Potency and specificity of the pharmacological action of a new, anti-asthmatic, topically administered soft steroid, etiprednol dicloacetate (BNP-166)

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List of non-standard abbreviations:
BALF - broncho-alveolar lavage fluid; GR - glucocorticoid receptor; GRE -
glucocorticoid response element; ICS - inhaled corticosteroids; PAS - periodic-acid
Schiff staining; PMA - phorbol-myristile-acetate; TAT - tyrosine-amino-transferase;

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Abstract

In the present study the pharmacological effects of etiprednol dicloacetate (BNP-166) a new soft steroid, intended to use for the treatment of asthma, were investigated in an animal model of allergen sensitized and challenged Brown Norway rats using local treatment. The examinations involved the determination of the effect of the compound on the extent of allergen induced broncho-alveolar fluid and lung tissue eosinophilia, goblet cell hyperplasia and mucus production, perivascular edema formation and airways hyperresponsiveness. The activity of etiprednol dicloacetate was compared to that of budesonide. Using in vitro methods the soft character of etiprednol dicloacetate was investigated together with its capability to dissociate transrepressing and transactivating properties. We found that combining all the examined parameters etiprednol dicloacetate was at least equipotent with budesonide in the animal model, but in several investigated variables it surpassed the activity of budesonide. The effect of etiprednol dicloacetate in vitro was shown to be the function of the quantity of the serum, present in the assay, it was also strongly affected by the incubation time and decreased significantly when it was pre-incubated with human plasma. These features are characteristics of a soft drug which is quickly inactivated in the systemic circulation. In addition it was revealed that while the transrepressing potential of etiprednol dicloacetate remained high, its transactivating activity was greatly reduced. These data indicate that the strong local effect of the compound will very likely be accompanied with a significantly reduced systemic activity predicting favorable selectivity in the pharmacological action of etiprednol dicloacetate.
Since the introduction of inhaled corticosteroids (ICS) for the treatment of asthma in the early 1970-s the therapeutic index of their use increased significantly. Most of the immediately apparent systemic (side) effects of the corticosteroids such as fluid retention, weight gain, and hyperglycemia virtually disappeared while the local activities responsible for the clinical efficacy are preserved (Boushey, 2000). Inhaled glucocorticoids have become the recommended and baseline treatment for all forms of persistent asthma (National Asthma Education and Prevention Program, 1997; GINA: Global Initiative for Asthma, 2002). Confidence over the safety of inhaled corticosteroid therapy has however been shaken lately by reports of weak but statistically significant associations of their lasting use with cataracts and glaucoma in the elderly (Cumming et al., 1997), with osteoporosis (Ebeling et al., 1998) and with the reduction of growth rate of preadolescent children (Doull et al., 1995). Now it is clear that all available ICS are systemically absorbed after topical administration to a variable degree, depending on the glucocorticoid molecule and the inhalation device, they thus have the potential to induce systemic effects and side effects. Retaining high local potency while reducing the systemic bioavailability are essential goals in the current design of new inhaled corticosteroids. The importance of the design of better and safer ICS is indicated by the fact that only in Europe for example approximately 3% of the population takes an inhaled corticosteroid and may do so for many years (Wong et al., 2000). It must also be added that inhaled glucocorticoids are often underused because of concerns about side effects (Volcheck and O’Connel, 1998), which again points to the significance and the necessity of safer drugs.
To further improve the therapeutic index, that is the ratio of the toxic to the therapeutic dose of a drug, is at least theoretically possible by changing both pharmacokinetics and pharmacodynamic parameters. Pharmacokinetics can deliberately be altered by using the “inactive metabolite approach” (Bodor, 1982, 1984, 1991) where one can design a soft analog of a drug which is active at the site of action (e.g.: in the lung in case of inhaled medications) but undergoes a one-step, predicted metabolism in the circulation and will be transformed to the very inactive metabolite from which its creation had been started (Bodor, 1999a). This process happens after the drug achieves its therapeutic role at the site of action and thus prevents the rest of the body to be exposed to the active drug or to various active or reactive metabolic products. Pharmacodynamic possibility to separate beneficial and deleterious effects of steroids is to try to dissociate the two main activities of glucocorticoids, which are transactivation and transrepression. As the glucocorticoid receptor (GR) is the effector of both of these activities, for a long time the separation of these two actions was not considered as a realistic selective target. However it has been shown recently that by mutating individual amino acids in different domains of the GR transactivation and transrepression became two separable functions (Heck et al., 1994), and studies with synthetic glucocorticoid derivatives have proved that it is possible to dissociate these two properties of the steroid molecule (Vayssiere et al., 1997).

Etiprednol dicloacetate (BNP-166; ethyl-17α-dichlo-roacetoxy-11β-hydroxyandrosta-1,4-diene-3-one-17β-carboxylate) is a new soft steroid designed based on Δ¹-cortienic acid (11β,17α-dihydroxy-androsta-1,4-diene-3-on-17β-carboxylic acid) which is a major metabolite of hydrocortisone and lacks corticosteroid activity (Bodor
Etiprednol dicloacetate was produced by modifying both the carboxyl (β) and hydroxyl (α) groups at position 17 of Δ1-cortienic acid. In this study the effects of etiprednol dicloacetate on allergen induced airways inflammation, remodeling and hyperreactivity was examined and compared to those of budesonide in vivo in an asthma model of Brown Norway-rat. Using in vitro methods the soft-character of etiprednol dikloacetate was investigated together with its capability to dissociate transrepressing and transactivating properties. Our data indicate that the strong local effect of etiprednol dicloacetate will very likely be accompanied with a significantly reduced systemic activity which predicts a favorable tolerability of the compound.
Methods

Human tissues

Human nasal polyps, derived from routine surgery, were obtained from the department of otolaryngology of St. Rókus Hospital (Budapest, Hungary). Patients were informed about the procedure and were asked to sign a written consent.

