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28-Day oral toxicity study with soft corticosteroid BNP-166 in rats and dogs, followed by a 14-day recovery period

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The aim of the present study was to evaluate the soft corticosteroid BNP-166 in rats and dogs treated orally with 0.2, 2.0, and 20.0 mg/kg for 28 days and the reversibility of any abnormalities during a 14-day post-dosing period. The test substance, BNP-166, was well tolerated during the 28-day treatment period. The observed changes were all characteristic for the pharmacological actions of a glucocorticoid. Treatment related changes occurred in the adrenals and thymus, and, to a lesser extent, in the lymph nodes, spleen and liver. There were no statistically significant reductions in the cortisol levels of all groups in the 0.2 and 2 mg/kg treatments. Significant reductions were observed in the high-dose group (20 mg/kg), but levels returned to normal by the end of the 14-day recovery period. Based on the results, the No Observable Adverse Effect Level (NOAEL) of BNP-166 soft corticosteroid in rat and dog after 28-day oral administration is 2 mg/kg. This value is approximately 40 times higher than that of budesonide. Pharmacodynamic and receptor binding studies have shown BNP-166 to have a similar potency to budesonide; therefore, BNP-166 can be considered safer when administered orally than other corticosteroids such as prednisolone or budesonide.

1. Introduction

Soft drugs are new therapeutic agents that undergo predictable metabolism to inactive metabolites after exerting their therapeutic effect [1, 2]. Corticosteroids represent an important class of drug used to treat inflammatory conditions and allergies as they are among the most effective anti-inflammatory compounds and offer the broadest range of treatment [3]. However, a number of contraindications, mostly resulting from general systemic corticosteroid side effects, severely limit their usefulness. Besides the consequences that result from the suppression of the hypothalamic-pituitary-adrenal (HPA) axis, prolonged therapy with corticosteroids is also limited by complications as fluid and electrolyte abnormalities, hypertension, hyperglycemia, increased susceptibility to infection, osteoporosis, myopathy, behavioral disturbances, cataracts, growth arrest, fat redistribution, and others [3]. Within the corticosteroid field, various attempts have been made to separate local and systemic effects, and soft steroids represent the most successful ones [2, 4]. The aim of the present study was to evaluate the soft corticosteroid BNP-166 in rats and dogs treated orally with 0.2, 2.0, and 20.0 mg/kg for 28 days and the reversibility of any abnormalities during a 14-day post-dosing period.

2. Investigations and results

2.1. Acute limit tests

Prior to the 28-day toxicity studies, acute limit tests were carried out in both rats and mice. Six animals per sex per group were administered BNP-166 by either the oral or the subcutaneous route at a dose of 2000 mg/kg. Animals were observed for 14 days before necropsy. No animals died during these studies, and no clinical effects were reported. Reduced weight gain was seen in all BNP-166-treated groups after subcutaneous administration. The reduced weight gain in the orally treated females was seen during the first 7 days, thereafter the weights of the animals increased to the point where at 14 days there was no difference between the treatment groups and the control groups. Reduced weight gain in the subcutaneously treated animals persisted for the full 14 days. No effects on thymus and adrenal weight were observed in orally treated animals, whereas in the subcutaneously treated animals these organs had statistically significantly decreased weights.

Following oral administration much of the feces were whitish in color. This is possibly unchanged test article. In the subcutaneously treated animals, residual test article was observed at the injection site indicating that the animals had been exposed to a depot of material that was gradually being absorbed over the 14 day study period.

2.2. 28-Day oral toxicology study in rats

The test substance, BNP-166, was well tolerated during the 28-day treatment period. No animal died and no clinical symptoms were observed during the study. The treatment did not cause intolerable changes in any dose group. The observed changes were all characteristic for the pharmacological actions of a glucocorticoid and were observed in the highest dosing groups (20 mg/kg).

The treatment with test substance influenced the body weight of both males and females in the 20 mg/kg dose groups. Body weight gain of animals as well as food consumption significantly decreased during the treatment period. During the recovery, animals in the treatment groups consumed significantly more food and gained more body weight than the control animals; therefore, the body weight of both the males and the females normalized to the end of the 14-day treatment-free period.

