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Anti-inflammatory effect and soft properties of etiprednol dicloacetate (BNP-166), a new, anti-asthmatic steroid

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In vitro and *in vivo* anti-inflammatory properties and soft characteristics of etiprednol dicloacetate (BNP-166) a new steroid, which has been developed for the treatment of asthma, were investigated in this study. The compound effectively decreased cytokine production in lipopolysaccharide stimulated lymphocytes and attenuated lectin-induced proliferation of blood mononuclear cells in tissue culture. In an animal model of allergen sensitized and challenged Brown Norway rats, using topical treatment, etiprednol dicloacetate substantially attenuated the extent of allergen induced bronchoalveolar fluid eosinophilia. At every examined parameter its pharmacological effects were comparable to those of budesonide. By means of *in vitro* biological and analytical methods the soft character of BNP-166 was also investigated. The anti-inflammatory effect of etiprednol dicloacetate *in vitro* was shown to be the function of the quantity of serum components, present in the assay. This loss of activity was most likely the result of the fast metabolism of etiprednol dicloacetate, which in the presence of sera could have been demonstrated by LC/MS/MS. Our data indicate that the significant local effect of the compound will very likely be accompanied with a drastically reduced systemic activity indicating an encouraging selectivity of the pharmacological action of etiprednol dicloacetate.

1. Introduction

The prevalence of allergic diseases of the upper and lower airways, rhinitis and asthma, has increased in most industrialized countries during the past few decades. Allergic rhinitis now afflicts 10–30% of the general population (Howarth and Holmberg 1995; Berger 2003), and is frequently associated with bronchial asthma (Bousquet et al. 2003) which is one of the most common chronic disorders not only in industrialized countries but also in several developing states and has significant effects on patients' health and quality of life (Martin et al. 1997). Although the causes of the continuous increase in prevalence, severity, and mortality of allergic airways diseases in industrialized countries are still debated and several hypotheses have been assessed (Ring et al. 2001; Wills-Karp et al. 2001) the increase in the number of diseased patients highlights the deficiency present in existing treatments. Of the several drugs currently available neither doctors nor are patients completely satisfied.

Corticosteroids are among the most effective drugs serving modern medicine and topically administered corticosteroids have, in fact, revolutionized the treatment of allergic airway diseases and have now become the mainstay of therapy for these disorders (GINA 2002; CCD 2002). Many of the patients however are worried about the side effects of glucocorticoids. As it has been learned from the accumulating pieces of evidence: all available inhaled corticosteroids can be systemically absorbed after topical administration to a variable degree; they thus have the potential to induce both systemic and topical side effects. As a consequence, many patients are reluctant to use steroids in any form including topical, and parents may be especially hesitant to sanction their use in children. Therefore inhaled glucocorticoids are often underused and this way the presumed side effects indirectly but severely influence the fulfillment of the real potential of the otherwise extremely effective drugs (Volcheck and O'Connel 1998). There is no doubt that inventing and producing drugs as effective as glucocorticoids, but without their side effects would be highly treasured.

Among the several approaches intended to improve the safety of corticosteroid therapy the so called "retrometabolic design method" (Bodor 1982; 1984; 1991) is unique in the sense that instead of trying to enhance effectivity, it improves the therapeutic index (ratio of activity and toxicity) of a drug. The principle of one of these drug designs (soft drugs) is that it starts from a known inactive metabolite of an effective drug and than, by chemical modifications, it produces an active compound from this. The soft design, in addition to activity, incorporates the most desirable way in which the molecule is inactivated and detoxified having exerted its biological effect.