Animals

Male Brown Norway rats, weighing 140-170 g at the beginning of the experiments, were purchased from Charles River Hungary LTD (Budapest, Hungary). Upon arrival the animals were inspected for overt signs of ill health then quarantined for a week before use. They were kept by five in standard animal cages on a constant 12 hours light/dark cycle. The animals had free access to tap water and standard laboratory chow, also purchased from Charles River. Animals were treated according to the European Communities Council Directive (86/609/EEC) and all the experimental procedures were approved by the Institutional Animal Care Committee.

Drugs and chemicals

Chemicals were purchased from SIGMA (St. Louis, MO, USA), unless indicated otherwise. Etiprednol dicloacetate (BNP-166, Figure 1, panel A) was synthesized at the Department of Chemistry of IVAX-DRI. Bordatella pertussis vaccine was a generous gift of Mr. Mihály Garamvölgyi (Human LTD, Gödöllő, Hungary). Urethane, Primazin (2% xylasine), Ketalar (10% ketaminum), and the Unopette® kit were purchased from Reanal (Budapest, Hungary), Alfasan International BV (AB Woerden, The Nederland’s), Parke Davis (London, England) and Becton-Dickinson (Franklin Lakes, NJ, USA) respectively.
Sensitization, treatment and challenge of animals

Animals were randomly assigned into various treatment groups (4-5 animals per group), weighed and numbered. They were sensitized with ovalbumin precipitated on alum (25 µg ovalbumin + 20 mg Al(OH)₃ in 0.5 ml saline / animal) administered subcutaneously on the back on days 0, 14 and 21. Simultaneously on each occasion 0.25 ml (10⁹ cells/ml) heat-inactivated *Bordatella pertussis* vaccine was injected intraperitoneally. On the 28th day different doses (0.1, 1.0, 10.0 and 100.0 µg/kg) of the glucocorticoids tested were administered intratracheally 2 hours prior to the challenge. Intratracheal drug application was performed under short lasting general anaesthesia plus muscle relaxation produced by intramuscularly given xylazine (10 mg/kg) and ketamine (10 mg/kg). Animals were kept in supine position and a special cannula (Vasocan® Braunüle) was led through the larynx and was advanced into the mid-portion of the trachea. 10 mg of powdered solid substance (vehicle [lactose monohydrate] and active drug) was puffed into the lung by a 5 ml syringe. Control animals were treated with vehicle only. Antigen challenge was carried out by exposing the animals for 1 hour to vaporized 1% aqueous solution (saline) of ovalbumin administered via the “nose only inhalation system” (Nose Only Exposure System for Rodents; Technical and Scientific Equipment GmbH, Bad Homburg Germany).

*Bronchoalveolar lavage (BAL)*

48 hours after challenge animals were sacrificed by an overdose of urethane then BAL fluid (BALF) was obtained. After tracheotomy a polyethylene catheter was inserted and advanced to the bifurcation of the trachea. Then the airways were washed by 3 ml of Hank’s balanced salt solution pre-warmed to 37°C. Washing was repeated three times
with the same volume of buffer, and the washouts were collected into a centrifuge tube containing sodium citrate. Total eosinophil number was counted in the collected BALF, after phloxine B staining (Unopette® kit).

**Measurement of airway hyperreactivity ex vivo**

Tracheae were removed from the animals, and after careful cleaning from the adhesive tissues they were cut into single rings. Ring preparations were suspended into organ bath chambers containing Krebs’ buffer and were maintained at 37 °C with continuous aeration. For the recording of isometric tension changes rings were placed under 1.0 g tension and after an equilibration period of 30 minutes, cumulative concentration response to acetylcholine was determined. Maximal response of control (sensitized, unchallenged and non-treated) tracheal rings was obtained at 10^{-3} M acetylcholine. The magnitude of this response was defined as 100%. All other contractions were expressed as a percentage and related to the control response. Concentrations of acetylcholine necessary to cause contraction equal to 50% to that of the control were determined for each preparation using linear regression. Two to three rings were investigated from each animal.

**Histochemistry**

Lung specimens of all lobules from each animal were collected after the broncho alveolar lavage. Samples were fixed in phosphate-buffered 8% formalin for 2 weeks, and then were routinely processed for histochemistry. 5µm thick sections were cut and mounted on surface-treated slides. Perivascular and peribronchial eosinophilia were determined on modified May-Grünwald-Giemsa stained sections, by counting all eosinophils within visual fields in all peribronchial and perivascular lung tissue at a magnification of 630x.
Perivascular edematous areas were determined on Periodic acid-Schiff (PAS) stained specimens which were counterstained with haematoxylin. 50 randomly chosen microvessels of each experimental group were digitally exposed (Zeiss Axiocam - Axiovert 200 system) at a magnification of 400x. Measurement of the area of perivascular edema was performed by Zeiss Axiovision 3.1 software (Carl Zeiss Vision GmbH). Extension of the perivascular edema is expressed as a percentage of the area of the respective microvessel. Mucus production and goblet cell hyperplasia were determined on similarly prepared (PAS+hematoxylin) lung tissue sections counting all epithelial cells of each airway segment in the whole preparations at a magnification of 400x. Changes in the number of mucus producing cells are expressed as the ratio of PAS positive goblet cells to all epithelial cells counted in all lobules of tissue sections.