Hematology parameters were in most cases within the physiological limits. The observed significantly higher erythrocyte count, hemoglobin concentration, and hematocrit values, which occurred mostly in the 20 mg/kg treated both males and females groups, was regarded as the pharmacological effect of the test substance. The decreased lymphocyte count recorded in the 20 mg/kg treated groups of both sexes is also due to the pharmacological effect of the test substance. The lower lymphocyte count normalized during the 14-day treatment free period; however, in the case of females, the lymphocyte count remained slightly below the control values.

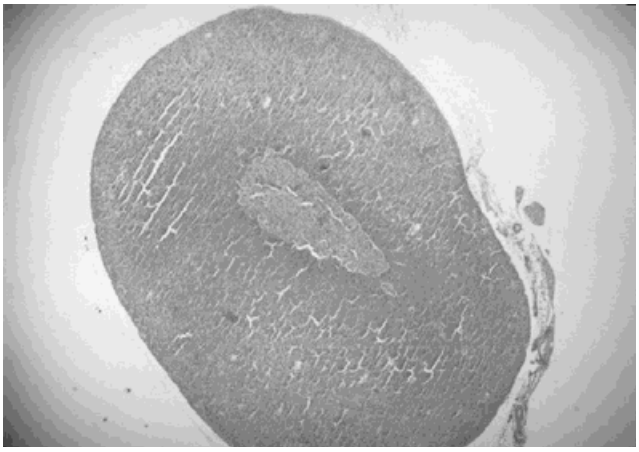

Fig. 1: Normal rat adrenal. Magnification 26×

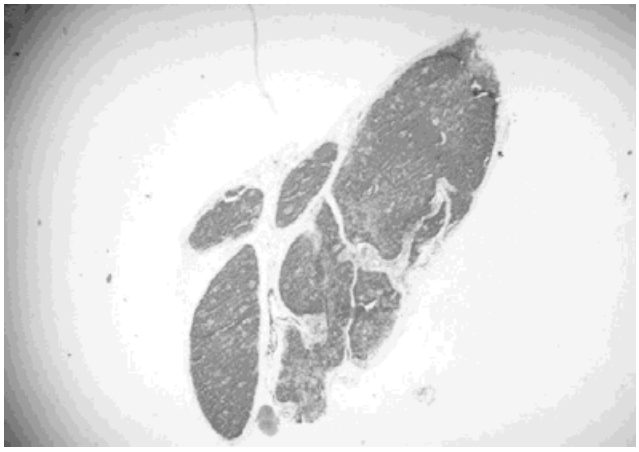

Fig. 4: Thymus of rat treated with 20 mg/kg BNP-166. Magnification 26 ×

No biologically significant differences were recorded among the clinical chemistry parameters of the control groups treated with the test substance. The higher blood glucose levels in females in the 20 mg/kg dose group is a consequence of the pharmacological effect of the test substance.

Gross pathological observations were in agreement with the histopathological findings. All were characteristic of the pharmacological actions of a glucocorticoid.

Important decreases in the absolute and relative weights of thymus, adrenals, and spleen were recorded in the 20 mg/kg dose groups. After the 14-day recovery period, in most cases these organ weights were fully normalized. The absolute and relative adrenal weights in the females were still lower than the controls, but had increased relative to the 28-day values.

During the histopathological examinations, treatment related changes occurred in the adrenals (Figs. 1, 2) and thymus (Figs. 3, 4), and, to a lesser extent, in the lymph nodes (Figs. 5, 6) and spleen. Functional atrophy was observed in the adrenals. In the lymphoid organs of the affected animals cellularity decreased, and the structure of some

