Title: USE OF CARBOXY COMPOUNDS SUCH AS 2-(4-ACETOXYPHENYL)-2-CHLORO-N-METHYL-ETHYLAMMONIUM CHLORIDE AS ANTI-INFLAMMATORY AGENTS

Abstract: The present invention relates to the use of specific carboxy compounds, more specifically to the use of 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, in the treatment of inflammatory diseases. Part of the invention is also a composition, preferably a pharmaceutical composition, comprising as active ingredient at least 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride together with (pharmaceutical) acceptable excipients.
Use of carboxy compounds such as 2(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride as anti-inflammatory agents

Field of the invention

The present invention relates to the use of specific carboxy compounds, more specifically to 2(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, to treat inflammatory diseases.

Background of the invention

Synthetic glucocorticoids remain among the most effective agents for the treatment of chronic inflammatory diseases. However, major side effects severely limit their therapeutic use. Physiologic and therapeutic activities of glucocorticoids are mediated by a nuclear receptor belonging to a superfamily of ligand-inducible transcription factors that, in addition to directly regulating their cognate gene programs, can also interfere with other signalling pathways, such as those utilizing NF-κB.

NF-κB is an inducible transcription factor complex which regulates the expression of various genes involved in inflammatory and immune responses. It is activated upon exposure of cells to pro-inflammatory cytokines (TNF, IL-1), oxidants (hydrogen peroxide, ozone, superoxide anions), bacterial compounds (LPS), viral products (dsRNA, HTLV-I Tax protein), PKC activators (phorbol esters, platelet activating factor) and UV- or γ-irradiation.

NF-κB is a promising target for anti-inflammatory and immunosuppressive therapies. Inhibition of NF-κB activity by glucocorticoids (GC) has been well documented, although gene stimulatory effects by GC have also been observed.

Although GC remain, as mentioned above, among the most potent immunosuppressive and anti-inflammatory drugs currently available, and are especially effective in the treatment of chronic asthma or rheumatoid arthritis, side effects such as hypothalamic-pituitary-adrenal axis insufficiency, diabetes, altered lipid metabolism, steroid myopathy, osteoporosis, and infectious and neuropsychiatric complications limit the therapeutic use of classical glucocorticoid agonists. Therefore there is a need to investigate and search for novel compounds that have anti-inflammatory properties without having severe side-effects.

Compound A (CpdA) or 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride is a stable analogue of the hydroxyphenylaziridine precursor found in the
Namibian shrub Salsola tuberculiformis Botschantzhev (Louw, 1997). Contraceptive properties have been attributed to plants of the Salsola genus as early as 1902 and recognized and passed on through oral Bushman tradition (Brondegaard, 1973). Feeding experiments with the shrub indeed caused contraceptive effects in rats and prolonged gestation in sheep (van der Merwe, 1976; Basson, 1969). A study by van der Merwe et al. (1976) led also to the isolation of an active but very labile HPLC fraction from the dried plant material, a hydroxyphenylaziridine, and synthesis of a more stable but biologically active analogue, CpdA (Louw, 1997). Recent work performed by the group of Louw and co-workers set out to unravel the contraceptive mechanism of action and the molecular targets of this desert plant derivative analogue. Multiple levels of interference with endogenous glucocorticoid action were observed. From their results, it was concluded that CpdA disrupts the oestrus cycle of rats by interacting with glucocorticoid-binding proteins such as steroidogenic enzymes and plasma steroid-binding globulins, thereby altering the interaction between the hypothalamus, pituitary, adrenal gland and gonads (HPA axis versus HPG axis) (Louw, 1997 and 1999).

The present invention relates to the surprising finding that carboxy compounds such as CpdA display a specific anti-inflammatory effect to a similar extend as glucocorticoids without having the severe side effects of glucocorticoids.

**Brief description of figures**

**Fig. 1.** L929sA cells with the stably integrated reporter gene construct p1168hu.IL6P-luc+ were either treated with 1µM of DEX, 0.1 or 1µM of CpdA, as indicated, in absence or presence of 2000IU/ml TNF. DEX or CpdA was added 2 hours before TNF for a total induction period of 8 hours. Cell lysates were assayed for luc activities and normalized for protein content. Promoter activities are expressed as ‘induction factor’, i.e. the ratio of expression levels recorded either under induced and non-induced conditions. Assays were performed in triplicate and results are representative of at least two independent induction experiments.

**Fig. 2.** L929sA cells with a stably integrated p1168(kBmut)IL6P-luc+ construct were either treated with 1µM DEX, 0.1, 1 or 10µM CpdA, in absence or presence of 60nM
Staurosporine (STS). Induction protocol and plotting of the results is similar as in Fig. 1.

**Fig. 3.** L929sA cells with the stably integrated reporter gene construct p1168(AP-1-mut)IL6P-luc+ were either treated with 1µM of DEX, 1 or 10µM of CpdA, as indicated, in absence or presence of 2000IU/ml TNF. DEX or CpdA was added 2 hours before TNF for a total induction period of 8 hours. Induction protocol and plotting of the results is similar as in Fig. 1.

**Fig. 4.** L929sA cells with the stably integrated reporter gene construct p(IL6κB)₃50hu.IL6P-luc+ were either treated with 1µM of DEX, 0.1, 1 or 10µM of CpdA, as indicated, in absence or presence of 2000IU/ml TNF. DEX or CpdA was added 2 hours before TNF for a total period of 8 hours. Induction protocol and plotting of the results is similar as in Fig. 1.

**Fig. 5.** L929sA cells with a stably integrated pE-selectin-Luc construct were either treated with 1µM DEX, 1 or 10µM CpdA, in absence or presence of 2000IU/ml TNF. Induction protocol and plotting of the results is similar as in Fig. 1.

**Fig. 6.** L929sA cells with a stably integrated pICAM-Luc construct were either treated with 1µM DEX, 1 or 10µM CpdA, in absence or presence of 2000IU/ml TNF. Induction protocol and plotting of the results is similar as in Fig. 1.

**Fig. 7.** L929sA cells with a stably integrated p(GRE)₂-50-luc+ construct were induced with 1µM DEX, in absence or presence of 0.1 or 1µM CpdA for a total induction period of 8 hours. Plotting of the results is similar as in Fig. 1.

**Fig. 8.** A murine IL-6 ELISA was performed using the supernatant culture medium of subconfluent induced cells. Cells were treated with 1µM DEX or 10 µM CpdA, in absence or presence of 2000IU/ml TNF. DEX or CpdA was added 2 hours before TNF, for a total induction period of 8 hours. This figure is representative of two independent experiments.