Assay for cytokines

Commercially available human cytokine ELISA sets were used. TNF-α and GM-CSF sets were purchased from BD Pharmingen, (San Diego, CA, USA) IL-1β set was obtained from R&D Systems (Minneapolis, MN, USA). ELISA was performed according to the manufacturer’s protocol. Cell-free supernatants were tested in duplicate. The detection limits were 7.8 pg/ml for TNF-α, 4.7 pg/ml for GM-CSF and 3.9 pg/ml for IL-1β. Results were expressed as mean percentage of inhibition. IC₅₀ values for test compounds were calculated by linear regression.

TNF-α production of lipopolysaccharid stimulated human blood

Peripheral blood from healthy donors was collected aseptically into sterile heparinized tubes. Whole blood samples from each individual were parallel used both undiluted and after 5-fold dilution with RPMI-1640 medium in every experiment. Blood samples were
distributed into 24-well plates and incubated with serial concentrations of the test compounds and 1 μg/ml lipopolysaccharid for 24 hours at 37°C in a CO₂ thermostat. Controls were treated with lipopolysaccharid and the vehicle (PBS or 0.01% dimethylsulphoxide in PBS). After incubation cell-free supernatants were separated by centrifugation at 1000 g for 10 minutes and stored at -20°C until tested for the amount of TNF-α. Test compounds were examined in blood samples from five different individuals. Two parallel cultures per treatments were run.

**Pre-incubation of the experimental compounds with serum and measurement of their effect on TNF-α production of lipopolysaccharid stimulated human peripheral blood mononuclear cells**

Mononuclear cells from peripheral blood of healthy donors were isolated on Ficoll-gradient. One million cells in 0.9 ml RPMI-1640 medium were distributed into 24-well plates and serial concentrations of the test compounds, made in fresh human serum either instantly or 18 hours previously, were added (0.05 ml) together with the lipopolysaccharid (0.05 ml, 1μg/ml final concentration). Pre-incubation of the compounds with serum was carried out at 37°C. Processing of the cultures was done as described above.

**GM-CSF production of lipopolysaccharid-stimulated dispersed human nasal polyp cells and nasal polyp-derived fibroblasts**

Human nasal polyp cells were isolated and cultured as described before (Crampette et al., 1996). Tissue, obtained from surgery, was cut into small pieces and digested for two hours in RPMI-1640 medium containing protease (from Streptomyces griseus; 2 mg/ml), DNAse-I (from bovine pancreas; 2000 units/ml) and collagenase (from Clostridium
hystolyticum; 2 mg/ml). The digested tissue was pushed through a 100 μm mesh nylon cell strainer (Falcon), and the digestion process was stopped by adding fetal calf sera containing RPMI-medium. Erythrocytes were lysed; the dispersed cells were washed three times with the culture medium, and were plated at a density of 3x10^5 cell/well into 24-well tissue culture plate. Stock solutions of test compounds were made in dimethylsulphoxide, and were further diluted in medium. Compounds were added to the culture 30 minutes before stimulation with lipopolysaccharid (10 μg/ml). After incubation for 1, 2 or 5 days supernatants were taken from the cultures, and were kept at –70 ºC until GM-CSF concentrations were determined. Primary fibroblasts were produced by culturing the dispersed nasal polyp cells, and enzymatically passaging the adherent cells for at least 3 times. Cultures with typical fibroblast morphology were used in the experiments. Cells were plated at a density of 2x10^4 cell/well into 24-well tissue culture plate 48 hours before adding drugs. Cells were stimulated with lipopolysaccharid (10 μg/ml). After 24 and 48 hours of incubation supernatants were taken from the cultures, and after centrifugation they were kept at –70 ºC until their GM-CSF concentrations were determined. IC50 values were determined by linear regression.

**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 % fetal calf sera, 5x10^{-5} M 2-mercapto-ethanol, 2 mM glutamine and antibiotics, and were split in every 3rd days. To examine the effect of test compounds 2x10^6 cells/well (24 well plates in 1ml/well volume) were stimulated with 1 μg/ml lipopolysaccharid 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 % dimethylsulphoxide. Two parallel cell cultures per treatment groups were run in three independent experiments. IL-1β levels in cell-free supernatants were determined by ELISA. Transrepressing activities of the compounds were expressed relative to dexamethasone.

