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Integration of *in vivo* and *in vitro* approaches to characterize the toxicity of Antalarmin, a corticotropin-releasing hormone receptor antagonist

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Abstract

Non-clinical studies were conducted to evaluate the toxicity of Antalarmin, a corticotropin-releasing hormone type 1 receptor antagonist being developed for therapy of stress-related pathologies.

Antalarmin was not genotoxic in bacterial mutagenesis assays, mammalian cell mutagenesis assays, or *in vivo* DNA damage assays. In a 14-day range-finding study in rats, Antalarmin doses ≥ 500 mg/kg/day (3000 mg/m²/day) induced mortality. In a 90-day toxicity study in rats, no gross toxicity was seen at doses of 30, 100, or 300 mg/kg/day (180, 600, or 1800 mg/m²/day, respectively). Antalarmin (300 mg/kg/day) induced mild anemia, increases in serum γ -glutamyl transferase activity, and microscopic hepatic pathology (bile duct hyperplasia and epithelial necrosis, periportal inflammation). Microscopic renal changes (cortical necrosis, inflammation, hypertrophy, nephropathy) were observed in rats at all Antalarmin doses. In a 14-day range-finding study in dogs, Antalarmin doses ≥ 50 mg/kg/day (1000 mg/m²/day) induced repeated emesis and bone marrow suppression. In a 90-day toxicity study in dogs, Antalarmin (4, 8, or 16 mg/kg/day [80, 160, or 320 mg/m²/day, respectively]) induced bone marrow and lymphoid depletion, but no gross toxicity. Comparative *in vitro* studies using rat, dog, and human neutrophil progenitors demonstrated that canine bone marrow cells are highly sensitive to Antalarmin cytotoxicity, while rat and human bone marrow cells are relatively insensitive. As such, the bone marrow toxicity observed in dogs is considered likely to over-predict Antalarmin toxicity in humans. The hepatic and renal toxicities seen

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in rats exposed to Antalarmin identify those tissues as the most likely targets for Antalarmin toxicity in humans.

Keywords

Antalarmin; corticotropin-releasing hormone receptor antagonist; preclinical toxicology; bone marrow cytotoxicity

1. Introduction

Stress responses are mediated primarily by corticotropin-releasing hormone (CRH; also known as corticotropin-releasing factor or CRF) via the hypothalamic-pituitary-adrenal axis (Rivier and Vale, 1983; Vale et al., 1981). CRH binds to and activates the CRH type 1 receptor (Chen et al., 1993), and thereby stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary. CRH antagonists can ameliorate the neuroendocrine and psychopathological symptoms of severe depression and anxiety by binding to the CRH type 1 receptor, thereby suppressing the effects of unrestrained CRH secretion (De Bellis et al., 1993; Holsboer, 1999; Nemeroff et al., 1984; Zobel et al., 2000).

Antalarmin (*N*-butyl-*N*-ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]amine) (Fig. 1) is a non-peptide (small molecule) antagonist for the CRH type 1 receptor (Webster et al., 1996). Antalarmin was first synthesized by Chen (1994), and binds to the CRH Type 1 receptor with high affinity and specificity, demonstrating K_i values of 1.9, 1.3 and 1.4 nM in pituitary, cerebellum and frontal cortex homogenates, respectively (Webster et al., 1996). Furthermore, Antalarmin is orally bioavailable, and appears to provide a long duration of pharmacologic action *in vivo* (Webster et al., 1996). In rats, administration of Antalarmin suppresses the release of ACTH (Plotsky, 1997; Webster et al., 1996), and in primates, Antalarmin can attenuate the behavioral, neuroendocrine, and autonomic responses to stress (Deak et al., 1999; Habib et al., 2000; Ayala et al., 2004). On the basis of its activity in experimental model systems, Antalarmin is being developed as a model CRH receptor antagonist for possible clinical evaluation.

As part of the preclinical development of Antalarmin, subchronic (90-day) studies were performed to evaluate the toxicity of Antalarmin in rodent and non-rodent species, and to determine the reversibility of any induced toxicities following a 30-day recovery period. In addition to the *in vivo* toxicity studies, genetic toxicology studies (*Salmonella* reverse mutation, mouse lymphoma, and *in vivo* rat bone marrow micronucleus assays) were performed to identify the possible mutagenic or clastogenic effects of Antalarmin. *In vitro* cytotoxicity assays were performed in human, rat, and canine bone marrow cells in order to characterize the possible myelotoxic effects of this agent, and to determine which test species more closely resembles human responses. The results of these *in vivo* and *in vitro* preclinical toxicology studies were designed to support an overall characterization of the toxicology of Antalarmin, identify sensitive target organs for Antalarmin toxicity, investigate possible species differences in toxicity, and support the selection of initial doses of Antalarmin to be used in “first in man” clinical trials.