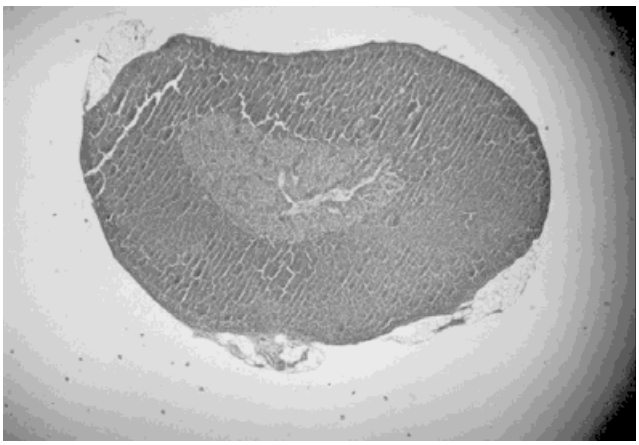

Fig. 2: Adrenal of rat treated with 20 mg/kg BNP-166. Magnification 26 ×

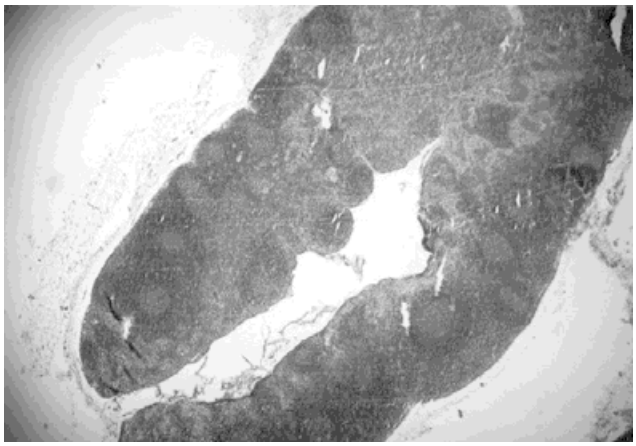

Fig. 5: Normal rat lymph node. Magnification 26×

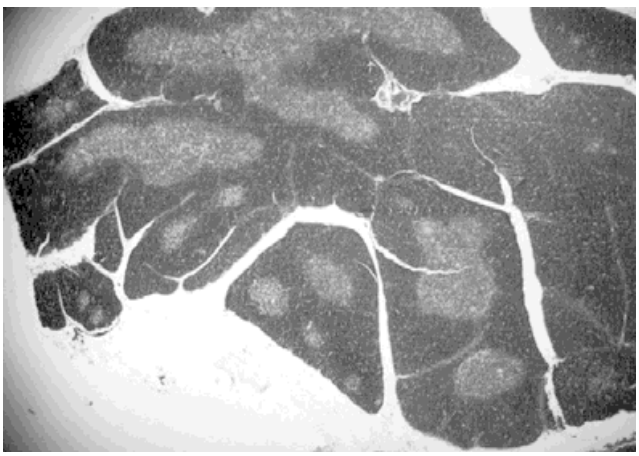

Fig. 3: Normal rat thymus. Magnification 26×

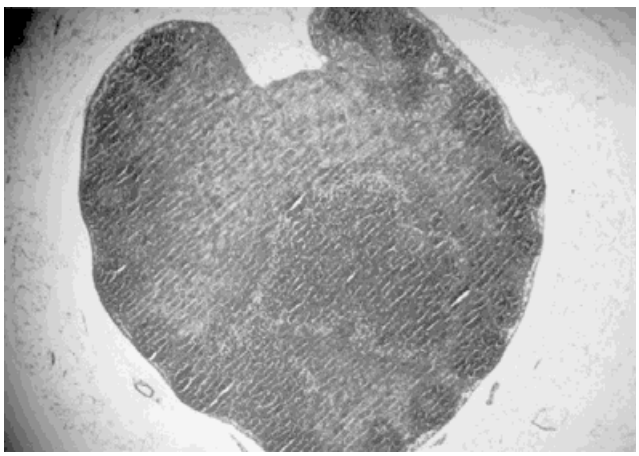

Fig. 6: Lymph nodes of rat treated with 20 mg/kg BNP-166. Magnification 26×

thymuses disintegrated. In the lymph nodes and in a single spleen only, slight diminution of the lymphoid tissue was observed. The low score value and the complete normalization indicated only slight effects. Based on the results, the No Observable Adverse Effect Level (NOAEL) of BNP-166 in rat after 28-day oral administration is 2 mg/kg.

2.3. 28-Day oral toxicology study in dogs

Clinical symptoms occurred rarely during the study. Vomitus in both sexes was observed occasionally before and after treatment in all groups. Because of the sporadic appearance, this observation was not considered treatment related. Mean body weights and food consumption in all treated groups were not statistically significantly different from those in the control groups.

In both groups treated with 20 mg/kg, there was a reduction in body weight gain during the 4-week treatment period. This effect was more pronounced in females, but did not achieve statistical significance in either group. During the 14-day recovery period, there was an increase in body weight gain compared to control animals, which was statistically significant in males but not in females.