**Measurement of tyrosine-amino-transferase activity in rat hepatoma cells**

Trans-activating properties of the compounds were determined by measuring the steroid-induced increase in the activity of tyrosine-amino-transferase (TAT, involved in glucocorticoid-dependent stimulation of neoglucogenesis), by the method of Diamondstone (1966), using a rat hepatoma cell line (HTC, ECACC 93129198, European Collection of Cell Cultures, Salisbury, Wiltshire, UK) and treatment conditions as described before (Vayssiere et al., 1997). HTC cells were cultured in DMEM supplemented with 10% fetal calf sera. Semiconfluent cultures were treated with the compounds overnight in serum-free medium. Cells, collected by centrifugation, were resuspended in appropriate aliquots of the reaction buffer (0.2 M KH₂PO₄ buffer, pH 7.3) and centrifuged again with 40,000xg for 20 minutes at 4°C. The supernatants were collected, their protein contents were determined with Bradford reagent, and the samples obtained were either stored at -20°C or used immediately. Tyrosine transaminase activity was assayed by measuring the conversion of p-hydroxyphenylpyruvic acid (pHPP) to p-hydroxibenzaldehyde (pHBA). 200 µl of the standard reaction mixture (34.5 µM L-tyrosine, 84 µM pyridoxal phosphate, 7.8 mM diethyldithiocarbamate) was added to a 200 µl aliquot of cell cytosol, vortexed and preincubated for 5 min at 37°C. The reaction was started with the addition of α-ketoglutarate (10 mM), and was allowed to proceed for 2 to 30 min at 37°C. After the required incubation, 10 N NaOH was added which stopped
the reaction and started the conversion of pHPP to pHBA. After 30 min at room
temperature the absorbances were read at 310 nm against a zero-time control prepared by
adding the NaOH just before the α-ketoglutarate. Two parallel measurements were made
in three independent experiments. At each molar concentration dexamethasone activity
was taken as 100% and for the other steroids relative activities were calculated.

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, or with Student’s t-test; differences between treatments (e.g. etiprednol
dicloacetate vs. budesonide) was analyzed by 2-way ANOVA. All the calculations were
done with a Statistica for Windows software version 5.1 (StatSoft Inc., Tulsa OK, USA).
Results

Effect of etiprednol dicloacetate and budesonide on allergen-induced cellular infiltration. Antigenic challenge caused significant increase in the number of eosinophils both in the broncho-alveolar fluid, and at various sites in the lung of the experimental animals. In the BALF of the vehicle-treated and challenged Brown Norway rats three to four-fold increase was seen compared to sensitized non-challenged controls, while peribronchially (Figure 2 panel A, B and E) and perivascularly 20-50-fold elevation in the number of eosinophils could be observed. Both etiprednol dicloacetate and budesonide significantly attenuated peribronchial eosinophilia in the lung of the animals (Figure 3 panels C, D and E). The 50% effective doses of both compounds were around the 1 µg/kg dose; etiprednol dicloacetate (0.62 µg/kg) was slightly more effective than budesonide (1.61 µg/kg) in these examinations, and this difference was reflected by the comparative statistics of the treatments as well (p < 0.0001, 2-way ANOVA). In accord with this both compounds effectively decreased antigen-induced cellular infiltration into bronchial cavities of the experimental animals. In case of etiprednol dicloacetate already at the lowest applied dose (0.1 µg/kg) the inhibition of eosinophil immigration into the broncho-alveolar space was statistically highly significant and the rate of inhibition was more than 60%. Budesonide also successfully and dose-dependently decreased the BALF eosinophil numbers, however at the 0.1 µg/kg dose it was not yet effective (data not shown). Both steroids decreased the number of eosinophils at the perivascular area of the lung tissue as well; however the extent of this decrease were much less significant.
compared to their peribronchial effect (no inhibition at 0.1 µg/kg, and around 40% inhibition at the maximal doses of either etiprednol dicloacetate or budesonide).

**Effect of etiprednol dicloacetate and budesonide on allergen-induced epithelial cell mucus production.** As a result of the allergic stimulation, goblet cell hyperplasia, increased mucus secretion, and epithelial cell desquamation was seen with high frequency at the epithelial lining of the bronchi of Brown Norway-rats (Figure 3, panels A and B). This alteration was quantitated by determining the ratio of PAS-positive cells to all epithelial cells and in general at least 10-fold increase could be detected in this ratio (Figure 3, panel E). Both etiprednol dicloacetate and budesonide effectively decreased the number of mucus secreting cells as it is demonstrated on panels C and D of figure 3 showing data at the 10 µg/kg doses. Graphical presentation of all of the data (Figure 3, panel E) reveals the quantitated effectivity of the compounds. Almost all of the applied doses of etiprednol dicloacetate were more effective than similar doses of budesonide; the calculated 50% effective doses were 13.8 and 32.7 µg/kg respectively, and there was a statistically significant difference (p = 0.0042, ANOVA) between the effect of the two compounds.

**Effect of etiprednol dicloacetate and budesonide on allergen-induced perivascular edema formation.** Due to the release of inflammatory mediators, ovalbumin challenge caused a significant edema around the small vessels (both in arteries and veins) in the lung tissue of the sensitized animals. In challenged animals the area of the edematic loose connective tissue around the small vessels increased at least four-fold relative to the same area in the control (sensitized, non-challenged) animals (Figure 4). The applied morphometrical procedure allowed the quantification of the increase and the
effect of drug treatment on this alteration. Both budesonide and etiprednol dicloacetate successfully decreased the antigen-induced formation of perivascular edema (Figure 4). Again etiprednol dicloacetate showed slightly better results with an approximate ED_{50} of 2.9 µg/kg while in case of budesonide the 50% effective dose was about 8.5 µg/kg. The two treatments statistically significantly differed from each other (p = 0.0041, ANOVA).