2. Materials and methods

2.1. Antalarmin

Antalarmin (calculated molecular weight 378.56) was synthesized by Ash Stevens, Inc. (Detroit, MI), and was supplied by the National Institute of Mental Health (Bethesda, MD).

Antalarmin lot numbers PB-V-205 (purity 99.53%) and BM-03-38 (99.76%) were used for these studies. The chemical structure of Antalarmin is shown in Fig. 1.

2.2. Animal welfare

Prior to the initiation of *in vivo* experimentation, study protocols involving experimental animals were reviewed and approved by the IIT Research Institute Animal Care and Use Committee. Studies were performed in a laboratory facility that is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Program elements involving animal care and use were performed in compliance with United States Department of Agriculture regulations and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

2.3. Salmonella reverse mutation assay

The *Salmonella* reverse mutation assay was conducted in accordance with the methods described by Maron and Ames (1983). Five *Salmonella typhimurium* tester strains were used in this assay; 4 tester strains (TA 98, TA 100, TA 1535 and TA 1537) detect histidine reversion at G-C sites, and one tester strain (TA 102) provides enhanced detection of oxidizing mutagens and hydrazines. All tester strains were obtained from Moltox (Boone, NC), and were evaluated both in the presence and absence of a rat liver enzyme metabolic activation system (Aroclor 1254-induced rat liver S9 fraction [Moltox]).

A 25 mg/ml stock solution of Antalarmin was prepared in dimethyl sulfoxide (DMSO) immediately prior to each assay. Based on the results of a cytotoxicity range-finding assessment, Antalarmin was tested for mutagenicity in triplicate cultures ($\pm$ S9) at doses of 5, 50, 150, 625 and 1250 μ g/plate; triplicate cultures exposed to vehicle only and to appropriate positive control mutagens were included in each assay. Positive control mutagens for studies including S9 were 2-aminoanthracene (TA 98, TA 100, TA 1535 and TA 1537); 2-aminofluorene (TA 98 and TA 100); and danthron (TA 102). Positive control mutagens for studies without S9 were daunomycin (TA 98); methylmethanesulfonate (TA 100); cumene hydroperoxide (TA 102); sodium azide (TA 1535); and ICR-191 (TA 1537).

The number of revertant colonies in each plate was determined using an automatic colony counter. Criteria for a valid assay included: a spontaneous revertant frequency within the historical range from this laboratory; a strain-specific positive response to a positive control mutagen; a minimum of 1×10^7 cfu/plate; no cytotoxicity in cultures incubated with at least five concentrations of Antalarmin; and a phenotypically normal background bacterial lawn (in comparison to vehicle control cultures). The test material was considered mutagenic if at least one non-toxic concentration induced a reproducible two or more-fold increase in the number of revertant colonies per plate (compared to vehicle control), and/or a reproducible dose-response trend was identified in one or more tester strains.

2.4. Mouse lymphoma [L5178Y TK^(+/-)] assay

The L5178Y TK^(+/-) (clone 3.7.2C) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in log phase growth by serial subculturing. To reduce the frequency of spontaneous TK^(-/-) mutants, pre-existing TK^(-/-) mutants were removed from cell cultures by exposure to thymidine, hypoxanthine, methotrexate, and glutamine (THMG) for approximately 24 h; this agent combination selects against the TK^(-/-) phenotype.

A stock solution of Antalarmin (25 mg/ml) was prepared in DMSO immediately prior to each assay. For metabolic activation of promutagens, Aroclor 1254-induced rat liver S9 fraction (Moltox) was used. Methyl methanesulfonate served as the positive control article for

incubations without S9, and benzo[α]pyrene was used as the positive control article for incubations in which +S9 was included.

Based on cytotoxicity observed in a dose range-finding assay, Antalarmin concentrations of 0.9, 1.9, 3.9, 7.8, 15.6, 31.3, 62.5 and 125 $\mu\text{g/ml}$ were tested in the definitive mutation assay without S9. Antalarmin concentrations of 1.9, 3.9, 7.8, 15.6, 31.3, 62.5, 125 and 250 $\mu\text{g/ml}$ were tested in the definitive mutation assay in the presence of S9. Exposure times in the definitive mutation assays were 24 h (without S9) and 4 h (with S9).