The activity of γ-GT and alkaline phosphatase was increased in both males and females from the 20 mg/kg treated group during treatment. The increase of alkaline phosphatase activity is due to the production of so-called steroid-induced alkaline phosphatase. These increases were regarded as a pharmacological rather than hepatotoxic effect of BNP-166; a conclusion supported by the histopathological results. Values of albumin and triglycerides increased in both males and females in the 20 mg/kg group by the end of the 28-day treatment. This is a pharmacological effect on metabolism that is characteristic of the glucocorticoid class of compound.

Changes at autopsy, observed at the end of the dosing period, in livers from females of 2 mg/kg group and from both sexes of 20 mg/kg groups as well as changes in the thymus in males from 20 mg/kg group were considered treatment related. The liver was usually light/yellowish colored, swollen, and easy to tear. Thymuses showed fatty infiltration. While the reddish areas in the small intestinal mucosa from one female in the 20 mg/kg treatment group did not reveal any pathologic alterations when examined microscopically, a relationship to the treatment could not be excluded. The pathological examination of tissues from animals that were in the 14-day recovery groups showed no differences between the treated and control animals, demonstrating that the observed alterations following 28 days of treatment with BNP-166 at 20 mg/kg were reversible.

Adrenal weights at the end of the 28-day treatment were statistically significantly lower in animals from both groups treated at the 20 mg/kg level and in females from 2 mg/kg group compared to controls. This is a characteristic effect of glucocorticoids through negative feedback mechanism on the HPA axis. Following a 14-day recovery period, adrenal weights from males in 20 mg/kg treatment group had recovered partially but they were still statistically significantly lower than those of animals from the control group. Liver weights from both males and females in 20 mg/kg group were higher than those of the control group, but the differences were not statistically significant. This observation was thought to be due to the glycogen deposition, which was observed histopathologically. At the end of the 14-day recovery period, liver weights in the treated animals were similar to those of controls. Thymus weights from both males and females in 20 mg/kg groups and from females in 2 mg/kg groups were reduced at the end of the 28-day treatment period. These decreases were only statistically significant in the high dose females. Following the 14-day recovery period thymus weights in the high dose females had recovered but were still lower than in control females.

There were no histopathology findings that were unrelated to the known pharmacological action of BNP-166. Histopathology findings were in good agreement with the results of other tests carried out during this study. Treatment-related histopathology changes were observed in the 2 mg/kg and 20 mg/kg dose groups, primarily in the adrenals and thymus and to a lesser extent in the lymph nodes, spleen, and liver. Changes in the liver were attributable to the high glycogen content of the organ. The adrenals (Figs. 7, 8) showed a narrowing of the fascicular and reticular zones with smaller cells. This change was described as functional atrophy as the changes were either completely or nearly completely normalized in the 14-day recovery period. Thymuses (Figs. 9, 10) and lymph nodes (Figs. 11, 12) from the high dose group animals showed decreased cellularity or in the most severe cases disintegration of the thymic structure. Atrophy of the thymus was also observed in a single female from the 2 mg/kg group. The observed changes were nearly or completely normalized by the end of the 14-day recovery period.

