Potency and Specificity of the Pharmacological Action of a New, Antiasthmatic, Topically Administered Soft Steroid, Etiprednol Dicloacetate (BNP-166)

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ABSTRACT

In the present study, the pharmacological effects of etiprednol dicloacetate (BNP-166; ethyl-17α-dichloroacetoxy-11β-hydroxyandrost-1,4-diene-3-one-17β-carboxylate), 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 with 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 preincubated with human plasma. These features are characteristics of a soft drug that 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 1970s, 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 (available at http://www.ginasthma.com)]. Confidence in the 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’Connell, 1998), which again points to the significance and the necessity of safer drugs.

ABBREVIATIONS: ICS, inhaled corticosteroids; GR, glucocorticoid receptor; BNP-166, ethyl-17α-dichloroacetoxy-11β-hydroxyandrost-1,4-diene-3-one-17β-carboxylate; BALF, broncho-alveolar lavage fluid; PAS, periodic-acid Schiff staining; ELISA, enzyme-linked immunosorbsent assay; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; TAT, tyrosine-amino-transferase; ANOVA, analysis of variance; GRE, glucocorticoid response element; GM-CSF, granulocyte macrophage-colony stimulating factor.
To further improve the therapeutic index, that is the ratio of the toxic to the therapeutic dose of a drug, it 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) in which one can design a soft analog of a drug that 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 (Vaysseiere et al., 1997).

Etiprednol dicloacetate (BNP-166; ethyl-17α-dichloroacetoxysteroxy-11β-hydroxyandrosta-1,4-diene-3-one-17β-carboxylate) is a new soft steroid designed based on Δ⁴-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, 1999b). Etiprednol dicloacetate was produced by modifying both the carboxyl (β) and hydroxyl (α) groups at position 17 of Δ⁴-cortienic acid. In this study, the effects of etiprednol dicloacetate on allergen induced airways inflammation, remodeling, and hyperreactivity was examined and compared with those of budesonide in vivo in an asthma model of the Brown Norway rat. Using in vitro methods, the soft character of etiprednol dicloacetate 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.

Materials and Methods

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

Animals. Male Brown Norway rats, weighing 140 to 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 in standard animal cages (five to a cage) on a constant 12-h 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-Aldrich (St. Louis, MO), unless indicated otherwise. Etiprednol dicloacetate (BNP-166; Fig. 1, A) was synthesized at the Department of Chemistry of IVAX-DRI. Bordatella pertussis vaccine was a generous gift of Dr. Mihály Garamvölgyi (Human LTD, Gödöllő, Hungary). Urethane, Primazin (2% xylazine), Ketalar (10% ketamine), and the Unopette kit were purchased from Reanal (Budapest, Hungary), Alfasan International BV (AB Woerden, The Netherlands), Parke Davis (London, UK), and BD Biosciences (Franklin Lakes, NJ), respectively.

Sensitization, Treatment, and Challenge of Animals. Animals were randomly assigned into various treatment groups (4–5 animals/group), weighed, and numbered. They were sensitized with ovalbumin precipitated on alum (25 μg of ovalbumin + 20 mg of Al(OH)₃) in 0.5 ml of saline/animal) administered subcutaneously on the back on days 0, 14, and 21. Simultaneously, on each occasion, 0.25 ml (10⁹ cells/ml) of 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 h before the challenge. Intratracheal drug application was performed under short lasting general anesthesia 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 midportion of the trachea. Powdered solid substance (10 mg) [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 h 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. Forty-eight hours after challenge, animals were sacrificed by an overdose of urethane, and then bronchoalveolar lavage fluid (BALF) was obtained. After a tracheotomy, a polyethylene catheter was inserted and advanced to the bifurcation of the trachea. The airways were then washed by 3 ml of Hank’s balanced salt solution prewarmed 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. Trachea were removed from the animals, and after careful cleaning from the