At 12–15 days after exposure to Antalarmin, vehicle only, or positive control mutagens, the number of large and small mutant colonies in each plate were counted using a BIOTRAN II automatic colony counter (New Brunswick Scientific, Edison, NJ). The cloning efficiency, relative cloning efficiency, relative total growth, and mutation frequency were calculated for each plate. A positive mutagenic response was defined as a statistically significant increase in the number of small and/or large colonies in one or more groups treated with Antalarmin versus vehicle control, and/or a dose-related increase in the number of colonies versus vehicle control. A valid assay also required a vehicle control response within the laboratory historical range and a statistically significant ($p \leq 0.05$) elevation of the number of colonies in cultures exposed to the appropriate positive control.

2.5. *In vivo* rat bone marrow micronucleus assay

Male and female CD rats [CrI:CD[®](SD)IGSBR] were received at approximately 6 weeks of age from virus-free colonies maintained at Charles River Laboratories (Kingston, NY); animal husbandry was as described below in section 2.6. Bone marrow micronucleus assays were performed on six rats/sex/group using the same Antalarmin dosing formulations and dose levels (0 [vehicle], 30, 100, and 300 mg/kg/day) used in the subchronic toxicity bioassay. Antalarmin or vehicle was administered orally (by daily gavage) to rats for 14 consecutive days; cyclophosphamide (30 mg/kg) served as the positive control article, and was administered via intravenous injection to the positive control group once on study day 14. All study animals were euthanized by CO₂ overdose on study day 15. The femur was excised and the bone marrow was flushed using fetal bovine serum (FBS). Bone marrow cells were suspended in FBS and centrifuged. The supernatant was removed except for a small volume (0.1–0.5 ml) and the cells were resuspended in this volume. Three wedge smears of each suspension were made on glass slides, fixed in methanol, and air dried before staining with acridine orange (Hayashi et al., 1983). Slides were coded and analyzed by a reviewer who was unaware of the group identity of any slide. At least 2000 polychromatic erythrocytes (PCE) per animal were screened for the presence of micronuclei using a fluorescent microscope and filters for fluorescein isothiocyanate. In order to stabilize the variance, the micronucleus frequency per 2000 PCEs was transformed by adding one to each count and then taking the log of the adjusted number. The transformed micronucleated PCE data were then analyzed by comparing Antalarmin dose groups versus the vehicle control group using a paired *t*-test.

A positive response was defined as a statistically significant increase in the number of micronucleated cells in one or more groups treated with Antalarmin versus vehicle control and/or a dose-related increase in the number of micronucleated PCE versus vehicle control. A valid assay also required that the vehicle control response fall within the historical range for this laboratory, and a statistically significant ($p \leq 0.05$) increase in the number of micronucleated cells in the positive control group (versus vehicle controls).

2.6. Subchronic oral toxicity study in rats

Male and female CD rats [CrI:CD[®](SD)IGSBR] were received at approximately 5 to 6 weeks of age from virus-free colonies maintained at Charles River Laboratories (Portage, MI). Rats

were housed individually in suspended stainless steel cages in a temperature-controlled room maintained on a 12 h light/dark cycle, and were held in quarantine for two weeks prior to the initiation of drug administration. With the exception of scheduled fasting periods, rats were allowed free access to Certified Rodent Diet 5002 (PMI Nutrition International, Inc., Brentwood, MO). City of Chicago drinking water was supplied to rats *ad libitum* using an automatic watering system.

After release from quarantine, animals were assigned to experimental groups using a computer-based randomization procedure that blocks for body weights. Groups of 20 rats/sex received daily oral (gavage) exposure to Antalarmin at doses of 30, 100, or 300 mg/kg/day (180, 600, and 1800 mg/m²/day, respectively) for 90 days, or to vehicle only (0.5% [w/v] aqueous carboxymethylcellulose/0.2% [w/v] Tween 80) for the same period. Antalarmin dose levels used in the 90-day toxicity study were selected on the basis of a 14-day range-finding toxicity study. In that study, administration of Antalarmin at 500 or 1000 mg/kg/day for 14 days induced mortality, whereas no limiting toxicity was observed in rats receiving Antalarmin at doses of ≤ 250 mg/kg/day.