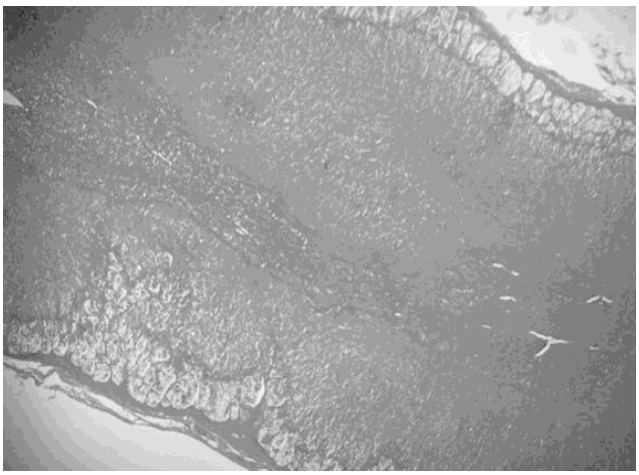

Fig. 7: Normal dog adrenal. Magnification 26×

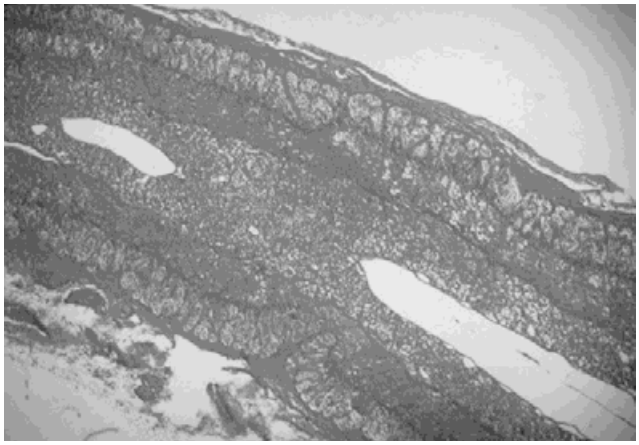

Fig. 8: Adrenal of dog treated with 20 mg/kg BNP-166. Magnification 26×

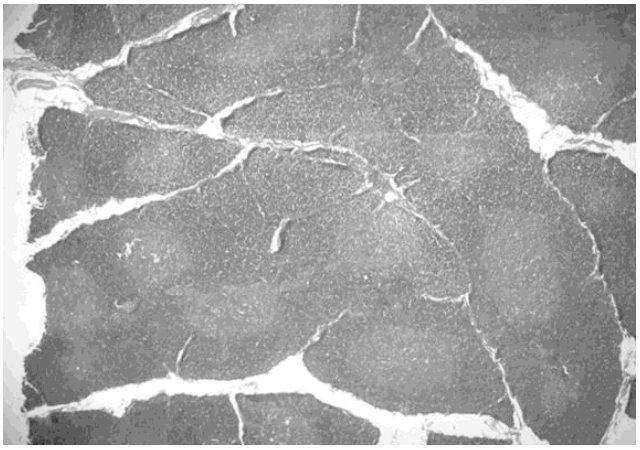

Fig. 9: Normal dog thymus. Magnification 26×

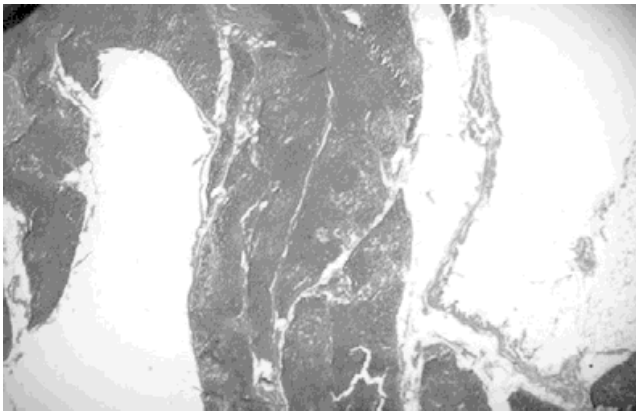

Fig. 10: Thymus of dog treated with 20 mg/kg BNP-166. Magnification 26×

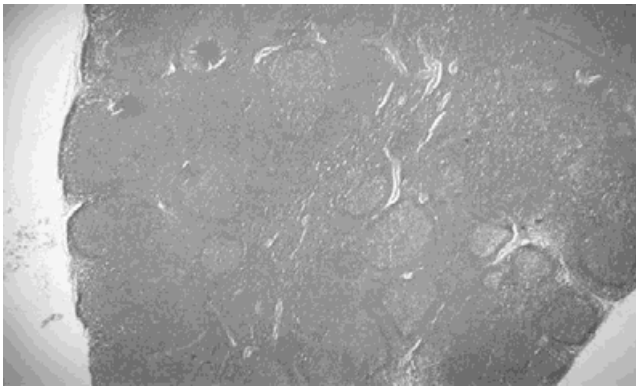

Fig. 11: Normal dog lymph node. Magnification 26×

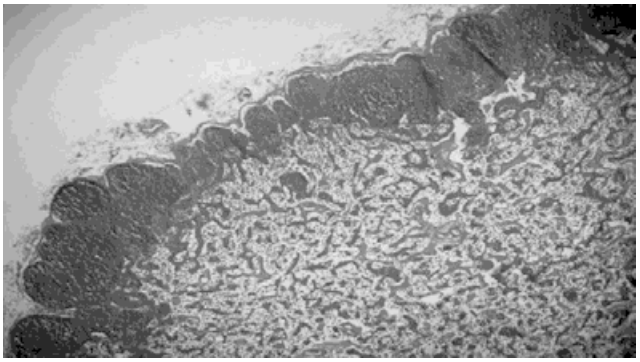

Fig. 12: Lymph node of dog treated with 20 mg/kg BNP-166. Magnification 26×

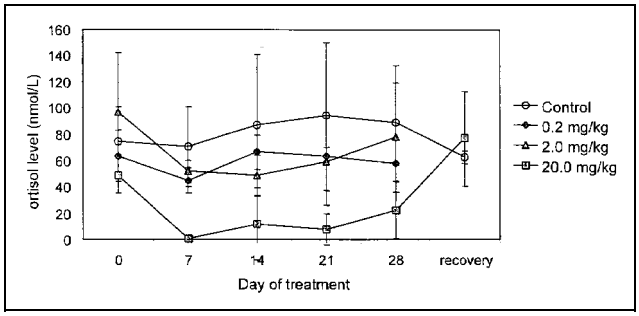

Fig. 13: Cortisol levels after BNP-166 treatment in male beagle dogs. Data represent mean ±SD for N = 8 (control and 20.0 mg/kg) and N = 4 (0.2 mg/kg and 2.0 mg/kg animals per group

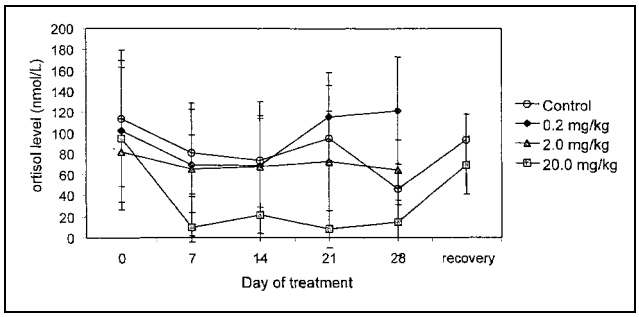

Fig. 14: Cortisol levels after BNP-166 treatment in female beagle dogs. Data represent mean ±SD for N = 8 (control and 20.0 mg/kg) and N = 4 (0.2 mg/kg and 2.0 mg/kg animals per group

Serum cortisol levels were significantly lower in both males and females (Figs. 13, 14) in the 20 mg/kg treatment groups. This is a characteristic pharmacological effect of the glucocorticoid class of drug. Standard deviations of this parameter were high due to highly variable of individual responses. The greatest decrease was observed at day 7 in both sexes, and serum cortisol levels were below the limits of detection in most animals. There were no statistically significant reductions in the cortisol levels of all groups in the 0.2 and 2 mg/kg treatments. Significant reductions were observed in the high-dose group (20 mg/kg), but levels returned to normal by the end of the 14-day recovery period. Based on the results, the NOAEL of BNP-166 soft corticosteroid in dogs, as in rats, after 28-day oral administration is 2 mg/kg.