**Effect of etiprednol dicloacetate and budesonide on allergen-induced airway’s hyperreactivity.** Airway’s hyperreactivity was measured using isolated tracheal rings of the sensitized or the sensitized and in addition challenged Brown Norway rats. The acetylcholine dose-response curve (Figure 5) showed the characteristic increase in the contractile response of the isolated organ pieces derived from challenged animals. The hyperreactive response was normalized by glucocorticoids as it is shown on the figure by one in vivo applied dose of etiprednol dicloacetate. Quantitative analysis of the data (Table 1) indicated that etiprednol dicloacetate was more effective in normalizing the antigen induced decrease in the acetylcholine dose necessary to produce a fixed contraction, but budesonide was better in decreasing the high acetylcholine dose (10^{-3}M) provoked contraction (Table 1).

**Effect of the concentration of serum, length of incubation time and pre-incubation with serum on the activity of etiprednol dicloacetate in vitro.** To assess systemic biological stability of etiprednol dicloacetate its activity was measured in vitro under varying circumstances. The effect of different concentrations of sera components on the activity of etiprednol dicloacetate was measured in a simple in vitro system, where cells of whole human blood were stimulated with lipopolysaccharid and TNF-α production was measured. By using the blood in undiluted and in diluted form it was
possible to compare the effect of sera components (proteins, enzymes) on the activity of the examined compounds. It was found that 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, when the duration of incubation was the same (Figure 6). The effect of the incubation time and the pre-incubation with sera on the activity of etiprednol dicloacetate and other glucocorticoids were also examined (Table 2). By using two different cell-types and measuring the production of GM-CSF (panel A) it was seen that while the activity of budesonide or dexamethasone did not or only slightly changed with the time of incubation, it dramatically affected the activity of etiprednol dicloacetate. In case of dispersed nasal polyp cells the decrease in activity was almost 100-fold during the 5 days incubation while we could not detect any changes in the effectivity of budesonide. The activity of etiprednol dicloacetate on GM-CSF production of nasal polyp-derived fibroblasts decreased more than 20-fold while only a two-fold reduction was seen with dexamethasone. Preincubation with human sera caused a radical drop in the activity of etiprednol dicloacetate (panel B). 18 hours of incubation with human sera triggered more than a hundred-fold decrease in the effectivity of etiprednol dicloacetate, while the same treatment resulted in only a two-fold reduction in the activity of dexamethasone.

**Dissociation of transactivating and transrepressing effect of etiprednol dicloacetate and other glucocorticoids.** Transrepressing and transactivating activity of the compounds were determined by measuring their inhibition in IL-1β production of a stimulated human monocyte cell line and by evaluating glucocorticoid induced increase in the activity of tyrosine-amino-transferase of a rat hepatoma cell line. All the measured
activities were expressed relative to dexamethasone. Comparisons were made at two molar concentrations where the effect of dexamethasone was most pronounced. Transrepressing activity of both etiprednol dicloacetate and budesonide was comparable to that of dexamethasone at the concentration of 1 µM, however at 100 nM concentrations both compounds proved to be approximately twice as active in this setup (Figure 7). There was no statistically significant difference between the activities of the two compounds. At the same time transactivating activity of budesonide and etiprednol dicloacetate at either examined concentrations was considerably lower than that of dexamethasone, but repetitively in three independent experiments etiprednol dicloacetate was the least potent in increasing TAT activity (p<0.03 compared to budesonide; Figure 7).
Discussion

The prevalence of asthma has been on the rise in recent years especially at the
developed part of the world and it was reported to affect more than a hundred million
people on our globe (Global Strategy for Asthma Management and Prevention, 2002).
According to the most recent report of the Centers for Disease Control and Prevention
(CDC, 2002), of the U.S. Department of Health and Human Services, asthma still
remains a critical clinical and public health problem. In spite of the early indications of
certain success in current asthma intervention programs (e.g., limited decreases in asthma
hospitalization and death rates) other data in this report indicate the serious need for
continued surveillance and targeted interventions in the US and perceptibly in other
countries worldwide. Medical treatment of asthmatics to effectively alleviate symptoms
or to prevent progression of the disease and the deterioration of the patients’ state will
inevitably be necessary for an extended period of time.

The introduction of ICS is the single most important advance in the management
of asthma patients with persistent symptoms. The treatment has resulted in substantially
reduced asthma exacerbation rates, leading to reduced asthma hospital care and reduced
asthma death frequency (Lanes et al., 2002). Furthermore ICS therapy improves asthma
symptoms and lung function on a long term basis and improves the quality of life of
asthma patients. Because concerns about the potential risk is probable the most
significant impediment to the use of inhaled steroids several approaches have been tried
to decrease the risk; for example by titrating down the daily dose to the minimal effective
level, and by optimizing the frequency of the treatment (Toogood, 1998). However in
spite of these efforts the need for the effective but safe, “perfect” steroids still exists. As it is almost impossible to overcome pharmacological effectivity of present day synthetic steroids the emphases in the research and development has lately been shifted toward the safety issue. One of the most recent approaches to produce an effective but safe drug is the so-called retrometabolic drug design. Retrometabolically designed drug candidates have already been under investigation in widely different pharmacological fields intended to use as anti-glaucoma agents (Bodor, 1999b), atimicrobials (Bodor et al., 1980a), anti-tumor compounds (Bodor and Kaminski, 1980) or as short acting anticholinergics (Bodor et al., 1980b). As the site of intended action and the site of administration coincide in inhalation therapy the potential for the application of the retrometabolic approach in the treatment of asthma with ICS is especially promising.