Throughout the study, rats were observed a minimum of twice daily to monitor their general health status; detailed clinical examinations and measurements of body weight and food consumption were performed once weekly. To identify possible neurotoxicity, functional observational battery (FOB) evaluations were conducted on 5 rats per sex per group during quarantine (pre-test), during weeks 4, 8 and 12, and during the last week of the recovery period. FOB evaluations in rats included home cage observation, handheld observation, audition (click) reflex response, body temperature, open field (mobility/gait), tail pinch, pupil response, vision, hindlimb extension, catalepsy, grip strength (forelimb and hindlimb), aerial righting reflex, and foot splay. Indirect funduscopy ophthalmic examinations were performed on all study animals during the quarantine period (pre-test) and during the final week of the treatment period.

Blood samples for clinical chemistry, hematology, and coagulation evaluations were collected from fasted rats designated for necropsy at the end of the treatment and recovery periods (days 91 and 121, respectively), and from non-fasted rats during study weeks 5 and 9. Clinical pathology assays were performed using automated instruments (Synchron CX5 Clinical Chemistry Analyzer [Beckman Instruments, Brea, CA]; Advia System 120 Hematology Analyzer [Bayer Corp., Tarrytown, NY]; MLA Electra 900 Automatic Coagulation Timer [Hemoliance, Raritan, NJ]). Overnight urine samples were collected from fasted rats on days 91 and 121, and were analyzed by dipstick and microscopy.

On study day 91, 15 rats/sex/group were euthanized by CO₂ overdose and received a complete gross necropsy. All gross lesions plus approximately 45 tissues per rat were collected and fixed in 10% neutral buffered formalin. Remaining rats in each group (designated as recovery animals) were held for an additional 30 days without further treatment, and were euthanized and necropsied (as above) on study day 121. Histologic processing and histopathologic evaluations were performed on all tissues collected at the end of the dosing period from all rats in the high dose and vehicle control groups. Histopathologic evaluation of tissues collected from rats in the low and middle dose groups (euthanized on day 91) and from all rats in the recovery groups (euthanized on day 121) were limited to gross lesions and identified target tissues.

Continuous *in vivo* data from the rat toxicology study were compared by analysis of variance (ANOVA), followed by *post-hoc* analysis using Dunnett's test for comparisons of multiple treatment groups to a single control group. Comparisons of incidence data were performed

using by X^2 analysis or Fischer's exact test. A minimum significance level of $p \leq 0.05$ was used in all comparisons.

2.7. Subchronic oral toxicity study in dogs

Male and female purebred beagle dogs were received at approximately 6 to 7 months of age from Covance Research Products (Kalamazoo, MI), and were held in quarantine for three weeks prior to randomization into experimental groups. Dogs were housed individually in stainless steel cages in a temperature-controlled room maintained on a 12 h light/dark cycle. Dogs were provided with 400 g of Certified Canine Diet 5007 (PMI Nutrition International, Inc.) for a minimum of 2 h each day, and were permitted free access to City of Chicago drinking water supplied via an automatic watering system. Each dog received a supervised daily exercise period outside of its cage.

After release from quarantine, dogs were assigned to experimental groups using a computerized randomization program that blocks for body weight. Groups of 5 dogs/sex received daily oral (capsule) exposure to Antalarmin at doses of 4, 8 and 16 mg/kg/day (80, 160, and 320 mg/m²/day, respectively) for a minimum of 90 days, or to empty capsules only for the same period. Antalarmin dose levels used in the 90-day toxicity study were selected on the basis of a 14-day range-finding toxicity study. In that range-finding study, administration of Antalarmin at doses of ≥ 50 mg/kg/day was associated with repeated emesis, dose-related decreases in body weight and food consumption, duodenal inflammation, and dose-related bone marrow suppression; these doses were considered to be above the maximum tolerated dose for repeat-dose administration of Antalarmin in dogs. In the range-finding study in dogs, administration of Antalarmin at 10 mg/kg/day induced decreases in white blood cell and reticulocyte counts, lymphoid depletion, and minimal hepatic changes, but did not induce emesis or other clinical evidence of toxicity.

Throughout the study, dogs were observed a minimum of twice daily to monitor their general health status; detailed clinical examinations and measurements of body weight and food consumption were performed once weekly. To identify possible neurotoxic effects of Antalarmin, FOB evaluations were conducted on all dogs once during quarantine (pre-test), during weeks 4, 8 and 12, and during the last week of the recovery period. FOB evaluations in dogs included mental status, gait and posture, righting reflex (with and without sight placing responses), papillary reflex, core body temperature, hopping test, wheelbarrow test, hindlimb and forelimb flexor reflexes, perineal reflex, patellar reflex and menace reflex. Indirect funduscopic ophthalmic examinations were performed on all study animals during the quarantine period (pre-test), during the final week of the treatment period, and during the final week of the recovery period. Electrocardiograms (ECGs) were obtained from all dogs during the quarantine period (pre-test), the final week of the treatment period, and the final week of the recovery period; ECGs were evaluated for heart rate and rhythm, amplitude of the P wave and QRS complex, and duration of the P wave, PR, QRS and QT intervals.