3. Discussion

Patterns of toxicity of the soft corticosteroid BNP-166 in both the dog and the rat after 28 days administration are typical of the glucocorticoid class of compound. The primary target organs were the thymus and the adrenal and, to a lesser extent, the liver and lymphoid tissues.

Based on published data, one-month oral toxicology studies similar to our design were performed with budesonide in rats and dogs [5]. Forty-eight rats were divided into four groups of 6 per sex per group and treated with doses 0, 0.05, 0.5, and 50 mg/kg/day, by gastric tube. Increased mortality was seen in the high dose group. Atrophy of the adrenals and lymphoid system, gastric ulceration, and intestinal bleeding were found in the treated groups. A dose-related decrease of lymphocytes was also observed. In the dog one-month oral toxicology study, 1/sex/group were treated with doses of 0, 0.01, 0.1, and 1.0 mg/kg. Atrophy of the adrenal and lymphoid system occurred in the 1 mg/kg dose group. Increased glycogen

storage was observed in the liver. These effects were also observed in the mid-dose budesonide group (0.1 mg/kg) and were relatively minor in the low-dose budesonide group. No changes besides these typical corticosteroid effects were reported in the study.

BNP-166 binds to glucocorticoid receptors with a relative binding affinity in the same range of potency as budesonide. It is active in various models of inflammation and allergy. The No Adverse Effect Level (NOAEL) of soft corticosteroid BNP-166 administered orally for 28 days is 2.0 mg/kg. This result compares favorably with budesonide which, when administered orally produced pharmacological effects on the HPA axis at 0.05 mg/kg.