**Fig. 9.** A to D. HEK293T cells were transiently transfected with 100ng of p(IL6kB)₃50hu.IL6P-luc+ (A and B) or pNF-kB-Luc (C and D), 100ng of a β-Galactosidase control plasmid and 200ng of Mock DNA (A and C) or pSVhGRα (B and D). After transfection, cells were pre-incubated for 2 hours with 1µM DEX and 10µM CpdA and RU486, where appropriate. Subsequently, TNF (2000IU/ml) was added, where necessary, and inductions were continued for another 6 hours. Plotting of the results is as described for Fig. 1, except that the non-induced state is taken as 100.
**Fig 10.** L929sA cells were either left untreated or treated with DEX (1μM) and various concentrations of CpdA, as indicated (in μM), alone or together with TNF (2000IU/ml). DEX and CpdA were added 2 hours before TNF, for a total period of 4 hours. The total protein extract was incubated with a 32P-labeled IL-6 NF-κB response element and protein-DNA complexes were analyzed in an EMSA. Arrowheads indicate the activated κB complex, the constitutively expressed recombination binding protein (RBP)-Jκ and the free probe. Composition of NFκB complexes has been described in detail in (Vanden Berghe et al., 1999 J Biol. Chem. 274: 32091-32098, Plaisance et al., 1997 Mol. Cell. Biol. 17: 3733-3743).

**Fig 11.** L929sA cell lines with various stably transfected Gal4 constructs, as indicated in the graph, were transiently transfected with the p(Gal)2-50hu.II6P-luc+ reporter gene. After transfection, cells were either left untreated or treated with DEX (1μM) and various concentrations of CpdA, as indicated (in μM).

**Fig 12.** L929sA cells are treated with either 1μM DEX, 1 or 10μM CpdA and/or 2000IU/ml TNF, where indicated. Cell lysates were made and activated JNK, p38 or ERK were detected using the corresponding phospho-specific MAPK antibodies (Figure 12 A, B and C respectively). The upper band in 12A is a nonspecific, constitutive band which actually serves as an additional loading control.

**Detailed description of the invention**

The current invention concerns the use of a carboxy compound having the following formula (I) or its aziridine derivative as a medicament:

![Chemical Structure](image)

, wherein R is a hydrogen or carboxy group, X is a hydrogen, hydroxy or halogen such as chloride, bromide and fluoride and Y is a lower alkyl group such as methyl, ethyl, propyl and butyl. Preferably the R group is an acetoxy, X a halogen, most preferably a chloride, and Y a methyl. The latter compounds can surprisingly be used as anti-inflammatory agents to treat inflammatory diseases. These compounds can also be used as agent to treat diseased states related to NF-κB- and/or AP-1-mediated gene expression.

**The invention further relates to compositions, preferably pharmaceutical compositions, comprising as an active ingredient at least one of the compounds as defined in**
formula (I) together with (pharmaceutically) acceptable excipients. Unexpectedly, it appears that compounds having the formula (I), preferably those wherein the R group is an acetoxy, X is a halogen most preferably a chloride and Y is a methyl, demonstrate a strong inhibiting activity of NF-κB-driven genes, but exert a weak or no activity towards the glucocorticoid response element (GRE)-dependent genes. The preferred compound exerting this activity is 2(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride and has a molecular weight of 264.14. This compound is hygroscopic and sensitive to light; it should be kept in the freezer (-4°C) and may not be exposed to light. This compound indeed starts to cyclise to the corresponding aziridine, when kept in solution (especially at pH5 and above) as described by Louw et al. 1997 (Biochem Pharmacol 24:167-75).

The term ‘compound’ (or ‘drug-like compound’) is well known to those skilled in the art and includes compounds suitable for use in medicine, for example as the active ingredient in a medicament. The compounds of the present invention can be purified from plants as indicated above but may also be synthesized by any known technique in organic chemistry, molecular biology or biochemistry. More specifically, the compounds of the present invention can be synthesized as described by Louw et al. 1997 (Biochem Pharmacol 24:167-75).

It is clear that the compounds of the present invention can also be used as “lead compounds”. That is, they may provide a starting-point for the design of other compounds that may have other characteristics. The compounds of the present invention have anti-inflammatory, analgesic and antipyretic activities comparable with glucocorticoids, but avoid side effects and are useful in methods of treating diseases or conditions in which NF-κB/IL6 have been implicated. Such diseases and conditions include those in which inflammation or tissue injury is involved such as osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and other rheumatologic and pain indications. Other diseases in which inflammation is involved and in which the compounds of the present invention are useful include asthma, psoriasis, septic shock and inflammatory bowel disease. It is further believed that NF-κB is involved in diseases in which apoptosis is involved. As such, the compounds of the present invention are useful to limit tissue and/or cell damage and may be beneficial for ischaemic disease, neural injury and myocardial infarction. The compounds of the invention are useful in the treatment or prevention of
Alzheimer's disease. They delay the onset or slowing the progression of Alzheimer's disease, even when used in low amounts.

The aforementioned compounds of the invention or compositions (formulations) thereof may be administered by any conventional method including oral and parenteral (e.g. subcutaneous, intraperitoneal, intravascular or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time.

Thus, the present invention involves a method of treating a subject having inflammation or an inflammation-related disorder with a therapeutically effective amount of a compound of the present invention. The invention is useful for, but not limited to, the treatment of inflammation in a subject, and for treatment of other inflammation-associated disorders such as pain, headaches or fever. As already indicated above, the compounds of the present invention are useful to treat arthritis, including but not limited to rheumatoid arthritis, spondyloarthritis, gouty arthritis, osteoarthritis, systemic lupus erythematosus, and juvenile arthritis. The compounds of the present invention are also useful in the treatment of asthma, bronchitis, menstrual cramps, tendinitis, bursitis, and skin related conditions such as psoriasis, eczema, acne, burns and dermatitis. Moreover, the compounds of the present invention are useful to treat gastrointestinal conditions, such as inflammatory bowel disease, Crohn's disease, gastritis, irritable bowel syndrome and ulcerative colitis and for the prevention or treatment of cancer, such as colorectal, breast cancer, prostate cancer or leukemia. The compounds of the present invention are further useful in treating inflammation in such diseases as vascular diseases, migraine headaches, periarteritis nodosa, thyroiditis, aplastic anemia, Hodgkin's disease, chronic lymphocytic leukemia, scleroderma, rheumatic fever, type I diabetes, myasthenia gravis, multiple sclerosis, sarcoidosis, nephrotic syndrome, Bechet's syndrome, polymyositis, gingivitis, hypersensitivity, swelling occurring after injury, myocardial ischemia and the like. In addition, the compounds of the present invention are useful in the treatment of ophthalmic diseases, such as retinitis, retinopathies, conjunctivitis, uveitis, ocular photophobia, and of acute injury to the eye tissue. The compounds of the present invention are also useful in the treatment of pulmonary inflammation, such as that associated with viral infections and cystic fibrosis. The compounds of the present invention are further useful for the treatment of certain central nervous system disorders such as cortical dementia including Alzheimer's disease. It should be stressed that the compounds of the present invention are useful as anti-inflammatory
agents with the additional benefit of having significantly less harmful glucocorticoid side effects! Further, the compounds of the present invention may also be useful in the treatment of allergic rhinitis, respiratory distress syndrome, endotoxin shock syndrome, atherosclerosis and central nervous system damage resulting from stroke, ischemia and trauma. Besides being useful for human treatment, these compounds are also useful for treatment of mammals, including horses, dogs, cats, rats, mice, sheep, pigs, etc.