Blood samples for clinical chemistry, hematology and coagulation evaluations were collected from fasted dogs at pre-test, from fasted dogs designated for necropsy (during week 13 and during the final week of the recovery period), and from unfasted dogs during study weeks 4 and 9. Clinical pathology assays were performed using automated instruments (Synchron CX5 Clinical Chemistry Analyzer [Beckman Instruments]; Advia System 120 Hematology Analyzer [Bayer Corp.]; MLA Electra 900 Automatic Coagulation Timer [Hemoliance]). Urine samples were collected from fasted dogs at pre-test, week 13 and during the final week of the recovery period, and were analyzed by dipstick and microscopy.

On day 92, 3 dogs/sex/group were euthanized and necropsied with full tissue collection. The remaining 2 dogs in each group (designated as recovery animals) were held for an additional

29–30 days without further treatment, and were euthanized and necropsied on study days 120–121. At necropsy, all gross lesions and approximately 45 tissues were collected from each animal and fixed in 10% neutral buffered formalin for histopathologic evaluation. All tissues collected from all dogs were processed by routine histologic methods, stained with hematoxylin and eosin, and evaluated histopathologically.

Statistical evaluation of continuous *in vivo* data from the canine toxicology study was performed by ANOVA, with *post-hoc* analyses performed using Dunnett's test. Incidence data were compared by χ^2 analysis or Fischer's Exact Test. A minimum significance level of $p \leq 0.05$ was used in all comparisons.

2.8. *In vitro* comparative myelotoxicity assay

In order to determine if the observed species differences in susceptibility to Antalarmin myelotoxicity occurred at the level of the myeloid progenitor cell, and to compare the relative sensitivity of rat, dog, and human myeloid progenitor cells to Antalarmin cytotoxicity, studies were performed to characterize the bone marrow toxicity of Antalarmin using the colony forming unit-granulocyte macrophage (CFU-GM) assay. Human, rat, and canine bone marrow mononuclear cells were isolated and processed in Iscove's Modified Dulbecco's Medium (IMDM). Human bone marrow aspirates were obtained from four healthy volunteers (Poietics, Inc., Gaithersburg, MD), and bone marrow mononuclear cells were isolated via centrifugation over a Ficoll density gradient. Rat bone marrow cells were isolated following sterile dissection of femurs from male F344 rats (Battelle Memorial Institute, Columbus, OH); femurs were placed into 50 ml sterile cell culture tubes containing 20 ml nutrient media supplemented with gentamicin (20 $\mu\text{g}/\text{ml}$ final concentration). Dog bone marrow aspirates were collected sterilely from the iliac crest or femoral canal of donor beagle dogs into a 50 ml syringe containing sodium heparin and gentamicin to final concentrations of 10 IU/ml and 20 $\mu\text{g}/\text{ml}$, respectively. CFU-GM assays were performed as previously described (Erickson-Miller et al., 1997; Parchment, 1998; Parchment et al., 1993, 1994, 1998), except that species-specific recombinant GM-CSF was used as the sole cytokine in each culture.

A pilot study of Antalarmin was conducted using a single marrow donor from each species and final test concentrations of 0.01, 0.10, 0.33, 1.0, 3.3 and 10 $\mu\text{g}/\text{ml}$. This pilot study was followed by the conduct of a definitive myelotoxicity study in which eight concentrations of Antalarmin (1, 3.3, 6.7, 10, 25, 33, 67, and 100 $\mu\text{g}/\text{ml}$) were evaluated using bone marrow progenitors collected from three marrow donors per species. In each study, vehicle control cultures contained 0.05% (v/v) DMSO, without added Antalarmin.