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Fig. 1. Chemical structure of etiprednol dicloacetate (BNP-166) and Δ⁴-cortienic acid. A, etiprenol dicloacetate: ethyl-17α-dichloroacetoxystereoxy-11β-hydroxyandrosta-1,4-diene-3-one-17β-carboxylate; B, Δ⁴-cortienic acid: 11β,17α-dihydroxy-androsta-1,4-diene-3-on-17β-carboxylic acid.
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 of tension, and after an equilibration period of 30 min, cumulative concentration response to acetylcholine was determined. Maximal response of control (sensitized, unchallenged, and nontreated) tracheal rings was obtained at 10⁻⁸ 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 bronchoalveolar lavage. Samples were fixed in phosphate-buffered 8% formalin for 2 weeks, and then were routinely processed for histochromy. Thick sections (5 µm) 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 630×. Perivascular edematous areas were determined on periodic acid-Schiff-stained (PAS) specimens that were counterstained with hematoxylin. Fifty randomly chosen microvessels of each experimental group were digitally exposed (Zeiss Axiocam; Axiovert 200 system) at a magnification of 400×. Measurement of the area of perivascular edema was performed by Zeiss Axiovision 3.1 software (Carl Zeiss Vision GmbH, Jena, Germany). Extension of the perivascular edema is expressed as a percentage of the area of the respective microvessel. Mucus-producing goblet cells 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 400×. 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), and the IL-1β set was obtained from R&D Systems (Minneapolis, MN). ELISA was performed according to the manufacturer’s protocols. 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 the mean percentage inhibition and goblet cell hyperplasia were determined similarly by comparing the control response to the response of treated preparations. The control value was defined as 100%, and all values were expressed as a percentage and related to the control response. Contraction values were determined by linear regression.

**TNF-α Production of Lipopolysaccharide-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 lipopolysaccharide for 24 h at 37°C in a CO₂ thermostat. Controls were treated with lipopolysaccharide and the vehicle (PBS or 0.01% dimethyl sulfoxide in PBS). After incubation, cell-free supernatants were separated by centrifugation at 1,000 g for 10 min 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.

Preincubation of the Experimental Compounds with Serum and Measurement of Their Effect on TNF-α Production of Lipopolysaccharide-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 of 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 h previously, were added (0.05 ml) together with the lipopolysaccharide (0.05 ml; 1 µg/ml final concentration). Preincubation of the compounds with serum was carried out at 37°C. Processing of the cultures was done as described above. In the experiments, the amount of TNF-α produced was determined by ELISA sets were used. TNF-α levels in cell-free supernatants were determined by Bradford reagent, and the samples obtained were either stored at −20°C or used immediately. Tyrosine transaminase activity 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 neoglycogenesis), by the method of Diamondstone (1966) using a rat hepatoma cell line (ECACC 93129198; European Collection of Cell Cultures, Salisbury, Wiltshire, UK) and treatment conditions as described before (Vayssiere et al., 1997). Hepatoma cell line cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 5 × 10⁻⁵ M 2-mercapto-ethanol, 2 mM glutamine, and antibiotics, and were split every 3 days. To examine the effect of test compounds, 2 × 10⁶ cells/well (24-well plates in 1 ml/well volume) were stimulated with 1 µg/ml lipopolysaccharide and 25 µg/ml silica for IL-1β, as described previously (Németh et al., 1995). The test compounds were dissolved in RPMI 1640 medium or the medium containing 0.01% dimethyl sulfoxide. Two parallel cell cultures per treatment groups were run in three independent experiments. IL-1β levels in cell-free supernatants were determined by ELISA. Activities of compounds were expressed relative to dexamethasone.

**Measurement of Tyrosine-Amino-Transferase Activity in Rat Hepatoma Cells.** Transactivating 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 neoglycogenesis), by the method of Diamondstone (1966) using a rat hepatoma cell line (ECACC 93129198; European Collection of Cell Cultures, Salisbury, Wiltshire, UK) and treatment conditions as described before (Vayssiere et al., 1997). Hepatoma cell line cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Semi-confluent 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 40,000g for 20 min 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 L-tyrosine to L-dopa with the automated 200 µl (34.5 µM L-tyrosine, 84 µM pyridoxal phosphate, and 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 that stopped the reaction and started the conversion of p-hydroxyphenylpyruvic acid to p-hydroxybenzaldehyde. 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.

**ED₅₀ Calculation, Statistical Evaluation.** ED₅₀ values were calculated with GraphPad Prism software (GraphPad Software, Inc., San Diego CA). Statistical analysis between groups was done with Mann-Whitney U test or with Student’s t test; differences between treatments (e.g., etiprednol dicloacetate versus budesonide) was analyzed by two-way ANOVA. All the calculations were done with a Statistica for Windows software version 5.1 (StatSoft Inc., Tulsa OK).