The phrase “therapeutically-effective” is intended to qualify the amount of agent for use in the therapy which will achieve the goal of improvement in inflammation severity and avoiding side effects typically associated with alternative therapies.

Also embraced within this invention are compositions comprising the compound of the present invention in association with one or more non-toxic, (pharmaceutically-acceptable) carriers and/or diluents and/or adjuvants (collectively referred to herein as ‘excipients’) and, if desired other ingredients. The active compounds and compositions may, for example be administered orally, intra-vascularly, intra-peritoneally, sub-cutaneously, intra-muscularly or topically. For oral administration, the (pharmaceutical) composition may be in the form of, for example, a tablet, capsule, suspension, aerosol or liquid. The pharmaceutical composition is preferably made in the form of a dosage unit containing a particular amount of the active ingredient. Examples of such dosage units are tablets or capsules. The active ingredient may also be administered by injection as a composition wherein, for example, saline, dextrose or water may be used as a suitable carrier.

The amount of therapeutically active compounds that are administered and the dosage regimen for treating a disease condition with the compounds and/or compositions of this invention depends on a variety of factors, including the age, weight, sex and medical condition of the subject, the severity of the disease, the route and frequency of administration, and the particular compound employed, and thus may vary widely. The pharmaceutical compositions may contain active ingredients in the range of about 0.1 to 2000 mg, preferable in the range of about 0.5 to 500 mg and most preferably between about 1 and 100mg. A daily dose of about 0.01 to 100 mg/kg body weight, preferably between about 0.5 and 20 mg/kg body weight and most preferably between about 0.1 to 10 mg/kg body weight, may be appropriate. The daily dose can be administered in one to four doses per day.
In the case of psoriasis and other skin conditions, it may be preferable to apply a topical preparation of compounds of this invention to the affected area two to four times a day.

For inflammations of the eye or other external tissues, e.g. mouth and skin, the formulations are preferable applied as a topical ointment or cream, or as a suppository, containing the active ingredients in a total amount of, for example 0.075 to 30% w/w, preferably 0.2 to 20% w/w and most preferably 0.4 to 15% w/w. When formulated in an ointment, the active ingredients may be employed with either paraffinic or a water-miscible ointment base. Alternatively, the active ingredients may be formulated in a cream with an oil-in-water cream base. If desired, the aqueous phase of the cream base may include, for example at least 30% w/w of a polyhydric alcohol such as propylene glycol, butane-1-diol, mannitol, sorbitol, glycerol, polyethylene glycol and mixtures thereof. The topical formulation may desirably include a compound which enhances absorption or penetration of the active ingredient through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethyulsulfoxide and related analogs. The compounds of this invention can also be administered by a transdermal device. Preferably topical administration will be accomplished using a patch either of the reservoir and porous membrane or of a solid matrix variety. In either case the active agent is delivered continuously from the reservoir or microcapsules through a membrane into the active agent permeable adhesive, which is in contact with the skin or mucosa of the recipient. If the active agent is absorbed through the skin, a controlled and predetermined flow of the active agent is administered to the recipient. In the case of microcapsules, the encapsulating agent may also function as the membrane.

The oily phase of the emulsions of this invention may be constituted from known ingredients in a known manner. While the phase may comprise merely an emulsifier it may comprise a mixture of at least one emulsifier with a fat or an oil or with both a fat and an oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier that acts as a stabilizer. It is also preferred to include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make-up the so-called emulsifying wax, and the wax together with the oil and fat make up the so-called emulsifying ointment base that forms the oily dispersed phase of the cream formulations. Emulsifiers and emulsion stabilizers suitable for use in the formulation of the present invention include Tween60, Span80, cetostearyl alcohol, myristyl alcohol,
glyceryl monostearate, and sodium lauryl sulfate, among others. The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations is very low. Thus, the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters may be used. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredients are dissolved or suspended in suitable carrier, especially an aqueous solvent for the active ingredients. The anti-inflammatory active ingredients are preferably present in such formulations in a concentration of 0.5 to 20%, advantageously 0.5 to 10% and particularly about 1.5% w/w. For therapeutic purposes, the active compounds of this combination invention are ordinary combined with one or more adjuvants appropriate to the indicated route of administration. If administered per os, the compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and then tableted or encapsulated for convenient administration. Such capsules or tablets may contain a controlled-release formulation as may be provided in dispersion of active compound in hydroxypropylmethylcellulose. Formulations for parental administration may be in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions and suspensions may be prepared from sterile powders or granules having one or more of the carriers or diluents mentioned for use in the formulations for oral administration. The compound may be dissolved in water, polyethylene glycol, propylene glycol, ethanol, corn oil, cottonseed oil, peanut oil, sesame oil, benzyl alcohol, sodium chloride, and/or various buffers. Other adjuvants and modes of administration are well and widely known in the pharmaceutical art. Although these compounds have been
described with respect to specific embodiments, the details of these embodiments are not to be construed as limitations.

The invention is hereunder further explained by way of an ‘Examples’ section, without being restrictive in the scope of the current invention.

5

Examples

Materials & Methods

Cytokines and reagents

Recombinant murine TNF, produced in our laboratory, has a specific biological activity of $1.3 \times 10^8$ units/mg protein and contained $<1.8$ ng endotoxin/mg protein. The specific activity was determined by a standardized cytotoxicity assay on 164 WEHI cl 13 cells compared with an international standard TNF preparation (National Institute for Biological Standards and Control, Potters Bar, UK). Dexamethasone was purchased from Sigma. A stock solution of the reagent was routinely prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at 4°C. The same procedure was followed for RU38486, which was described previously (Vanden Berghe et al., 1999). Staurosporine (STS) (Tamaoki et al., 1986; Ruegg et al., 1989) was purchased from Calbiochem-Novabiochem International (San Diego, CA) and was stored as a 2mM solution in DMSO at −20°C. Compound A or 2(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride has a molecular weight of 264.14. A stock solution was prepared in DMSO, aliquoted and stored at 4°C in the dark.

Control experiments verified that the final concentration of organic solvent did not interfere with any of the assays.

The phospho-specific p38 (Thr-180/Tyr-182), p42/p44 (Thr-202/Tyr-204) and SAPK/JNK (Thr-183/Tyr-185) MAPK polyclonal rabbit antibodies detect only the dual phosphorylated form of MAPK. They were purchased from New England Biolabs (Beverly, MA, USA) as part of a kit, which also includes anti-rabbit IgG coupled to hors eradish peroxidase, used as a second antibody for Western blotting.

Luciferase (luc) reagent comprised 270μM CoA (Sigma), 470μM luciferin (Sigma), and 530μM ATP (Boehringer Mannheim) in 10mM TRICINE, 0.54mM (MgCO3)4Mg(OH)2, 1.34mM MgSO4, 0.05mM EDTA, 16.7mM DTT (all from Sigma).