CFU-GM colonies were defined as foci of clonal cell proliferation containing at least 64 myeloid cells. All colony morphologies (focal, focal diffuse, mixed) were included, and studies performed using marrow harvested from each donor were considered to be independent experiments. For each donor, colony counts from triplicate cultures were averaged to obtain a single data point at each concentration, and the percent inhibition was determined by comparing the number of colonies in the treatment group to the number of colonies in the vehicle control cultures exposed to DMSO only (vehicle without added drug). Colony counts (normalized as a percentage of control) at each concentration were then plotted to generate the concentration-response curve. As previously reported (Erickson-Miller et al., 1997; Parchment et al., 1994, 1998; Pessina et al., 2003), the endpoint of the assay for quantifying inter-species differences in susceptibility to drug toxicity is the IC_{90} , which is the concentration that inhibits colony formation by 90%. IC_{90} values were calculated from log-linear regression on concentration-response between the flanking data points on either side of the 90% inhibition level. If the concentration-response curve did not reach the 90% effect level, then the IC_{90} was projected from the log-linear regression line on the last two data points. In the present studies, insufficient

in vitro toxicity prevented the derivation of IC₉₀ values in myeloid progenitor cells of rat and human origin.

3. Results

3.1. *In vitro* and *in vivo* genetic toxicology studies

In range-finding cytotoxicity assays conducted prior to the definitive *Salmonella* reverse mutation assays (Ames tests), Antalarmin was not cytotoxic in any tester strain when bacteria were exposed to dose levels of up to 2500 µg/plate, either with or without metabolic activation (data not shown). In the definitive Ames tests, no increases of two-fold or greater in the frequency of revertant colonies were identified in any tester strain exposed to Antalarmin dose levels of up to 1250 µg/plate, either with or without S9 (Table 1). On this basis, it is concluded that Antalarmin is not mutagenic in a standard battery of Ames tester strains, either with or without metabolic activation.

In the preliminary mouse lymphoma assay (data not shown), 30% to 60% cytotoxicity was observed when L5178Y TK^(+/-) cells were incubated with Antalarmin at concentrations < 250 µg/ml + S9. In the absence of S9, cytotoxicity in L5178Y TK^(+/-) cells exposed to Antalarmin concentrations of < 250 µg/ml ranged from essentially non-cytotoxic to approximately 50%. Incubation of cells with Antalarmin concentrations ≥ 250 µg/ml was highly cytotoxic; levels of cytotoxicity at these concentrations of Antalarmin were above that which is suitable for further study. In the range-finding study, the mutation frequencies of all concentrations of Antalarmin tested (± S9) were within the range of mutation frequencies seen in cultures exposed to the vehicle control article (data not shown). In the definitive mouse lymphoma mutagenicity assay, cytotoxicity ranged from 0 to approximately 50% in the absence of S9, and 0 to approximately 60% in the presence of S9. At each Antalarmin concentration tested (± S9), mutation frequencies in Antalarmin-treated cells were comparable to those observed in vehicle controls (Table 2). On this basis, it is concluded that Antalarmin is not mutagenic in the mouse lymphoma [L5178Y TK^(+/-)] assay.

In the *in vivo* bone marrow micronucleus assay, no statistically significant increases from vehicle control were seen in the number of micronuclei in PCE cells observed in male or female rats exposed to any dose level of Antalarmin (30, 100, or 300 mg/kg/day; Table 3). Statistically significant ($p \leq 0.05$) decreases from vehicle control in the number of micronuclei were observed in mid dose males and females and in high dose males (Table 3). These decreases are not considered to be biologically significant. By contrast, statistically significant ($p \leq 0.05$) increases in the number of micronuclei were observed in both male and female rats exposed to the positive control article, cyclophosphamide. On the basis of these results, it is concluded that Antalarmin is not clastogenic in rat bone marrow cells.

3.2. Subchronic oral toxicity study in rats

Oral administration of Antalarmin to rats for 90 consecutive days at doses of up to 300 mg/kg/day (1800 mg/m²/day) induced no mortality or gross clinical evidence of toxicity in any study animal. No treatment-related clinical signs were observed in any rat treated with Antalarmin at any time in the study, and mean body weights, body weight gains, and food consumption were comparable in vehicle controls and in all Antalarmin-treated groups throughout the study (data not shown). FOBs performed in this study during weeks 4, 8, and 12 failed to identify any neurological deficits in rats receiving subchronic exposure to Antalarmin. The results of ophthalmic examinations were negative in all study groups.

Statistically significant elevations (67% to 433%) in serum γ-glutamyl transferase (GGT) activity were observed at weeks 5, 9, and 13 in both sexes of rats receiving the high dose of

Antalarmin (Table 4). Elevations in serum GGT were not seen in rats receiving the low or middle doses of the test article, and were not present in any group at the end of the recovery period. Smaller (< 30%) but statistically significant elevations in serum cholesterol were seen at all time points in female rats exposed to the high dose of Antalarmin; these changes were not present in female rats in the middle or low dose groups, or in male rats (Table 4). As was the case with GGT, serum cholesterol levels in female rats in the high dose group were comparable to vehicle controls at the end of the recovery period. As such, the effects of Antalarmin on these clinical pathology parameters in rats were reversible upon discontinuation of exposure.