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**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, 3 to 4-fold increase was seen compared with sensitized nonchallenged controls, while peribronchially (Fig. 2, A, B, and E) and perivascularly 20- to 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 (Fig. 2, C, D, and E). The 50% effective doses of both compounds were around the 1/10 g/kg dose; etiprednol dicloacetate (0.62 g/kg) was slightly more effective than budesonide (1.61 g/kg) in these examinations, and this difference was reflected by the comparative

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**Fig. 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 h later were challenged with ovalbumin aerosol. Forty-eight hours later their lungs were fixed in 8% formalin and were processed for histochemistry, as described in the materials and methods. 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 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 with vehicle treated challenged controls was p < 0.001; therefore, it was not labeled on the figure. Calculated ED₅₀ 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 two-way ANOVA).
statistics of the treatments as well (p < 0.0001; two-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 bronchoalveolar 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 with 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 (Fig. 3, A and B). This alteration was quantitated by determining the ratio of PAS-positive cells to all epithelial cells, and in general, at least a 10-fold increase could be detected in this ratio (Fig. 3, E). Both etiprednol dicloacetate and budesonide effectively decreased the number of mucus secreting cells, as it is demonstrated in Fig. 3, C and D, showing data at the 10 μg/kg doses. Graphical presentation of all of the data (Fig. 3, E) reveals the quantitated efficacy of the compounds. Almost all of the applied doses of etiprednol dicloacetate were more effective than similar doses of budesonide; the calculated

![Fig. 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 h later were challenged with ovalbumin aerosol. Forty-eight hours later their lungs were fixed in 8% formalin and were processed for histochemistry as described in the materials and methods. 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. 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 with vehicle treated challenged controls was p < 0.001; therefore, it was not labeled on the figure. Calculated ED_{50} 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 two-way ANOVA).]
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 4-fold relative to the same area in the control (sensitized, nonchallenged) animals (Fig. 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 (Fig. 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 Hyperreactivity.** Airway 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 (Fig. 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 Preincubation 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.

![Fig. 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 h later were challenged with ovalbumin aerosol. Forty-eight 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 with vehicle treated challenged controls are: *, p < 0.05; **, p < 0.01; and ***, p < 0.001. Calculated ED$_{50}$ 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 two-way ANOVA).

![Fig. 5. Effect of etiprednol dicloacetate on antigen-induced airway hyperresponsiveness of Brown Norway rats. Sensitized rats were treated intratracheally with etiprednol dicloacetate and 2 h later were challenged with ovalbumin aerosol, as described under Materials and Methods. Forty-eight hours later their tracheas 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, nonchallenged 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).]
The effect of different concentrations of sera components on the activity of etiprednol dicloacetate was measured in a simple in vitro system in which cells of whole human blood were stimulated with lipopolysaccharide 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 and 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 3-fold if the examination was carried out in undiluted specimens, when the duration of incubation was the same (Fig. 6). The effect of the incubation time and the preincubation 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 (Table 2), 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 efficacy of budesonide. The activity of etiprednol dicloacetate on GM-CSF production of nasal polyd-derived fibroblasts decreased more than 20-fold, while only a 2-fold reduction was seen with dexamethasone. Preincubation with human sera caused a radical drop in the activity of etiprednol dicloacetate (Table 2). It triggered more than a 100-fold decrease in the efficacy of etiprednol dicloacetate, while the same treatment resulted in only a 2-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 (Fig. 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

TABLE 1
Effect of glucocorticoids on the ovalbumin-stimulated tracheal hyperresponsiveness. Sensitized Brown Norway rats were treated intratracheally and 2 h later were challenged with ovalbumin aerosol as described under Materials and Methods. Forty-eight hours later their trachea were isolated, and contraction of tracheal rings to different concentrations of acetylcholine (ACh) was determined.