Plasmids. p1168hu.IL6P-luc+, p1168(AP-1mut)IL6P-luc+, p1168(κBmut)IL6P-luc+ and p(IL6κB)50hu.IL6P-luc+ have been described previously (Plaisance, 1997; Vanden
Berghe, 1998). pE-selectin-Luc was a kind gift from Dr. D. Goeddel (Tularik). pSVhGRα and pMMTV-Luc were a generous gift from Dr. W. Rombauts (Leuven). pNFκB-Luc was purchased from Stratagene Cloning Systems (La Jolla, CA). p(GRE)_{2}50hu.IL6P-luc+ was obtained by replacing the κB motifs in p(IL6κB)_{3}50hu.IL6P-luc+ with a linker region, containing two consensus GRE (underlined) sites flanked by a BglII and PstI restriction site. The following oligonucleotides were annealed: AGATCTCTCTGCTGTACAGGATGTTCATACGGAT CCTGCTGTACAGGAGTTCTAGCTACCTGAC and TCTAGAGAGACGACATGTGC TACAAGATCGCCTAGGACGACATGTCTCTACAGATCGATGGACGTC. The 10 plasmids pGal4, pGal4-p65 and pGal4-VP16 were generously provided by Dr. M. L. Schmitz (DKFZ, Heidelberg). p(Gal)_{2}-50hu.IL6P-luc+ was previously described (De Bosscher, 1997).

**Transfection procedure.** L929sA mouse fibroblast cells were stably transfected by the calcium phosphate precipitation procedure according to standard protocols (Graham et al., 1973; Vanhoenacker et al., 1994) using a 10-fold excess of the plasmid of interest over the selection plasmid pPGKβGeobpA. Transfected cells were selected in 500μg/ml G418 for two weeks. Subsequently, resistant cell clones were pooled in order to level out individual clonal variation in expression and thus providing a reliable response upon induction. The cotransfected plasmid pPGKβGeobpA, conferring resistance to G418 and expressing constitutive β-galactosidase enzymatic activity, was further used as an internal control for calculating the protein concentration. L929sA cells were transiently transfected with the DEAE-dextrane transfection method, essentially as described earlier (De Bosscher et al., 1997). HEK293T human embryonic kidney cells were transiently transfected with the calcium phosphate precipitation technique. Briefly, 10^{5} actively growing cells were seeded in 24-well plates 24 hr before transfection and 400ng of total DNA was transfected. 16 hr after transfection the medium was replaced with fresh medium, containing 5% fetal and 5% newborn calf serum (Life Technologies, Paisley, UK), containing the appropriate inducing agents. Cells were lysed with lysis buffer (TROPIX, Bedford, MA) and samples were assayed for their protein or β-galactosidase content and luciferase activity.

**Reporter Gene Analysis.** Luciferase assays were carried out according to the instructions of the manufacturer (Promega Biotech). Light emission was measured in a
luminescence microplate counter (Top-Count; Packard Instrument Co., Meriden, CT). Luciferase activity, expressed in arbitrary light units, was corrected for the protein concentration in the sample by normalization to the co-expressed β-galactosidase levels. β-galactosidase protein levels were quantified with a chemiluminescent reporter assay Galacto-Light kit (TROPIX, Bedford, MA). Promoter activities are expressed as relative ‘induction factor’, i.e. the ratio between the expression levels of induced versus the non-induced state, with the latter taken to be 1.

Cell culture. 10^5 cells were plated out in 24-well plates and inductions were done at least in triplicate for each independent experiment, which was carried out twice. Inductions with DEX or CpdA at the indicated concentrations were added at –2h for a total of 8h, while TNF (2000IU/ml) or STS (60nM) was added at time point zero and left on the cells for 6h.

Lysates were made by washing the cells once with PBSA followed by addition of 100μl of lysis buffer (TROPIX, Bedford, MA) per 24-well. Luciferase and β-galactosidase levels were measured as stated above.

IL-6 ELISA. A murine IL-6 ELISA was performed by using a kit and the assay was performed according to the manufacturer’s instructions (R&D systems, UK). By making a standard graph, OD measurements could be correlated with levels of IL-6 present in the supernatant of the induced cells. IL-6 levels were expressed in pg/ml.

EMSA. An electrophoretic mobility-shift assay or EMSA was performed essentially as previously described (De Bosscher et al., 1997). DNA-binding activity was tested with an oligonucleotide containing the NF-κB site which comprises the sequence 5'-AGCTATGTGGGATTCCATGAGC-3' (underlined: single κB motif derived from the IL-6 promoter). DEX (1μM) and CpdA (1 and 0.1μM) were added 2 hours before TNF (2000 IU/ml). For supershift assay, 1μl polyclonal anti-p65 antibody from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding reaction.

MAPK activation assay. The assay was performed essentially as described by Boone et al., 1998. Briefly, L929sA cells were seeded at 250.000 cells/well in 6-well plates. After 24 h, cells were either left untreated, or treated with 1μM DEX or with CpdA (1 or 10 μM, as indicated on the figure) for 2 h and/or 2000 IU/ml TNF for 15 minutes. At the end of the incubation period, cells were washed in PBS. Cell extracts were essentially prepared as described in the protocol of a PhosphoPlus p38 MAPK antibody kit (New England Biolabs). One fifth of the total cell lysate (20μl) was separated by 12% SDS-
and blotted onto a nitrocellulose membrane. Western blot analysis was performed to detect phosphorylated MAPK proteins.

Example 1

CpdA transrepresses TNF-induced IL-6 promoter activity

In order to investigate whether the inhibitory action of CpdA is directed at the transcriptional level of IL-6 gene induction, we tested the effect of CpdA on an IL-6 promoter-driven reporter gene construct. Figure 1 shows that the induction with TNF of a p1168hu.IL6P-luc+ construct stably integrated in L929sA cells, can efficiently be inhibited with 1μM of DEX. A downmodulatory effect of CpdA at 0.1μM is already apparent, while a 1μM concentration of CpdA in combination with TNF lowers the relative luciferase induction factor to almost the same extent as 1μM DEX.

Example 2

CpdA inhibits a STS-induced AP-1-driven IL-6 promoter variant

Glucocorticoids can also exert transrepression of AP-1-driven genes. To see whether the inhibitory action of CpdA can also be directed to AP-1 activity, the κB-site of the IL-6 promoter was mutated. For this promoter variant, previous results have shown a complete loss of inducibility by TNF (Vanden Berghe et al., 1998, J.Biol.Chem, 273, 3285-3290). For this reason, the effect of CpdA on STS-induced promoter activity was examined. Figure 2 shows that STS can strongly activate a p1168(κBmut)IL6P-luc+ construct, stably integrated in L929sA cells, and that DEX at 1μM efficiently inhibits the AP-1-mediated transactivation. CpdA is also capable of inhibiting the STS-induced transactivation at concentrations of 0.1, 1 and 10μM.