Antalarmin induced a mild, dose-related anemia in both sexes (Table 4). When compared to vehicle controls, small (< 10%) but statistically significant reductions in one or more red cell parameters (hemoglobin, hematocrit, mean cellular hemoglobin, and/or mean cell volume) were seen in both male and female rats exposed to the middle or high doses of Antalarmin at all time points evaluated (weeks 5, 9, and 13). Antalarmin did not induce generalized bone marrow suppression: platelet counts were increased by approximately 25 to 30% in both sexes at various times during the treatment period, and histopathologic evaluation of the bone marrow indicated normal cellularity. As was the case with clinical chemistry endpoints, Antalarmin-induced anemia was largely reversible upon discontinuation of exposure, as most red cell parameters had returned to control levels by the end of the recovery period.

Small (9% to 21%) but statistically significant increases in activated partial thromboplastin time (APTT) were seen in both sexes of rats exposed to the middle or high doses of Antalarmin (Table 4). Because prothrombin time and fibrinogen levels were within normal ranges in both sexes (data not shown), and the effects of Antalarmin on APTT were generally reversible, these modest effects on coagulation are not considered to be toxicologically significant.

Statistically significant increases in urine volume (~200%) with accompanying decreases in specific gravity and refractive index were seen at week 13 in both sexes of rats exposed to the high dose of Antalarmin (data not shown). Because these effects were not present at the end of the recovery period, they could reflect a reversible reduction in renal concentrating function in rats receiving high dose exposure to Antalarmin.

At the terminal necropsy on day 91, statistically significant alterations in the absolute and/or relative (normalized to body weight) weights of the liver, kidneys, spleen, testes, and adrenals were identified in rats receiving subchronic exposure to Antalarmin (Tables 5 and 6). Perhaps the most important of these effects were the dose-related increases in liver weights seen in both sexes. When compared to vehicle controls, increases in mean absolute liver weights in male rats were increased by 24%, 35%, and 55% in the low, middle, and high dose groups, respectively. Similarly, female rats exposed to the low, middle, and high doses of Antalarmin demonstrated increases in mean absolute liver weights of 15%, 49%, and 101%, respectively. Although absolute organ weights had returned to control levels by the end of the recovery period (Table 5), increases in mean relative liver and kidney weights in high dose males and females persisted throughout the recovery period, as did increases in mean relative spleen weight in females (Table 6).

At necropsy on day 91, 3/15 females in the high dose group presented with grossly detectable hepatomegaly; this gross necropsy observation was correlated with both the finding of increased mean liver weight and the microscopic finding of centrilobular hypertrophy. All other gross lesions observed at necropsy were interpreted as incidental findings typically present in rat toxicology studies.

Histopathologic evaluation of tissues collected on day 91 identified drug-related alterations in the liver and kidney. Dose-related histopathologic findings in the liver included centrilobular

hyperplasia, bile duct hyperplasia and epithelial cell necrosis, and periportal inflammation. Dose-related centrilobular hypertrophy was seen in both sexes; this diagnosis was correlated with both the gross finding of hepatomegaly and increases in both absolute and relative liver weights. Bile duct hyperplasia was observed in 1/15 male rats in the middle dose group and 11/15 male and 14/15 female rats in the high dose group. Bile duct epithelial cell necrosis was observed in 10/15 high dose males and 8/15 high dose females, but was not seen at lower doses. These changes were correlated with increases in serum GGT activity seen in both sexes in the high dose group (Table 4). Periportal inflammation was observed in both sexes across all dose groups, but increased in severity with increasing Antalarmin dose.

Histopathologic lesions observed in the kidneys of Antalarmin-treated rats included inner cortical inflammation, necrosis, cytomegaly, and basophilic staining. All of these changes were dose-related, increased in severity with increasing Antalarmin dose, and increased in incidence to near 100% in the high dose group; however, they were not associated with significant alterations in kidney-related clinical pathology parameters such as BUN or creatinine. Nephropathy was observed in male rats in the control and all treatment groups, but was more severe in groups exposed to Antalarmin. Nephropathy was of minimal severity in female rats, regardless of exposure to Antalarmin.