Asthma is a complex multifactorial disease characterized by airway inflammation reversible airways obstruction and non-specific airways hyperreactivity (Barnes, 1999). Although asthma does not exist as a natural disease among animals, several animal models are available in which it is possible to demonstrate the pharmacological action of new anti-asthmatic drug candidates (Szelenyi, 2000). Ovalbumin sensitized and challenged Brown Norway rat is a widely used animal model in the development of agents with anti-asthmatic properties (Trifilieff et al., 2002; Huang et al., 2002). Allergic challenge in this animal causes extensive inflammation in the lung with characteristic increase in the number of eosinophils and mucus-producing goblet cells and in the extent of perivascular edema (Schneider et al., 1997; Taylor et al., 1997). In addition airway hyperreactivity also develops, which together with the above mentioned parameters also cardinal feature of asthma. To evaluate the clinical potencies of etiprednol dicloacetate,
we compared its effectivity in this model with budesonide which is probably the clinically most widely used inhaled corticosteroid (O’Connell, 2002).

In vivo activity of etiprednol dicloacetate in ovalbumin sensitized and challenged Brown Norway rat, in the applied experimental setup, was equal to that of budesonide considering all measured parameters. As for the alleviation of allergen-induced inflammation however, etiprednol dicloacetate was statistically significantly more effective than budesonide. It decreased tissue peribronchial eosinophilia (Figure 2), epithelial cells mucus production (Figure 3) and perivascular edema formation (Figure 4) to a greater extent than budesonide. Etiprednol dicloacetate also successfully decreased antigen-induced tracheal hyperreactivity (Figure 5) and in general its activity was similar to that of budesonide (Table 1). The above data demonstrated that etiprednol dicloacetate is a highly potent compound with a very good prospective to treat asthma.

In parallel with the remarkable in vivo activity etiprednol dicloacetate showed unique properties in vitro, when its effect was examined in the presence of different amount of human plasma, the interval of incubation time has been varied or the compound was pre-incubated with human sera. The effectivity of etiprednol dicloacetate decreased in parallel with the increase of the amount of serum proteins in the assay (Figure 6). Quick inactivation of the compound could also be observed in experiments where etiprednol dicloacetate was incubated with the cells for varying length of time in the presence of sera, or were pre-incubated with sera before adding into the cell culture (Table 2). In the applied systems, etiprednol dicloacetate significantly lost its activity either as a function of incubation time or as a result of pre-incubation. In the same arrangements neither dexamethasone nor budesonide, behaved similarly. The quick
inactivation of etiprednol dicloacetate which clearly depends on the amount of serum proteins present in the assays indicate that once this steroid reaches the systemic circulation it loses its activity therefore it might not cause serious side effects. Indeed half-life of the original compound in the sera of rats was found less than one hour after intravenous administration (Pátfalusi M., data not shown). The identification of the putative degrading enzyme of etiprednol dicloacetate (most likely a carboxylesterase) is presently under investigation, the two main metabolites of the compound, the 17α–OH and the 17β–COOH derivatives of BNP-166, has already been identified. The demonstrated in vivo effectivity at the site of administration combined with the quick systemic inactivation predicts high selectivity of the pharmacological action of etiprednol dicloacetate. The 28 days oral toxicity study with etiprednol dicloacetate in rats and dogs seems to support this prediction. The No Adverse Effect Level (NOAEL) for both species was found to be 2.0 mg/kg which compares favorably to that of budesonide, about 40-times lower (Miklos et al., 2002), and is several fold higher than the expectable effective dose.

In addition to the affirmative pharmacological and pharmacokinetical properties, pharmacodynamic features of etiprednol dicloacetate were also encouraging. The compound proved to be a dissociated glucocorticoid, showing a reduction in transactivating activity while preserving transrepressive abilities. Glucocorticoids are signaling through one receptor (GR) but the result of the signal can be either increase (activation) or inhibition (repression) of gene expression. Transactivation is mediated by binding of the hormone-activated receptor to a defined DNA sequence, called glucocorticoid response element (GRE). This process may account for some of the
unwanted effects of glucocorticoids via the increase in expression of genes involved in
gluconeogenesis and development of arterial or ocular tensions (Pedersen and O’Byrne,
1997; Sorkness, 1998). Although glucocorticoids activate some genes the products of
which are clearly anti-inflammatory, the main mechanism by which glucocorticoids
suppress inflammation is transrepression. Transrepression may be the result of binding to
negative GREs but it occurs mainly by interaction with transcription factors (AP-1, NF-
κB) which control the gene of many inflammatory mediators from IL-1β to RANTES.
Domain-swapping (Pearce and Yamamoto, 1993) and mutational studies (Heck at al.,
1994) have proved that the activation and the repressor functions of the receptor are
clearly separable. It was shown that dimerization of GR is not required for
transrepression of AP-1 ability which is probably resulted from an inhibitory protein-
protein interaction. The nature of the ligand also proved to be a conformation-
determining factor; therefore it was possible to produce compounds with dissociated
properties (Vayssiere et al., 1997) opening up the possibility to design selectively acting
glucocorticoids.