Example 3

CpdA transrepresses TNF-induced IL-6 promoter activity via negative interference with the transcription factor NF-κB

Previous studies in our group have designated NF-κB as the most important transcription factor involved in TNF-mediated IL-6 gene induction. However, TNF induction of the IL-6 promoter activates not only NF-κB, but also AP-1. To rule out the additional effect of TNF on AP-1 activity and to investigate the actual transcription factor target of CpdA, the AP-1 binding site was mutated. Figure 3 shows that TNF activates a p1168(AP-1mut)IL6P-luc+ construct stably integrated in L929sA cells, and that DEX at 1μM can efficiently inhibit this NF-κB-mediated transactivation. Again, CpdA at concentrations of 1μM and 10μM is capable of significantly inhibiting the TNF-
induced promoter activity. Figure 4 shows that CpdA inhibits, to a similar extent as DEX, the TNF-activated recombinant reporter gene construct p(IL6×B)50hu.IL6Pluc+.

**Example 4**

**CpdA downregulates TNF-induced E-selectin promoter activity**

The downregulatory effect of CpdA was investigated on other physiological promoters, such as of the E-selectin and ICAM genes. Figure 5 demonstrates that CpdA-mediated inhibition can also be demonstrated on TNF-induced pE-selectin-luc+, stably integrated in L929sA cells. CpdA at 10μM inhibits the TNF-activated reporter to the same extent as 1μM DEX, i.e. to background levels. 1μM CpdA in combination with TNF is also effective, though to a lesser extent. Similar results were obtained with the ICAM promoter (Figure 6).

**Example 5**

**CpdA exhibits no transactivation potential on a GRE-dependent reporter gene**

CpdA demonstrates a similar transrepressive action on NF-κB and AP-1-driven genes as glucocorticoids. We were interested to know whether CpdA mediates these effects by binding to and modulating the glucocorticoid receptor in the same manner as the steroidal ligand, DEX. Hereto we investigated whether CpdA can stimulate a GRE-driven promoter coupled to luciferase.

Figure 7 demonstrates the lack of activity of CpdA at 0.1 and 10μM on a p(GRE)2-50-luc+ construct, stably integrated in L929sA cells. In contrast, DEX is able to transactivate this construct at least 8-fold. Stimulation with both agents together indicates competition for GR molecules present in the cell.

**Example 6**

**CpdA inhibits TNF-induced IL-6 protein production**

Corticosteroids exert their anti-inflammatory action by downmodulating the expression of pro-inflammatory genes. We were interested to know if CpdA could repress the pro-inflammatory cytokine IL-6 as compared to dexamethasone, a synthetic steroid ligand for GR.

Figure 8 shows that TNF is able to stimulate endogenous murine IL-6 production in L929sA cells, and that CpdA at 1μM and at 10μM is able to efficiently lower these TNF-induced levels.
Example 7

CpdA repression of NF-κB-driven genes is dependent on the presence of GR and the constitution of the TATA-box

In HEK293T cells the amount of endogenous GR is negligible, therefore, we used these cells to test whether the effect of CpdA depends on the presence of GR. Figure 9 (panel A and B) demonstrates that, only when GR is present, TNF-activated p(IL6-κB)50hu.IL6P-luc+ can be repressed by DEX and CpdA (compare lanes 4 and 8 of panel A with lanes 12 and 16 of panel B). This result represents evidence that CpdA does work via the activated GR. Previously we have observed that glucocorticoid repression is dependent on the identity of the TATA box. We found that a NF-κB-Luc construct carrying an E1B TATA could be activated by TNF, but no longer showed responsiveness to GC repression, however, the molecular mechanism behind this remarkable phenomenon is not known yet. We were interested to find out whether CpdA repression was also influenced by the TATA box context.

Interestingly, similarly as observed with DEX, also CpdA is not able to mediate repression of pNF-κB-Luc, suggesting CpdA and DEX work via a similar mechanism. (Figure 9, panel C and D)

Example 8

CpdA does not interfere with the DNA-binding activity of NF-κB

The mechanism of action by which CpdA inhibits NF-κB-driven gene expression can take place at several levels. Previously, we demonstrated that in L929sA cells DEX represses NF-κB-driven genes without affecting the DNA-binding capacities of NF-κB. Since some effects of CpdA are reminiscent of GR-mediated gene repression, we investigated the effect of CpdA on NF-κB DNA-binding activity. Figure 10 shows however that also for CpdA no change of NF-κB DNA-binding activity is observed at all concentrations indicated. Similar results were obtained for TC10 mouse endothelial cells (data not shown). This result suggests that the repressive mechanism of CpdA is not effected by abolishing the NF-κB DNA-binding activity and lets presume a mechanism reminiscent of that published for GC-mediated repression of IL-6-driven genes (De Bosscher, 1997).

Example 9

CpdA inhibits the transactivation potential of p65

Our previous data demonstrated that glucocorticoids mediate transrepression of NF-
κB via a nuclear interference mechanism between this transcription factor and the activated GR (De Bosscher, 1997). Since CpdA, similar to DEX, does not affect the DNA-binding activity of NF-κB, we tested its effect on the transactivation capacities of p65, by making use of a Gal4-p65 chimeric protein. This fusion protein stimulates a gal4-dependent reporter gene. Figure 11 demonstrates that DEX as well as CpdA at 10μM is able to halffmaximally inhibit the activity of Gal4p65. Specificity of repression is demonstrated by using Gal4 fused to the viral activator VP16 as a negative control.

Example 10

CpdA does not inhibit JNK kinase activity

It has been reported that part of the mechanism by which GC can mediate gene repression of AP-1-driven genes is by inhibiting the JNK kinase activity. DEX is able to prevent phosphorylation and subsequent activation of JNK, at its turn unable to phosphorylate the c-Jun component of AP-1 (Caelles, 1997). Our aim was to find out whether CpdA could also inhibit activated MAPK, in a similar way as DEX. We performed MAPK assays with lysates from L929sA cells and this lead to the following interesting results (Figure 12). Unlike DEX, which is able to inhibit the amount of phosphorylated JNK, CpdA does not affect the amount of activated JNK p46 and p54. The amount of activated, phosphorylated p38 and ERK MAPK kinases was not affected by either DEX or CpdA. From this result we can conclude that CpdA does not mediate AP-1-dependent gene repression by interference with the JNK kinase pathway.

Example 11

11.1 Beneficial activities of Cpd A in cotton-pellet granuloma and croton oil-induced ear oedema test

The anti-inflammatory activities of CpdA are evaluated in two animal models, the cotton-pellet granuloma test performed with female Wistar rats and the croton oil-induced ear oedema test with mice.