Loteprednol etabonate was the first representative of this approach developed for different anti-inflammatory applications (Howes 2000; Szelenyi et al. 2000). In the present paper, we show some characteristic pharmacological and pharmacokinetical features of a new soft-steroid etiprednol

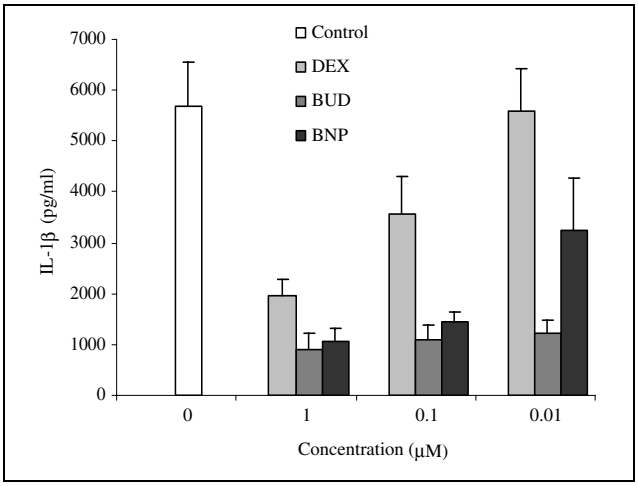

Fig. 1: Effect of glucocorticoids on the IL-1βproduction of LPS and silica-stimulated human mococyte (THP.1) cells. THP.1 cells were stimulated as described in the Experimental section, and were incubated together with different concentrations of the test compounds, dexamethasone, budesonide or etiprednol dicloacetate. After 18–20 h incubation IL-1-β levels in cell-free supernatants were determined by ELISA. The values represent mean ± S.E.M. for three independent experiments

dicloacetate (BNP-166) intended to use for the treatment of inflammatory airway diseases. Etiprednol dicloacetate has been designed based on Δ^1-cortienic acid which is a major metabolite of hydrocortisone and lacks corticosteroid activity (Bodor 1999). Etiprednol dicloacetate was produced by modifying both the carboxyl (β) and hydroxyl (α) groups at position 17 of Δ^1-cortienic acid. The detailed pharmacodynamics of etiprednol has recently been described (Kurucz et al. 2003).

2. Investigations and results

2.1. In vitro anti-inflammatory effect of etiprednol dicloacetate

Etiprednol dicloacetate significantly inhibited the liberation of IL-1β from the human monocyte cell line THP-1. Its effect was much more pronounced than that of dexamethasone and up to 10^{-7} M, it was equal to that of budesonide. At 10 nanomolar concentration, where dexamethasone did not exert any inhibition, etiprednol was still close to 50% effective. The other corticosteroid, budesonide however at the lowest examined concentration still showed almost maximal inhibition (Fig. 1).

In the inhibition of the proliferation of lectin stimulated human peripheral lymphocytes etiprednol dicloacetate was the most active. The median of the calculated IC_{50}-value of etiprednol dicoacetate was less than half of what was calculated for dexamethasone (Table).

Table: Effect of glucocorticoids on the lectin-induced proliferation of human mononuclear cells

Glucocorticoid	Mean of IC_{50} (nM)	Range of IC_{50} (nM)	N
Dexamethasone	72	7–120	6
Budesonide	39	26–74	6
Etiprednol dicloacetate	30	15–55	6

Human peripheral mononuclear cells were isolated on Ficoll-gradient and were stimulated with concanavalin A in the presence of serial concentrations of glucocorticoids. After 72 h incubation (^{3}H)-Thymidine was added to the cultures and the rate of proliferation was determined. IC_{50}-values were calculated using non-linear regression

