we used this test to screen the compounds and to differentiate in vivo the effect of a combination and an association of the separate compounds.

Male OF1 mice (20-22 g, Charles River) were injected with 250 mg/kg NMDA (iv) 1 h after administration by oral route of the substances. Time of survival was measured.

Thus, the combination is much more active than the separate compounds, alone or in association. It also shows a synergism with the combination.

COMPOUNDS	DOSE (mg/kg IP)	% OF PROTECTION
Salicylic acid	4	-
L-NMMA	5.7	6
Salicylic acid + L-NMMA	(4.0 + 5.7)	6
Example 2	10.0	66
Salicylic acid	0.4	-
L-NMMA	0.57	3
Salicylic acid + L-NMMA	(0.4 + 0.57)	3
Example 2	1	63
sulindac	6.4	18.2
L-NMMA	3.4	5.4
sulindac + L-NMMA	(6.4 + 3.4)	20
Example 7	10	54.5

indomethacin	6.55	-
L-NMMA	3.40	4.5
indomethacin + L-NMMA	(6.55 + 3.40)	4.5
Example 10	10	33.5
Salicylic acid	3.7	-
L-NAME	6.3	5
Salicylic acid + L-NAME	(3.7 + 6.3)	5
Example 14	10	26

9- *In vivo* effect on neuronal death after focal cerebral ischemia in mice :

Systemic intraperitoneal (ip) administration of the compounds was carried out 5 hours after cortical infarction induced by the occlusion of the middle cerebral artery in male Swiss mice (20-22 g), according to Duverger et al. - Pharmacology of cerebral ischemia in Krieglstein and Oberpichler, Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1990, 409-413. Four days later, the mice were decapitated and their brain removed and frozen to be then sectioned in coronal slices of 10 µM thickness. Area infarction was measured by image analysis. Reduction of infarcted volume was measured and compared with non treated animal. The percentage indicated the mean reduction of infarct from six animals per group - MK 801, a NMDA antagonist was used as a control substance. The obtained results are as follows:

	Reduction of infarct
MK 801 (3 mg/kg)	-51 %
example 1 (3 mg/kg)	-68 %
example 2 (3 mg/kg)	-55 %
exampie 3 (3 mg/kg)	-70 %
example 10 (3 mg/kg)	-64 %
example 17 (3 mg/kg)	-69 %

10- *In vivo* effect on endotoxin treated pithed rat :

As previously reported, the association of an inhibitor of NO synthase and an inhibitor of cyclooxygenase have a synergistic effect to restore the blood pressure and vascular reactivity in endotoxinic or septic animal.

Male Sprague Dawley rats (body weight 280-320 g) were pithed. One hour after pithing animals were given endotoxin (EDTX, Escherichia Coli, lipopolysaccharide, O III, B_4=300µg/kg/h) for 60 minutes. This resulted in an important hypotension and a loss of vascular reactivity to vasopressor agents, such as methoxamine. After 60 minutes of perfusion of the compounds with endotoxin, a dose response curve to methoxamine was constructed in a cumulative fashion and an ED50 value (50 % of the effective dose to restore normal activity to methoxamine) was determined by regression analysis for each animal. The obtained results are as follows:

Compounds	Dose mg/kg/h	vascular reactivity to methoxamine DE_{50} (µg/kg)
control		79 ± 9
EDTX treated animal		278 ± 34
L-NMMA	50	189 ± 15
aspirin	150	136 ± 29
example 1	30	82 ± 18
example 2	30	98 ± 35
example 3	30	76 ± 12
example 4	30	72 ± 11

Toxicology :

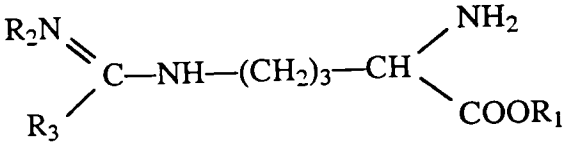
Products were administered per os (p.o.) or by intraperitoneal route (i.p.), in groups of 10 mice with increasing doses. LD_{50} (lethal dose 50 %) of animals are comprised of from 100 and 1000 per os, and of from 150 and 500 by i.p.

Posology :

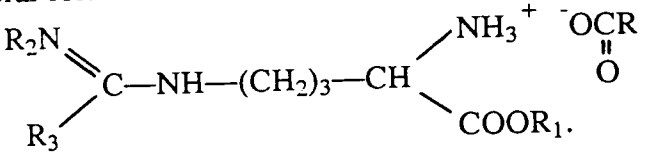
The compounds of the invention may be administered at a dose of from 1 to 300 mg per diem.