The cotton-pellet granuloma assay is performed as described by Meier R et al. (1950: Experientia 6:469–477), Vayssiere BM et al. (Mol Endocrinol. 1997 Aug;11(9):1245-55) and Vanden Berghe W et al. (Mol Pharmacol. 1999 Oct;56(4):797-806). Two cotton pellets (10 mg each) are inserted subcutaneously into the upper dorsal area of female Wistar rats (weight range 90–100 g, Iffa Credo, France). Test compounds are administered orally once a day for 4 days. Twenty-four hours after the last treatment,
the animal is killed and the pellet, along with the surrounding granuloma, is carefully dissected from the animal and its dry weight is determined. For this, granuloma and pellet are heated at 60 °C overnight. By subtracting the initial weight of the pellet, the dry weight of the granuloma is determined. The anti-inflammatory potential of the compounds is evaluated by their ability to prevent granuloma formation and expressed as the compound doses at which the granuloma formation is decreased by 50% (ED50, given in milligrams/kg) estimated from dose-response curves. In the same test, the thymus is removed, and thymolysis is measured. The CpdA doses needed to obtain 50% reduction of the thymus weight (ED50) are given in milligrams/kg.

The croton oil-induced ear oedema test is carried out as described by Tonelli G et al 1965 (Endocrinology 77:625–634), Vayssiere BM et al. (Mol Endocrinol. 1997 Aug;11(9):1245-55) and Vanden Berghe W et al. (Mol Pharmacol. 1999 Oct;56(4):797-806) on groups of eight male OF1 mice weighing 18–22 g (Iffa Credo, L'Arbresle, France). Oedema is induced on one ear by the application of a solution of croton oil (2% vol/vol) in pyridine-water-ether 4:1:14.6 (by volume). Animals are killed 6 h later, and the ears are removed and weighed. Edema is determined from the difference in weight between the irritant-treated and the contra-lateral ear. The compound to be tested is dissolved in the croton oil solution and topically applied on the ear during 6 hours. ED50 values correspond to the doses reducing the control edema by 50%. The ED50 values are expressed in micro-grams/ear.

11.2. Beneficial effects of CpdA in autoimmune disease models in mice
Experimental autoimmune disease mice models for encephalomyelitis (EAE), uveitis (EAU), collagen-II arthritis (CIA), thyroiditis, nephritis, myasthenia gravis, orchitis, lupus, diabetes and osteoarthritis as described in detail by Matthys P et al. (J Leukoc Biol. 2000 Oct;68(4):447-54), Matthys P et al (J Immunol. 1995 Oct 15;155(8):3823-9), Matthys P et al. (Eur J Immunol. 1993 Sep;23(9):2209-16), Eynon EE et al (Immunol Rev. 1999 Jun;169:5-10) and Kollias G et al. (Immunol Rev. 1999 Jun;169:175-94) are being used to test the beneficial effects of CpdA in autoimmune disease. CpdA is administered systemically (iv or ip) and/or locally (intra-articular) by injection. In case of low stability/rapid clearance of the active compound, a peristaltic pump is used for obtaining a continuous flow and an accumulation of CdpA at the specific tissue under investigation. Cpd A is administered at a dose preferably 10 times more concentrated
than the prednisolone, most preferably in the same dose range as described in detail in Alegre et al. (J. Immunol. 1991, vol 146, p.1184).

11. 3. Beneficial effects of Cpd A on TNF lethality models in mice

Female C57Bl/6 mice (Charles River, Sulzfeld, Germany), eNOS-deficient mice (Huang et al., Nature 1995, vol 377, p. 239-242), or iNOS-deficient mice (Laubach et al., Proc. Natl. Acad. Sci. USA, 1995, vol 92, p. 10688-10692) (Jackson Laboratories) are being used at the age of 7–12 weeks at the beginning of the experiment. The animals are housed in temperature-controlled, air-conditioned facilities with 12 hr dark/light cycles and food and water ad libitum. All experiments are approved by and performed according to the guidelines of the animal ethics committee from the Universities of Ghent, Belgium, and Maastricht, The Netherlands. Four different TNF lethality models are used. In model I, a lethal dose of mTNF is injected i.v. in healthy, non-sensitized mice. The sensitization models II, III, and IV are performed as described in detail before (Cauwels et al., J. Immunol. 1995, vol 154, p. 2753-2763; J. Immunol. 1996, vol 156, p. 4686-4690). Briefly, in model II mice bearing an i.m. LLCH61 tumor with a diameter of about 15 mm are challenged with 20 μg hTNF, while in model III mice are sensitized by a BCG infection 2 weeks before being challenged by a 10–15 μg hTNF injection. Model IV consists of a daily i.p. treatment with 1 μg mlL-12 during 5 consecutive days, followed after a 2 day interval by a lethal hTNF challenge of 5–10 μg. All TNF injections are performed i.v. (200 μl, diluted in endotoxin-free PBS) and 100% lethal. The LD100 is determined with exactly the same lot of TNF and mice before the start of each individual experiment. Lethality is generally scored up to 7 days after TNF challenge. Cytokines, cell lines, and the reagents recombinant hTNF, mTNF, and mIFNg are produced in Escherichia coli and purified to homogeneity in our laboratory. The endotoxin content is 0.02 ng/mg, as assessed by a chromogenic Limulus amoebocyte lysate assay (Coatest; Kabivitrum, Stockholm, Sweden). Live BCG organisms are provided by the Institut Pasteur du Brabant (Brussels, Belgium), and recombinant mlL-12 is generously provided by Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ). Murine LLC clone H61 and the B16BL6 melanoma subline are gifts from Dr. M. Mareel (University Hospital, Ghent, Belgium) by courtesy of Dr. G. Vaes and Dr. I. Fidler, respectively. Tumor cells are cultured in DMEM supplemented with 10% FCS, 50 U/ml penicillin G, 50 mg/ml streptomycin sulfate, and 2 mM L-glutamine. Before injection, cells are washed twice in PBS. Cpd A is injected
i.v. at a dose preferably 10 times more concentrated dose range of prednisolone, most preferably in the same dose range. CpdA is dissolved in solution suitable for i.v. injection. Injection solutions are diluted in endotoxin-free PBS, and, unless stated otherwise, injection volumes are 200 μl. CpdA is administered systemically (iv or ip) or locally (intra-articular) by injection. In case of low stability/rapid clearance of the active compound, use of peristaltic pump is beneficial for continuous flow and accumulation of the latter compound at tissue under investigation. Cpd A is administered at a dose preferably 10 times more concentrated than prednisolone, most preferably in the same dose range as described in detail by Alegre et al. (J. Immunol. 1991, vol 146, p.1184). More details of the above-described experiment can be found in Cauwels et al (Immunity. 2000 Aug;13(2):223-31), Freeman BD & Natanson C. (Expert Opin Investig Drugs. 2000 Jul;9(7):1651-63), Meduri GU. (J Chemother. 1999 Dec;11(6):541-50), and Kollias et al. (Immunol Rev. 1999 Jun;169:175-94).