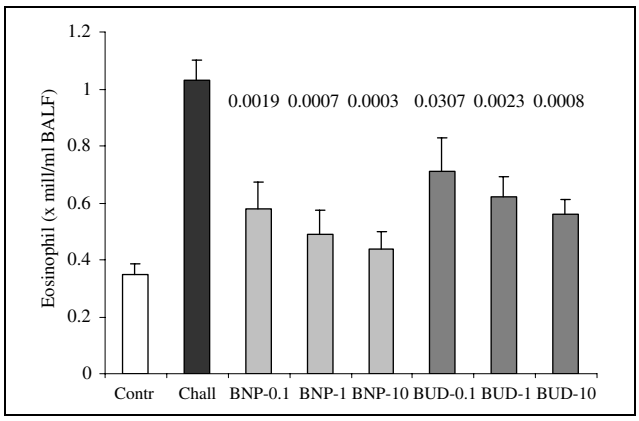

Fig. 2: Effect of etiprednol dicloacetate and budesonide on antigen-induced airway eosinophil infiltration into the bronchoalveolar tissues of BN rats. Sensitized rats were treated intratracheally with different doses (0.1, 1 and 10 μg/kg) of the drugs and 2 h later were challenged with OVA aerosol. 48 h later their lungs were lavaged and eosinophils were counted in the broncho-alveolar fluid. On the figure representation of peribronchial eosinophil numbers determined in samples derived from 8 (dug treated: BNP = etiprednol dicloacetate, BUD = budesonide) to 20 (Cont = negative and Chall = positive controls) animals for the treatment groups are shown. The level of significance (Mann-Whitney U-test) in case of each drug-treated group compared to vehicle treated challenged controls is shown on the figure. Approximate ED_{50}-values were: etiprednol dicloacetate was = 0.1 μg/kg and budesonide = 0.18 μg/kg. Etiprednol dicloacetate-treatment was statistically significantly superior to budesonide-treatment ($p < 0.01$, with 2-way ANOVA)

2.2. Effect of etiprednol dicloacetate and budesonide on allergen-induced accumulation of eosinophil granulocytes in the BAL fluid of actively sensitized and challenged Brown Norway rats

Antigen challenge induced a significant increase in the number of eosinophil granulocytes in the BAL fluid (Fig. 2). The pretreatment with BNP-166 resulted in a significant and dose-dependent inhibition of eosinophil accumulation with an IC_{50} value of approximately 0.1 μg/kg intratracheally. Similarly, budesonide significantly and also dose-dependently reduced the extent of airway eosinophilia. Its IC_{50} amounted to 0.18 μg/kg in this intratracheal treatment series. There was a statistically significant difference ($p < 0.01$) between the effect of the two drugs as showed by the applied ANOVA.

2.3. Effect of the amount of sera on the activity of etiprednol dicloacetate in vitro, assessed by using LPS-induced TNF-α release in human blood

To assess systemic biological stability of etiprednol dicloacetate its activity was measured in an *in vitro* system, where cells of undiluted and diluted whole human blood were stimulated with LPS and TNF-α production was measured. While dexamethasone was equally effective in diluted and undiluted blood the activity of etiprednol dicloacetate decreased at least three-fold if the examination was carried out in undiluted specimens if the duration of incubation was the same (Fig. 3).

2.4. Effect of the incubation time on the amount of BNP and its metabolite as determined by LC/MS/MS

To determine whether the reason for the decreased activity of etiprednol dicloacetate in the presence of different amount of human plasma is a consequence of its decomposition, an analytical method was used. With the applica-

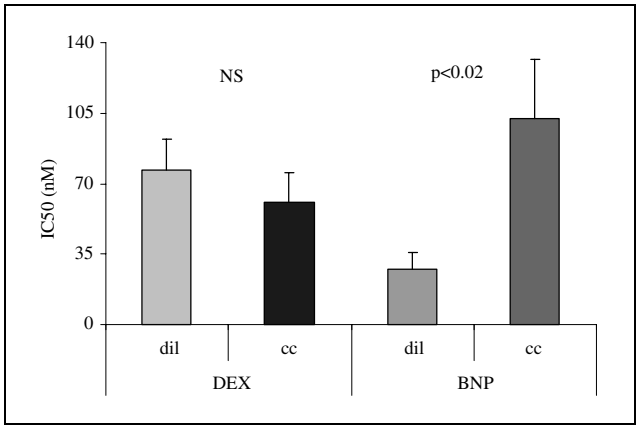

Fig. 3: Effect of the concentration of serum on the efficacy of dexamethasone and etiprednol dicloacetate in the inhibition of LPS induced TNF-α production by human blood. Blood samples after 1 : 5 dilution (dil) or without dilution (cc) were incubated together with the test compounds, dexamethasone (DEX), or etiprednol dicloacetate (BNP), and stimulated with 1 μg/ml LPS. After 18–20 hours incubation TNF-α levels in cell-free supernatants were determined by ELISA. IC_{50} values were calculated individually than the ratios of the IC_{50}-s obtained with diluted relative to undiluted samples were determined. The values represent mean ± S.E.M. for five individuals