| Treatment (n) | C50(ACh) Mean ± S.E.M. | Maximum Response Mean ± S.E.M. | p* | p  \\
|---------------|--------------------------|-----------------------------|-----|-----|
| µg/kg | log M | p  \\
| Sensitized (26) | −5.84 ± 0.16 | <0.0001 | 100 ± 0 | <0.0001 |
| Challenged (27) | −6.96 ± 0.17 | | 241 ± 32 | |
| BNP [0.1] (8) | −6.23 ± 0.27 | 0.0122 | 213 ± 31 | N.S. |
| BNP [1.0] (12) | −5.60 ± 0.33 | 0.0003 | 170 ± 30 | 0.0546 |
| BUD [0.1] (13) | −7.42 ± 0.24 | 0.0014 | 196 ± 30 | N.S. |
| BUD [1.0] (13) | −5.78 ± 0.33 | | 130 ± 18 | 0.0017 |

N.S., not significant.
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 nonchallenged animals.
c Student’s t-test.

Fig. 6. Effect of the concentration of plasma on the efficacy of dexamethasone and etiprednol dicloacetate in the inhibition of lipopolysaccharide 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 lipopolysaccharide. After an 18- to 20-h incubation, TNF-α levels in cell-free supernatants were determined by ELISA. Results are expressed as a percentage of inhibition. The values represent mean ± S.E.M. for five individuals. Ranges of TNF-α levels in nonstimulated and LPS-stimulated (NS/LPS; picograms per milliliter) control cultures were: <8–40 / 881-3087 (diluted blood) and <8–152 / 1989–4163 (undiluted blood).
TABLE 2
Effect of the time of incubation or preincubation with human serum on the effectivity of glucocorticoids

<table>
<thead>
<tr>
<th>Cells or Donors</th>
<th>Stimulation</th>
<th>Cytokine</th>
<th>Compound</th>
<th>Incubation Time (Days)</th>
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</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>Human nasal polyp cells</td>
<td>Lipopolysaccharide</td>
<td>GM-CSF</td>
<td>Budesonide</td>
<td>&lt;1</td>
</tr>
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<td></td>
<td></td>
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<td>&lt;1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;1</td>
</tr>
<tr>
<td>Human nasal polyp-derived</td>
<td>Lipopolysaccharide</td>
<td>GM-CSF</td>
<td>Dexamethasone</td>
<td>360</td>
</tr>
<tr>
<td>fibroblast cells</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>650</td>
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<tr>
<td>Donor 1</td>
<td>Lipopolysaccharide</td>
<td>TNF-α</td>
<td>Dexamethasone</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>1905</td>
</tr>
<tr>
<td>Donor 2</td>
<td>Lipopolysaccharide</td>
<td>TNF-α</td>
<td>Dexamethasone</td>
<td>&lt;3</td>
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<td></td>
<td>22</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>316</td>
</tr>
</tbody>
</table>

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 with budesonide; Fig. 7).

Fig. 7. Comparison of the transrepressing and transactivating properties of etiprednol dicloacetate, budesonide, and dexamethasone. Inhibition of IL-1β production of lipopolysaccharide and silica stimulated THP.1 cells (transrepression), and stimulation of tyrosine aminotransferase activity (transactivation) in HTC cells were determined as described under 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).

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 article indicate the serious need for continued surveillance and targeted interventions in the U.S. 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 efficacy 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 antiglaucoma agents (Bodor, 1989), antimicrobials (Bodor et al., 1980a), antitumor 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 nonspecific 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 antiasthmatic drug candidates (Szelenyi, 2000). Ovalbumin-sensitized and challenged Brown Norway rat is a widely used animal model in the development of agents with antiasthmatic properties (Huang et al., 2002; Trifiliieff 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 efficacy in this model with budesonide, which is probably the most widely used, inhaled clinical 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 (Fig. 2), epithelial cells mucus production (Fig. 3), and perivascular edema formation (Fig. 4) to a greater extent than budesonide. Etiprednol dicloacetate also successfully decreased antigen-induced tracheal hyperreactivity (Fig. 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 preincubated with human sera. The efficacy of etiprednol dicloacetate decreased in parallel with the increase of the amount of serum proteins in the assay (Fig. 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 preincubated 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 preincubation. 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, indicates that once this steroid reaches the systemic circulation it loses its activity; therefore, it might not cause serious side effects. Indeed, the half-life of the original compound in the sera of rats was found less than 1 h after intravenous administration (M. Patfaluski, 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 efficacy 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 and 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 et 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 monocyte 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 (Fig. 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
Acknowledgments

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References


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