11.4. Beneficial effects of Cpd A in acute and colitis mice models


Cpd A is administered at a dose preferably 10 times more concentrated than prednisolone, most preferably the same dose range as described in Steidler, L., W. Hans, et al. (2000, Science 289(5483): 1352-5)

11.5. Beneficial effects of Cpd A in liver shock model (hepatitis model)

An experimental shock model as described in detail in Van Mollee et al. (Infect Immun. 2000 Sep;68(9):5026-9) and Hochepied et al. (J Biol Chem. 2000 May 19;275(20):14903-9) is used in the present experiment. Female C57BL/6 mice (Iffa-Credo, Saint Germain-sur-l’Arbresle, France) are used at the age of 8–12 weeks. Rat a 1-AGP transgenic mice were generated as described below. They were generated by injecting genomic DNA into (C57BL/6 3 DBA/2)F1 zygotes, and the resulting
transgenic mice are back-crossed eight generations into a C57BL/6 background. Heterozygous transgenic mice from the line 9.5–5 constitutively produce about 2 mg/ml a 1-AGP. This is 10-fold more than wild-type (wt) animals. The colony was propagated by breeding heterozygous transgenic mice with C57BL/6 female mice; the offspring, containing heterozygous transgenics and wt littermates, was genotyped at weaning age by enzyme-linked immunosorbent assay. 100 µl of blood was collected by retro-orbital bleeding, after which serum was prepared. A 1-AGP was purified by phenol extraction and coated on the bottom of an enzyme-linked immunosorbent assay plate. After washing, rat a 1-AGP is detected using an anti-rat a 1-AGP polyclonal antibody (generated by H. Baumann in rabbits) (1/1,000) and an anti-rabbit antibody, conjugated to alkaline phosphatase (Sigma, St. Louis, MO; 1/5,000). The anti-rat a 1-AGP antibody does not cross-react with mouse a 1-AGP. About 50% of the offspring are heterozygous transgenic. Only female mice of 8–12 weeks are used in the experiments. Both transgenic and control (nontransgenic littermate) mice have comparable body weights. Mice are kept in a conventional, air-conditioned mouse room in 12-h light-dark cycles and received food and water ad libitum. Intraperitoneal injections have a volume of 0.5 ml. The reagents are diluted in pyrogen-free phosphate-buffered saline (PBS) immediately before injection. Mice are injected intramuscularly with bacteria (right thigh) in a volume of 100 µl. Mice are bled by retro-ocular bleeding or heart puncture under ether or tribromoethanol (160 mg/kg) anesthesia, respectively, and serum was prepared by clotting 30 min at 37 °C, removal of the clot, and centrifugation (15 min at 15,000 g). Bovine a 1-AGP, bovine serum albumin (BSA), alkaline phosphatase-conjugated anti-rabbit IgG, and p-nitrophenyl phosphate are obtained from Sigma. The a 1-AGP preparations contain 10 ng of endotoxin/mg of protein. a 1-AGP was 99% pure as mentioned by the manufacturer and as judged by polyacrylamide gel electrophoresis and subsequent Coomassie Blue staining. Recombinant mouse IL-1b was expressed in and purified from E. coli in our laboratory, had a specific activity of 3.65 $10^8$ units/mg, and contained less than 10 ng of endotoxin/mg of protein. K. pneumoniae (ATCC 43816), a strain that produces a lethal infection in normal mice, is inoculated in the right thigh muscle as described. An inoculum of 1-3 $10^6$ CFU/mouse is used except in the studies with rat a 1-AGP transgenic mice, where we use $10^5$ CFU/mouse. Survival is scored over a period of at least 5 days. Clearance of Bacteria: 36 h after injection of K. pneumoniae, mice are anesthetized by intraperitoneal injection of tribromoethanol. Blood is taken by heart
puncture. For preparation of plasma, 450 µl of blood is added to 50 µl of sodium citrate (0.1 M). Immediately thereafter, mice are killed by cervical dislocation. Then, mice are perfused with 10 ml of a 0.9% NaCl solution to wash out the blood. The liver, spleen, and kidney are removed aseptically, weighed, and homogenized mechanically in sterile saline. For homogenization, the liver is diluted (w/v) 2-fold; spleen and kidney are diluted 10-fold. The suspensions are diluted and plated out on sterile nutrient agar. After overnight incubation at 37 °C, CFU numbers are counted. Measurement of a 1-AGP—The concentration of a 1-AGP in mouse serum is measured using a home-developed sandwich enzyme-linked immunosorbent assay. A rat monoclonal antibody is coated (0.1 mg/ml) on 96-well Maxisorb plates. After blocking with 1% BSA and PBS, samples and a murine 1-AGP standard are titrated, after which the plates are incubated for 1 h at 37°C. A rabbit polyclonal antiserum (1/1,000) and subsequent alkaline phosphatase-conjugated anti-rabbit antibody (1/5,000) are used as secondary and third antibody. Human a 1-AGP is measured by nephelometry using a goat anti-human a 1-AGP polyclonal antibody. Effect of CpdA against a lethal infection with K. pneumoniae: to investigate whether CpdA confers protection, mice are pre-treated with CpdA 24 or 48 h before a lethal bacterial challenge (10^6 CFU unless otherwise stated) of K. pneumoniae. CpdA is administered systemically (iv or ip) or locally (intra-articular) by injection. In case of low stability/rapid clearance of the active compound, use of peristaltic pump is beneficial for continuous flow and accumulation of the latter compound at tissue under investigation. Cpd A is administered at a dose preferably 10 times more concentrated than prednisolone, most preferably in the same dose range as described in detail by Alegre et al. (J. Immunol. 1991, vol 146, p.1184).

11.6. Beneficial effects of Cpd A in atopic diseases (asthma, allergy)
An experimental in/ex vivo asthma model as described in detail by Hart et al. (Am J Respir Crit Care Med. 2000 Jan;161(1):224-31) is used in the present experiment. Briefly, it is determined whether inhaled CpdA therapy modulates the activity of the transcription factor, nuclear factor kappa B (NF-kB) during asthma. Individuals with mild asthma undergo bronchoalveolar lavage (BAL) with bronchial biopsies in a double-blind, placebo-controlled and crossover study after placebo or after inhaled CpdA (dose preferably 10 times more concentrated than for corticoids, most preferably same dose range i.e. 500 µg twice daily).
An experimental in/ex vivo (psoriasis) model as described in detail in Beyaert et al. (Eur J Immunol. 1992 Aug; 22(8):2181-4) is also used. Briefly fifty μl of PBS containing TNF+LiCl are injected subcutaneous into the back of mice which induces a psoriasiform inflammatory reaction. Effects of Cpd A will be analysed on skin biopsys. The animals are killed by cervical dislocation at different time intervals. Skin fragments with a diameter of 4 mm are removed and analysed by histo-immunochemistry or immersed in PBS and frozen at −20C. Later on, skin fragments are thawed and homogenized in PBS by 30 strokes with a Dounce homogenizer and centrifuged at 15000 x g for 10 min. Supernatants are tested for functional cytokine activity by appropriate ELISA or bioassay. Cpd A will be applied by a transdermal device at a dose preferably 10 times more concentrated than prednisolone, most preferably the same (therapeutic effective) dose.

11.7. Beneficial effects of Cpd A in virus replication models


An experimental, promyelocytic model of latency as described in detail by Critchfield et al. (Antivir Chem Chemother. 1999 Sep;10(5):275-84) is used in the present experiment. Cpd A is administered according to Critchfield et al. (Antivir Chem Chemother. 1999 Sep;10(5):275-84) at a dose preferably 10 times more concentrated than prednisolone, most preferably in the same dose range (preferentially 10-5 to 10-9M).