In this study we measured and compared transrepressive and transactivating
properties of etiprednol dicloacetate by determining inhibition of IL-1β production in a
human monocyctic cell line, and by monitoring activation of tyrosine aminotransferase
gene in a rat hepatoma cell line. IL-1β production is under the control of NF-κB, AP-1
(Jeon et al., 2000), and transcription of the TAT gene is induced by glucocorticoids in
vitro and in vivo due to the presence of GREs in the TAT gene promoter (Jantzen et al.,
1987). It was found that while etiprednol dicloacetate preserved its ability to suppress IL-
1β-production it showed negligible activity in activating TAT (Figure 7). Whether the
dissociation of glucocorticoid-dependent transactivation from transrepression may provide a chance to separate some of the negative side effects of the beneficial anti-inflammatory action of classic glucocorticoids is still not clear (Belvisi et al., 2001), but it is a likely advantage which may act favorably during patient treatment with etiprednol dicloacetate.

In summary: etiprednol dicloacetate is a new soft steroid with in vivo activity that reaches, or in some tests, surpasses the activity of the probably most well known second generation synthetic corticosteroid: budesonide. In addition the selectivity of etiprednol dicloacetate resulting from its pharmacokinetic and pharmacodynamic features compares favorably to those of other steroids and therefore looks forward to the clinical examinations with good prospects.
Acknowledgement:

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Legends for figures

Figure 1. Chemical structure of etiprednol dicloacetate (BNP-166) and $\Delta^1$-cortienic acid
A: Etiprenol dicloacetate: ethyl-17$\alpha$-dichloroacetoxy-11$\beta$-hydroxyandrosta-1,4-diene-3-one-17$\beta$-carboxylate; B: $\Delta^1$-cortienic acid: 11$\beta$,17$\alpha$-dihydroxy-androsta-1,4-diene-3-one-17$\beta$-carboxylic acid;

Figure 2. Effect of etiprednol dicloacetate and budesonide on antigen-induced airway eosinophil infiltration into the bronchoalveolar tissues of Brown Norway rats. Sensitized rats were treated intratracheally with different doses (0.1, 1, 10 and 100 µg/kg) of the drugs and 2 hours later were challenged with ovalbumin aerosol. 48 hours later their lungs were fixed in 8 % formalin and were processed for histochemistry as described in the materials and methods. Panel A to D show representative samples derived from sensitized (A), challenged (B) and budesonide (10 µg/kg, C) or etiprednol dicloacetate-treated (10 µg/kg, D) and challenged animals. In panel E graphic representation of peribronchial eosinophil numbers determined in samples derived from 4 to 25 animals for each treatment group are shown. (BNP = etiprednol dicloacetate, BUD = budesonide) The level of significance (Mann-Whitney U-test) in case of each drug-treated group compared to vehicle treated challenged controls was p<0.001 therefore it was not labeled on the figure. Calculated ED$_{50}$-values were: etiprednol dicloacetate = 0.62 µg/kg and budesonide = 1.61 µg/kg. Etiprednol dicloacetate-treatment was significantly superior to budesonide-treatment (p<0.0001, analysis of variance with 2-way ANOVA).
Figure 3. Effect of etiprednol dicloacetate and budesonide on antigen-induced goblet cell mucus production in the bronchoalveolar tissues of Brown Norway rats. Sensitized rats were treated intratracheally with different doses (0.1, 1, 10 and 100 µg/kg) of the drugs and 2 hours later were challenged with ovalbumin aerosol. 48 hours later their lungs were fixed in 8% formalin and were processed for histochemistry as described in the materials and methods. Panel A to D show representative samples derived from sensitized (A), challenged (B) and budesonide (10 µg/kg, C) or BNP-treated (10 µg/kg, D) and challenged animals. In panel E graphic representation of the ratio of PAS-positive (mucus producing) and total number of airway epithelial cells determined in samples derived from 4 to 25 animals for each treatment group are shown. (BNP = etiprednol dicloacetate, BUD = budesonide) The level of significance in case of each drug-treated group, except for BUD-0.1, compared to vehicle treated challenged controls were p<0.001 therefore it was not labeled on the figure. Calculated ED<sub>50</sub>-values were: etiprednol dicloacetate = 13.8 µg/kg and budesonide = 32.7 µg/kg. Etiprednol dicloacetate-treatment was significantly better than budesonide-treatment (p=0.0041, analysis of variance with 2-way ANOVA).

Figure 4. Effect of etiprednol dicloacetate and budesonide on antigen-induced perivascular edema-formation in the bronchoalveolar tissues of Brown Norway rats. Sensitized rats were treated intratracheally with different doses (0.1, 1, 10 and 100 µg/kg) of the drugs and 2 hours later were challenged with ovalbumin aerosol. 48 hours later their lungs were fixed in 8% formalin and were processed for histochemistry as described
in the materials and methods. Graphic representation of the ratio of edematous and the vascular area determined in samples derived from 4 to 25 animals for each treatment group are shown. (BNP = etiprednol dicloacetate, BUD = budesonide) The level of significance in case of each drug-treated group compared to vehicle treated challenged controls are: (*)-p<0.05; (**) -p<0.01; (***)-p<0.001. Calculated ED50-values were: etiprednol dicloacetate = 2.9 µg/kg and budesonide = 8.5 µg/kg. Etiprednol dicloacetate-treatment was significantly better than BUD-treatment (p=0.0042, analysis of variance with 2-way ANOVA).