tion of an HPLC/MS/MS-method the effect of the length of incubation and the amount of human plasma on the stability of etiprednol dicloacetate was determined. When the incubation was carried out in undiluted plasma (Fig. 4; a representative of two independent experiments which produced practically the same results) a significant decrease in the amount of etiprednol dicloacetate was seen already after 2 h of incubation and traces of the 17-α hydroxyl derivative, one of the proposed metabolites, could already be detected by this time. After 22 h only a small fragment of the original material could be detected but significant amount of the 17-α hydroxyl metabolite was seen. As it was expected decomposition of the material was less pronounced when the incubation was carried out in diluted plasma, but it happened with the same tendency albeit with slower speed.

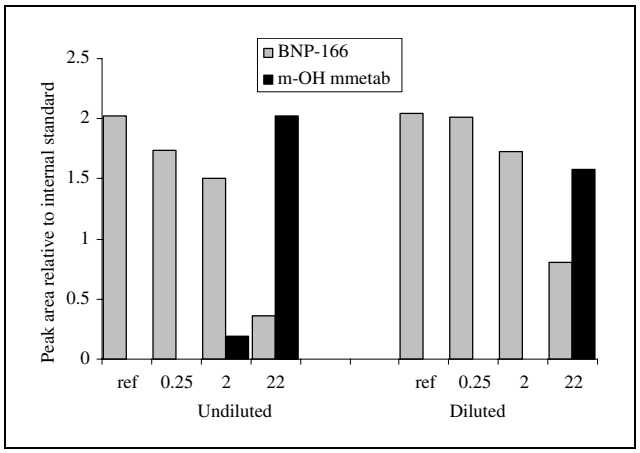

Fig. 4: Effect of the concentration of plasma and time of incubation on the stability of etiprednol dicloacetate. Etiprednol dicloacetate was added to freshly prepared human plasma of healthy donors at a concentration of 5 ng/ml, and were incubated at 37 °C for different intervals. After incubation the amount of the original drug and one of its main metabolite, were determined using a HPLC/MS/MS method as described in the experimental section. Results are expressed as peak areas normalized to the internal standard

3. Discussion

In spite of the impressive recent development in the types and numbers of the different new compounds intended to use for the treatment of allergic airway diseases (Szelenyi and Pahl 2002; Hele and Belvisi 2003), corticosteroids are still the cornerstone therapy for these disorders and improving their effectivity and selectivity seems to be one of the most fruitful medicine development strategy. Therapeutical advantage can be reached by the down-titration of their dose (Wilson and Robertson 2002), however using the lowest effective measured quantity is not without the risk of under-application, especially in case of inexperienced patients. With the introduction of designed changes into the chemical structure of glucocorticoids inactive compounds have been created which are activated on-site either by enzymes present in normal tissues or body fluids (pro-drugs; Dietzel et al. 2001) or they become active only in inflamed but not in normal tissues (disease activated drugs; Charpiot et al. 2001). The generation of non-steroidal glucocorticoid modulator molecules with strong anti-inflammatory activity but reduced glucocorticoid-like side effects (Coghlan et al. 2003) is a new direction to produce more selective compounds with high potential. However in all of these cases the metabolic fate of the ever so effective or selective compounds should be clarified and it might mean difficulties during drug development. For example with ciclesonide while the formation of active (first) metabolite is catalyzed by an esterase, in its further metabolism cytochrome P-450 enzymes are involved especially CYP3A4 (Hall 2000). The expression of this enzyme is marked with significant inter-individual variability (Lamba et al. 2002) and the amount of the enzyme is post-translationally down-regulated by grapefruit juice (Bailey et al. 1998). Cytochrome P4503A4 is inhibited by several drugs and commonly used compounds. Itraconazole, a known antifungal, markedly increased systemic effects of inhaled budesonide by inhibiting its P4503A4-mediated metabolism (Raaska et al. 2002). The unsurpassable advantage of the soft-approach is that once the compound exerted its effect locally, by passing the cells or getting into the systemic circulation a soft drug is converted into a compound which is already a biologically inactive major metabolite with known fate and the formation of which does not require the P450 system.