11.8. Test beneficial effects of Cpd A in parasite infection models

As TNF-induced NFkB is an important therapeutic target during parasitic infections, Cpd A is beneficial in treating parasitic infections. An experimental murine Plasmodium chabaudi chabaudi model as described in detail in Scorza et al. (Parasite Immunol. 1999 Nov; 21(11):545-54) is being used. Cpd A is administered systemically (iv or ip) or locally (intra-articular) by injection. In case of low stability/rapid clearance of the active compound, use of peristaltic pump is beneficial for continuous flow and accumulation of the latter compound at tissue under investigation. Cpd A is administered at a dose preferably 10 times more concentrated than prednisolone, most preferably in the same dose range as described in detail by Alegre et al. (J. Immunol. 1991, vol 146, p.1184).
11.9. Beneficial effects of Cpd A in cancer
As NFkB plays a role in cancer and apoptosis, NFkB inhibitors such as CpdA are beneficial as chemotherapy sensitizers.

An experimental ex vivo model CLL as described in detail in Van Causbroeck et al. Tijdschrift van de Belgische Vereniging voor Laboratoriumtechnologen December, 2000 is being used.

Cpd A is administered as described in Van Causbroeck et al. (Tijdschrift van de Belgische Vereniging voor Laboratoriumtechnologen December, 2000) at a dose preferably 10 times more concentrated than prednisolone, most preferably at the same dose range (preferentially $10^{-5}$ to $10^{-9}$M).
References


1. Use of a compound (I) with the formula

\[
\begin{array}{c}
\text{R} - \text{O} \quad \text{CH} \quad \text{CH}_2 \quad \text{N} \quad \text{Y} \\
\text{X} \\
\text{H}
\end{array}
\]

and/or its aziridine derivative,

wherein R is a hydrogen or carboxy group,

X is a hydrogen, hydroxy or halogen and

Y is a lower alkyl group

for the manufacture of a medicament to prevent and/or treat inflammation.

2. Use of a compound (I) according to claim 1 wherein R is an acetoxy group, X is chloride and Y is methyl.

3. Use of compound (I) with the formula as defined in claim 1 for the manufacture of a medicament to prevent and/or treat inflammatory diseases or inflammation-associated disorders chosen from the group comprising arthritis, asthma, bronchitis, tendinitis, psoriasis, eczema, dermatitis, burns, inflammatory bowel disease, cancer, vascular diseases, multiple sclerosis, ophthalmic diseases, pulmonary inflammation, infectious diseases and nervous system disorders.

4. Use of a compound (I) with the formula as defined in claim 2 for the manufacture of a medicament to prevent and/or treat inflammatory diseases or inflammation-associated disorders chosen from the group comprising arthritis, asthma, bronchitis, tendinitis, psoriasis, eczema, dermatitis, burns, inflammatory bowel disease, cancer, vascular diseases, multiple sclerosis, ophthalmic diseases, pulmonary inflammation, infectious diseases and nervous system disorders.
5. A composition comprising as active ingredient a compound having the formula (I) as defined in claim 1 or claim 2 and an acceptable excipient.

6. A composition according to claim 5, wherein said composition is a pharmaceutical composition.

7. A composition according to claims 5 or 6 for use as a medicament.

8. Use of a composition according to claims 5 or 6 for the manufacture of a medicament to prevent and/or treat inflammatory diseases or inflammation-associated disorders chosen from the group comprising arthritis, asthma, bronchitis, tendinitis, psoriasis, eczema, dermatitis, burns, inflammatory bowel disease, cancer, vascular diseases, multiple sclerosis, ophthalmic diseases, pulmonary inflammation, infectious diseases and nervous system disorders.
Fig. 7

![Bar graph showing induction factor with DEX and CpdA](image)

Fig. 8

![Bar graph showing pg/ml IL-6 with DEX, CpdA, and TNF](image)
Fig. 9

A: p(IL6κB)_50hl.IL6P-luc+

Induction Factor

GR  | DEX  | TNF  | CpdA
-----|------|------|------
-    | -    | -    | -    | 1 10 1 10

B: p(IL6κB)_50hl.IL6P-luc+

Induction Factor

GR  | DEX  | TNF  | CpdA
-----|------|------|------
+    | +    | +    | +    | + + + + + + + +

C: pNF-κB-luc

Induction Factor

GR  | DEX  | TNF  | CpdA
-----|------|------|------
+    | -    | +    | -    | - - + + - - + +

D: pNF-κB-luc

Induction Factor

GR  | DEX  | TNF  | CpdA
-----|------|------|------
+    | -    | +    | -    | - - + + - - + +

Fig. 10

DEX  | CpdA  | TNF
-----|------|------
1 10 | 1 10 1 10

IL-6-κB ➤
RBP-κ ➤

1 2 3 4 5 6 7 8 9 10
Fig. 11

![Graph showing Luc/β-Gal levels for Gal4-, Gal4p65, and Gal4VP16 with DEX and CpdA conditions.](image)

**Fig. 12**

**A**

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**B**

Phospho-JNK

- p54
- p46

**C**

Phospho-p38

Phospho-ERK

- p44
- p42

Legend:

- 1, 2, 3, 4, 5, 6, 7, 8: Sample numbers for each condition.
SEQUENCE LISTING

VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOL

USE OF CARBOXY COMPOUNDS SUCH AS
2-(4-ACETOXYPHENYL)-2-CHLORO-N-METHYL-ETHYLAMMONIUM
CHLORIDE AS ANTI-INFLAMMATORY AGENTS

CHA/COMP/047

99204433.9
1999-12-21

3

PatentIn Ver. 2.1

1

66

DNA

Artificial Sequence

Description of Artificial Sequence: annealed oligonucleotide

1

tagatctctcgtgtacagg atgttctagc ggtacctgct gtacaggatg ttctagctac 60
tgctcag 66

2

66

DNA

Artificial Sequence

Description of Artificial Sequence: annealed oligonucleotide

2

tctagagaga cgacagttcc tacaagatcg cctaggacga catgtcctac aagatcgtatg 60
gacgta 66
Artificial Sequence

Description of Artificial Sequence: part of oligonucleotide containing the NF-kappaB site

agctatgtgg gathttccca tgagc
A. CLASSIFICATION OF SUBJECT MATTER
IPCC 7 A61K31/137 A61P11/06 A61P17/00 A61P17/06 A61P19/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and where practical, search terms used)
EPO-Internal, CHEM ABS Data, EMBASE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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  - "A" document defining the general state of the art which is not considered to be of particular relevance
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  - "O" document referring to an oral disclosure, use, exhibition or other means
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- "I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "A" document member of the same patent family

Date of the actual completion of the international search
19 February 2001

Date of mailing of the international search report
27/02/2001

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Fax: +31-70 340-3016

Authorized officer
Siatou, E
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