CLAIMS

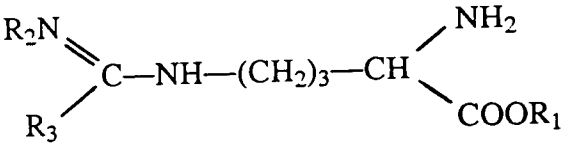
1. A salt of a cyclooxygenase inhibitor and the L-form of an arginine analogue,
the cyclooxygenase inhibitor having the general formula RCOOH wherein the carboxy group COOH is an accessible acidic function and R stands for the appropriate radical of the cyclooxygenase inhibitor,
the L-form of an arginine analogue having the general formula



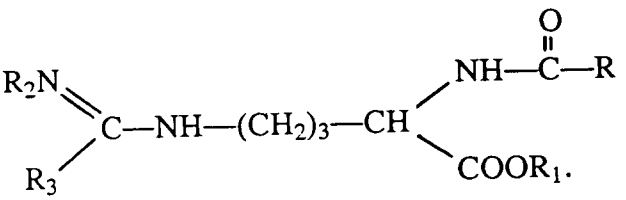
wherein R_1 represents a hydrogen atom or a methyl or ethyl group, R_2 represents a hydrogen atom or a nitro group and R_3 represents an amino, methylamino, ethylamino, hydrazino, methyl or ethyl group, with the proviso that if R_2 represents a hydrogen atom then R_3 does not represent an amino group,
the salt having the general formula



2. An amide of a cyclooxygenase inhibitor and the L-form of an arginine analogue, the cyclooxygenase inhibitor having the general formula RCOOH wherein the carboxy group COOH is an accessible acidic function and R stands for the appropriate radical of the cyclooxygenase inhibitor, the arginine analogue having the general formula



wherein R_1 represents a hydrogen atom or a methyl or ethyl group, R_2 represents a hydrogen atom or a nitro group and R_3 represents an amino, methylamino, ethylamino, hydrazino, methyl or ethyl group,
the amide having the general formula



3. A compound according to either preceding claim wherein the cyclooxygenase inhibitor is salicylic acid, acetylsalicylic acid, mefenamic acid, ibuprofen,

indomethacin or sulindac.

4. A compound according to any preceding claim wherein the arginine analogue is N-ω-monomethyl-L-arginine, N-ω-nitro-L-arginine or N-ω-nitro-L-arginine methyl ester.

5. The salt according to claim 1 of acetylsalicylic acid and N-ω-monomethyl--L-arginine.

6. The salt according to claim 1 of salicylic acid and N-ω-monomethyl-L-arginine.

7. The salt according to claim 1 of acetylsalicylic acid and N-ω-nitro-L-arginine.

8. The salt according to claim 1 of salicylic acid and N-ω-nitro-L-arginine.

9. The salt according to claim 1 of acetylsalicylic acid and N-ω-nitro-L-arginine methyl ester.

10. The salt according to claim 1 of indomethacin and N-ω-nitro-L-arginine.

11. The salt according to claim 1 of sulindac and N-ω-monomethyl-L-arginine.

12. The salt according to claim 1 of ibuprofen and N-ω-nitro-L-arginine.

13. The salt according to claim 1 of mefenamic acid and N-ω-nitro-L-arginine.

14. The salt according to claim 1 of indomethacin and N-ω-monomethyl-L-arginine.

15. The salt according to claim 1 of sulindac and N-ω-nitro-L-arginine methyl ester.

16. The salt according to claim 1 of mefenamic acid and N-ω-monomethyl-L-arginine.

17. The salt according to claim 1 of sulindac and N-ω-nitro-L-arginine.

18. The salt according to claim 1 of salicylic acid and N-ω-nitro-L-arginine methyl ester.

19. The salt according to claim 1 of indomethacin and N-ω-nitro-L-arginine methyl ester.

20. The amide according to claim 2 of acetylsalicylic acid and N-ω-nitro-L-arginine methyl ester.

21. The amide according to claim 2 of sulindac and N-ω-nitro-L-arginine methyl ester.

22. The amide according to claim 2 of ibuprofen and N-ω-nitro-L-arginine methyl ester.

23. A process for the preparation of a salt according to claim 1, the process comprising reacting, in substantially equimolar proportions in water or in a mixture of water and alcohol, at a temperature of from ambient temperature to the boiling point of the reaction mixture, a cyclooxygenase inhibitor as defined in claim 1 with an arginine analogue as defined in claim 1.

24. A process for the preparation of an amide according to claim 2, the process comprising reacting, in substantially equimolar proportions in acetonitrile in the presence of a base, at a temperature of from 0°C to ambient temperature, an arginine analogue as defined in claim 2 and a compound RCOX wherein R is as defined in claim 2 and X represents a halogen atom.

25. A pharmaceutical composition comprising a compound according to any of claims 1 to 22 in admixture with a pharmaceutically acceptable diluent or carrier.

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**** END OF REGISTER ENTRY ****

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