Etiprednol dicloacetate is a newly synthesized glucocorticoid with soft characters. In the present paper we have demonstrated that the *in vivo* activity of etiprednol dicloacetate in ovalbumin sensitized and challenged BN rat was at least equal to that of budesonide. These data are in concert with our previous publication (Kurucz et al. 2003) where a much more detailed *in vivo* investigation of the compound was carried out (peribronchial eosinophilia, epithelial cell mucus production, perivascular edema formation and the extent of airways hyperreactivity) leading to a very similar conclusion.

In parallel with this notable *in vivo* activity, etiprednol dicloacetate showed unique properties *in vitro*, when its effect was examined in the presence of different amounts of human serum The effectivity of etiprednol dicloacetate decreased in parallel with the increase in the amount of serum proteins present in the assay, and it was demonstrated by LC/MS/MS analysis that this decline in activity coincided with the disappearance of the original compound and the appearance of a supposed major metabolite.

In conclusion, etiprednol dicloacetate is a new soft steroid with a high *in vivo* anti-eosinophilic activity in experimental animals. In addition, etiprednol dicloacetate undergoes a rapid inactivation in the presence of human plasma so it can safely be assumed that once the compound is in the systemic circulation it will decompose with great speed. Thus, its side-effects inducing potential is considerably less than that of the known glucocorticoids. Clinical studies are ongoing.

4. Experimental

4.1. Drugs and chemicals

Chemicals were purchased from SIGMA (St. Louis, MO. USA), unless indicated otherwise. Etiprednol dicloacetate (ethyl 17α-dichloroacetoxy-11β-hydroxyandrosta-1,4-diene-3-one-17β-carboxylate, BNP-166) was synthesized at the Department of Chemistry of IVAX-DRI. *Bordetella pertussis* vaccine was a generous gift of Mr. Mihály Garamvölgyi (Human LTD, Gödöllő, Hungary).

4.2. Assay for cytokines

ELISA sets for human TNF-α (BD Pharmingen, San Diego, CA, USA) and IL-1β (R&D Systems Minneapolis, MN, USA) were used. ELISA was performed according to the manufacturer's protocol. Cell-free supernatants were tested in duplicate. Detection limits were 7.8 pg/ml for TNF-α and 3.9 pg/ml for IL-1β.

4.3. IL-1β production of stimulated THP.1 cells

THP.1 cells (human monocytic cell line; American Type Culture Collection, Rockville, MD, USA) were maintained in RPMI-1640 medium, supplemented with 10% FCS, 5×10^{-5} M 2-merkapto-ethanol, 2 mM glutamine and antibiotics, and were split in every 3rd days. To examine the effect of test compounds 2×10^6 cells/well (24 well plates in 1 ml/well volume) were stimulated with 1 μg/ml LPS and 25 μg/ml silica for IL-1β production as described previously (Németh et al. 1995). The test compounds were dissolved in RPMI-1640 medium or the medium containing 0.01% DMSO. Two parallel cell cultures per treatment groups were run in three independent experiments. IL-1 β levels in cell-free supernatants were determined by ELISA.

4.4. TNF-α production of LPS stimulated human blood

Peripheral blood from healthy donors was collected aseptically into sterile heparinized (Vacutainer™) tubes. Whole blood samples from each individual were parallel used both undiluted and after 5-fold dilution with RPMI-1640 medium in every experiment. TNF-α production was stimulated by LPS (1 μg/ml for 24 h) in the presence or absence of test compounds. After incubation cell-free supernatants were separated by centrifugation (1000 g for 10 minutes) and stored at −20 °C until determination of the amount of TNF-α.