Figure 5. Effect of etiprednol dicloacetate on antigen-induced airway hyperresponsiveness of Brown Norway rats. Sensitized rats were treated intratracheally with etiprednol dicloacetate and 2 hours later were challenged with ovalbumin aerosol as described in the materials and methods. 48 hours later their trachea were isolated, and contraction of tracheal rings to serial concentrations of acetylcholine was determined. Graphic representation of the responses (mean ± S.E.M.), expressed as percent of the maximal response of control samples, derived from three separate experiments are shown. Open triangles: sensitized, non-challenged animals (n=14); filled triangles: challenged vehicle-treated animals (n=14); closed circles: challenged animals, treated with etiprednol dicloacetate 1.0 µg/kg (n=12).

Figure 6. Effect of the concentration of plasma on the efficacy of dexamethasone and etiprednol dicloacetate in the inhibition of lipopolysaccharid induced TNF-α production by human blood. Blood samples after 1:5 dilution (filled circle) or without
dilution (open circle) were incubated together with the test compounds, dexamethasone (A), or etiprednol dicloacetate (B), and stimulated with 1 µg/ml lipopolysaccharid. After 18-20 hours incubation TNF-α levels in cell-free supernatants were determined by ELISA. Results are expressed as percentage of inhibition. The values represent mean ± S.E.M. for five individuals. Ranges of TNF-α levels in non-stimulated and LPS-stimulated (NS/LPS, pg/ml) control cultures were: <8-40 / 881-3087 (diluted blood), and <8-152 / 1989-4163 (undiluted blood).

Figure 7. Comparison of the transrepressing and transactivating properties of etiprednol dicloacetate, budesonide and dexamethasone. Inhibition of IL-1β production of lipopolysaccharid and silica stimulated THP.1 cells (transrepression), and stimulation of tyrosine aminotransferase activity (transactivation) in HAT cells were determined as described in the materials and methods. Activities were expressed relative to dexamethasone and comparison was done with two molar concentrations of the compounds. Means plus standard deviations calculated from three independent experiments are shown. Black bars = dexamethasone; gray bars = budesonide; white bars = etiprednol dicloacetate. There was no statistically significant difference between transrepressing activity of etiprednol dicloacetate and budesonide, but the transactivating properties of the two steroids differed significantly from each other at both of the applied concentrations (p<0.03; Student’s t-test).
Tables

Table 1. Effect of glucocorticoids on the ovalbumin stimulated tracheal hyperresponsiveness. Sensitized Brown Norway-rats were treated intratracheally and 2 hours later were challenged with ovalbumin aerosol as described in the materials and methods. 48 hours later their trachea were isolated, and contraction of tracheal rings to different concentrations of acetylcholine (Ach) was determined.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C$_{50}$(Ach)$^a$</th>
<th>Max. response$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[µg/kg itr.]</td>
<td>mean±SEM</td>
</tr>
<tr>
<td></td>
<td>(n) Log M</td>
<td>p$^c$</td>
</tr>
<tr>
<td>Sensitized (26)</td>
<td>-5.84 ± 0.16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Challenged (27)</td>
<td>-6.96 ± 0.17</td>
<td>-</td>
</tr>
<tr>
<td>BNP [0.1] (8)</td>
<td>-6.23 ± 0.27</td>
<td>0.0122</td>
</tr>
<tr>
<td>BNP-[1.0] (12)</td>
<td>-5.60 ± 0.33</td>
<td>0.0003</td>
</tr>
<tr>
<td>BUD-[0.1] (13)</td>
<td>-7.42 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>BUD-[1.0] (13)</td>
<td>-5.78 ± 0.33</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

$^a$ Concentration of acetylcholine necessary to provoke contraction equal to the half-maximal contraction of non-challenged animals;

$^b$ Measure of contraction at the highest applied acetylcholine dose, relative to non-challenged animals

$^c$ Student’s t-test;
Table 2. Effect of the time of incubation or pre-incubation with human serum on the effectivity of glucocorticoids. **Panel A**: serial concentrations of glucocorticoids were incubated together with different cells stimulated with lipopolysaccharid and after various time of incubation GM-CSF production were determined, and IC$_{50}$-values (shown in the table in nM) were calculated. Ranges of LPS-stimulated control levels (pg/ml) of the cytokine at the specified incubation periods were the following: (i) Human nasal polyp cells: 203-209 (day 1), 614-628 (day 2) and 2490-3270 (day 5); (ii) Human nasal cell derived fibroblasts: 84-86 (day 1) and 133-144 (day 5); **Panel B**: serial concentrations of glucocorticoids were incubated together with fresh human serum for 18 hours, than were added to human peripheral blood mononuclear cells stimulated with lipopolysaccharid and after 24 hours of incubation TNF-$\alpha$ production were determined and IC$_{50}$-values (shown in the table in nM) were calculated. Control cultures were set up without pre-incubating the drugs with serum. Ranges of basal and LPS-stimulated control levels (in pg/ml) of the cytokine were the following: Donor 1: 102 (unstimulated), 1637 (stimulated); Donor 2: 744 (unstimulated), 2979 (stimulated);
### A

<table>
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<th>Cells</th>
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<th>Compound</th>
<th>Incubation time (days)</th>
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<td>GM-CSF</td>
<td>Budesonide</td>
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<tr>
<td>Human nasal polyp derived</td>
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<td>Etiprednol</td>
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<td></td>
<td>dicloacetate</td>
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</tr>
<tr>
<td>Dexamethasone</td>
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<td></td>
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### B

<table>
<thead>
<tr>
<th>Donor</th>
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<th>Compound</th>
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<th>With pre-inc. incubation</th>
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Figure 1
Figure 2.
Figure 3.
Figure 6.