4. Experimental

4.1. Materials

BNP-166 used in this study was obtained from ALCHEM Laboratories Co. (Alachua, FL). Animal studies were performed at the IVAX Institute for Drug Research Central and Dunakeszi Toxicological Facilities, Hungary in accordance with the OECD and EC principles of GLP, Guidelines: OECD 407 and 409, (1995 and 1998) and EPA Nos. 870.3100, 870.3200 and 870.3150 (1998).

Two (males or females) Crl : (WI)BR rats were kept in individual cages with free access to food and water. Beagle dogs (IDR's colony) were kept alone from morning to the afternoon, then in twos (from same sex and dose group) if the symptoms stopped. Dog chow normal canine food was given daily, and the drinking water was available ad libitum.

4.2. Analytical procedures

EDTA-treated blood sample was used for hematology; sodium citrate treated sample for serum for clinical chemistry (centrifuge at 3000 RPM for 20 min). Serum samples were frozen at −18 °C for later processing. Hematology and clinical chemistry parameters were examined by Sysmex F 800, Cobas Mira Plus Chemistry System and KODAK Ektachem DT 60 Analyzer.

Serum cortisol levels were measured using ^{125}I RIA kit (Institute of Isotopes, Budapest, Hungary) by the Clinical Chemistry Laboratory of IDR according to valid operating procedures on all dogs before the treatment period (day 0), during the treatment once a week (day 7, 14, 21), after the treatment period (day 28), and on day 42. The untreated blood was left for 15 min and centrifuged at 3000 rpm for 20 min. Collected serums were frozen at −18 °C and examined within 8 days. Radioactivities of RIA samples were measured by a WALLAC WIZARD 1470 gamma counter. Evaluation of the results was performed by logit-log transformation with GraphPad Prism Version 2.0 software.

4.3. 28-Day oral toxicology study in rats

In the 28-day oral toxicity study, 60 rats per sex were divided into four groups of 10 or 20 rats per sex per group. Two groups of 10 rats/sex/group received either 2 mg/kg (mid dose) or 0.2 mg/kg (low dose) BNP-166 orally for 28 days prior to necropsy. In addition, two groups of 20 rats/sex/group received either 20 mg/kg (high dose) BNP-166 or 0 mg/kg (vehicle placebo) orally for 28 days. Half of the animals in the vehicle and high dose groups were necropsied following the 28-day dosing period. The rest of the animals of these two groups were sacrificed following a 14-day treatment-free period (recovery period). Drugs were administered orally by gavage.

4.4. 28-Day oral toxicology study in dogs

Twenty-four dogs per sex were divided into 4 groups of 4 or 8 dogs per sex per group. Two groups of 4 dogs/sex/group received either 2 mg/kg (mid dose) or 0.2 mg/kg (low dose) BNP-166 orally for 28 days prior to necropsy. In addition, two groups of 8 dogs/sex/group received either 20 mg/kg (high dose) BNP-166 or 0 mg/kg (vehicle placebo) orally for 28 days. At the end of the 28-day dosing period, half of the dogs in the high and vehicle placebo dosed groups were necropsied. The other 4 dogs/sex in these two dosing groups were sacrificed following a 14-day treatment-free period (recovery period). Drugs were administered orally in a gelatine capsule.

4.5. Histopathology

The tissues from each animal were preserved in 10% phosphate buffered formalin or Bouin. In addition part of the liver and the left kidney were preserved in Sanomiya fixative mixture. Bone marrow smears were fixed with methanol. The samples to be examined were embedded in paraffin wax, sectioned, stained with hematoxylin-eosin (as well as Oil-red-O and PAS in case of liver and kidneys) and examined by light microscope. Bone marrow smears were stained with May Grünwald-Giemsa.

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References

1 Bodor, N.: Chemtech **14**, 28 (1984)
2 Bodor, N.; Buchwald, P.: Med. Res. Rev. **20**, 58 (2000)
3 Schimmer, B. P.; Parker, K. L.; in: Hardman, J. G.; Limbird, L. E. (Eds.): Goodman & Gilman's The Pharmacological Basis of Therapeutics, p. 1459, McGraw-Hill, New York 1996
4 Bodor, N.; Buchwald, P.; in: Schleimer, R. P.; O'Byrne, P. M.; Szefler, S. J.; Brattsand, R., (Eds.): Airway Activity and Selectivity of Inhaled Steroids in Asthma, p. 541, Marcel Dekker, New York 2000
5 Astra USA, Inc., NDA 20–441 (1997)

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