4.5. Proliferation of lectin-stimulated peripheral mononuclear cells

Mononuclear cells from heparinized peripheral blood of healthy donors were isolated on suitable gradient (Optiprep solution, 1.077 g/ml). Serial dilutions of the test compounds (ranging from 2×10^{-5} to 2×10^{-8} M) were made in 100 μl of medium per well of sterile round-bottomed 96-well microtiter plate. Control wells contained culture medium only. 100 μl of cell suspension (10^6 cells/ml), containing concanavalin A (2 μg/ml) was added to each well. Proliferation background control cell suspension did not contain the lectin. All cultures were done in triplicate. Microtiter plates were incubated for 72 h at 37 °C, in 5% CO_2 containing humidified atmosphere. For the last 18 h of incubation, [^{3}H]thimidine was added to the cultures, at 0.1 μCi/well final concentration. At the end of the incubation, cells were harvested to glass microfiber filter (Whatman G/F) and associated radioactivity was determined by liquid scintillation.

4.6. Stability of etiprednol dicloacetate in the presence of human plasma

Etiprednol dicloacetate (BNP-166) was added to freshly prepared human plasma of healthy donors at a concentration of 5 ng/ml, and were incubated at 37 °C for different intervals. After incubation the amount of the original compound (ethyl-17α-dichloroacetoxy-11β-hydroxyandostra-1,4-diene-3-one-17β-carboxylate), and one of its main metabolite, M−OH (17α-11β-hydroxyandostra-1,4-diene-3-one-17β-carboxylate) were determined using an HPLC/MS/MS method (Mészáros et al., manuscript in preparation). In brief: Fluocinolone acetonide served as internal standard (20 ng/ml), samples were extracted with a liquid-liquid extraction on Extrelut® columns, and were separated on a Purospher STAR 30 × 2 mm (3 μm) reversed phase column at a flow rate of 0.3 ml/min, using a linear gradient with a mobile phase system containing acetonitrile, water and acetic acid. Determination were performed on a triple quadrupole mass spectrometer (Perkin-Elmer SCIEX API 2000) supplied with an electrospray interface operated in the positive ionization mode. The multiple ion monitoring, parent → doughter ion transitions of 485.2 → 265.2, 375.2 → 265.2 and 495.2 → 337.2 were used for the quantification of etiprednol dicloacetate, its M−OH metabolite and for the internal standard respectively. Results are expressed as peak areas normalized to the internal control.

4.7. In vivo experiments

Male Brown Norway (BN) rats, weighing 140–170 g at the beginning of the experiments, were purchased from Charles River Hungary LTD (Budapest, Hungary). They were kept under standardized laboratory conditions. All animal studies were conducted in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the Institutional Animal Care Committee.

Animals were sensitized with ovalbumin (OVA) precipitated on alum (25 μg OVA + 20 mg $Al(OH)_3$ in 0.5 ml saline/animal) administered subcutaneously on days 0, 14 and 21. Additionally, heat-inactivated *Bordetella pertussis* vaccine was injected intraperitoneally. On the 28th day, different doses of test compounds (0.1, 1 and 10 μg/kg) as powdered solid substances in vehicle were administered intratracheally 2 h prior to the challenge. Control animals were treated with vehicle (lactose) only. Antigen challenge was carried out by exposing the animals to vaporized OVA (1%, 60 min) administered via the "nose only inhalation system" (Technical and Scientific Equipment GmbH, Bad Homburg, Germany). 48 h after challenge animals were sacrificed by an overdose of urethane then a bronchoalveolar lavage (BAL) with pre-warmed Hank's solution was performed. Total eosinophil number was counted in the collected BAL fluid, after phloxine B staining (Unopette kit, Becton-Dickinson (Franklin Lakes, NJ, USA).

4.8. ED_{50} calculation, statistical evaluation

ED_{50}-values were calculated with GraphPad Prism software (GraphPad Software Inc., San Diego CA, USA). Statistical analysis between groups was done with Mann-Whitney U-test; difference between treatments was analyzed by 2-way ANOVA using the Statistica for Windows software version 5.1 (StatSoft Inc., Tulsa OK, USA).

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