Most microscopic lesions identified in rats exposed to Antalarmin were entirely or partially reversed during the 30-day recovery period. At the end of the recovery period, apparently non-reversible lesions included hepatic periportal fibrosis and chronic inflammation in the inner cortex of the kidney.

On the basis of the microscopic renal changes identified in both sexes at all Antalarmin doses, the No Observed Adverse Effect Level (NOAEL) could not be determined following oral administration of Antalarmin to rats for 90 days. Because administration of Antalarmin at doses ≥ 500 mg/kg/day induced mortality in the 14-day range-finding study, the 300 mg/kg/day (1800 mg/m²/day) dose of Antalarmin that was used in the present 90-day study approximates the Maximum Tolerated Dose (MTD) for this agent in rats.

3.3. Subchronic oral toxicity study in dogs

Oral (capsule) administration of Antalarmin to dogs for 91 consecutive days at doses of up to 16 mg/kg/day (320 mg/m²/day) induced no mortality or gross toxicity. Mean body weights, body weight gains, and food consumption were comparable in capsule controls and in all Antalarmin-treated groups at all times in the study. The results of FOBs failed to identify any evidence of neurotoxicity associated with Antalarmin exposure, and ophthalmology and electrocardiography results in Antalarmin-treated dogs were comparable to those seen in capsule controls (data not shown). However, in comparison to controls, diarrhea and emesis were observed with increased frequency in groups receiving the middle or high doses of Antalarmin.

The results of clinical chemistry assays, hematology assays, and urinalysis were comparable in both capsule controls and in groups exposed to Antalarmin, and no pattern of significant, dose-related effects on coagulation parameters were noted at any time during the dosing period. Similarly, no drug-related gross lesions were identified at either the terminal or the recovery necropsies, and no patterns of drug-related alterations in organ weights were observed in dogs treated with Antalarmin.

Histopathologic evaluation of tissues identified lymphoid depletion (mesenteric lymph nodes) and bone marrow depletion as probable drug-related effects of Antalarmin in dogs; hepatic changes (centrilobular degeneration/necrosis) were also seen in the high dose group, but were of minimal severity. Although lymphoid depletion was not clearly associated with Antalarmin

exposure in male dogs (observed in one control male dog and in one male dog in each Antalarmin dose group), lymphoid depletion was observed in one female in the low dose group and in three females in the high dose group. Bone marrow depletion was identified in one female dog in the high dose group, but was not seen in male dogs at any dose level or in female dogs in groups exposed to lower doses of Antalarmin. Because bone marrow depletion was observed in the 14-day range-finding study in both sexes of dogs exposed to Antalarmin at doses of 50, 100 or 200 mg/kg/day, this lesion in the high dose female from the current study is considered likely to be drug-related. By contrast to the results of the rat study, no microscopic evidence of Antalarmin toxicity was seen in the kidney.

On the basis of the bone marrow depletion and increased incidence of lymphoid depletion observed in female dogs in the high dose (16 mg/kg/day) group in the definitive study, and in both sexes exposed to Antalarmin at 10 mg/kg/day in the range-finding study, histopathology data suggest that the NOAEL for oral administration of Antalarmin to dogs is 8 mg/kg/day. However, occasional (but not dose-limiting) emesis was seen in dogs receiving Antalarmin at both the 8 and 16 mg/kg/day dose levels. Because emesis was not observed in dogs administered the low dose of Antalarmin, the NOAEL for subchronic administration of Antalarmin to dogs is defined as the low dose used in the present study (4 mg/kg/day [80 mg/m²/day]).

Oral administration of Antalarmin to dogs in the range-finding study was limited by repeated emesis and consequent body weight loss in dose groups exposed to doses of 50 mg/kg/day or greater. Because emesis was present (but not limiting) in dogs exposed to Antalarmin at 16 mg/kg/day (320 mg/m²/day) in the definitive study, the MTD for subchronic oral administration of Antalarmin to dogs falls between 16 mg/kg/day and 50 mg/kg/day.

3.4. *In vitro* bone marrow myelotoxicity studies

The pilot myelotoxicity study with Antalarmin was conducted using bone marrow from one donor per species (human, rat and dog), with the goal of identifying concentration-response relationships that could be studied in greater detail in definitive studies. The goal of the definitive studies was to derive IC₉₀ values on which interspecies comparisons of sensitivity to Antalarmin myelotoxicity could be based.

In the pilot myelotoxicity assay, the highest concentration of Antalarmin (10 µg/ml) tested completely inhibited colony formation by canine CFU-GM. By contrast, colony formation by human CFU-GM and rat CFU-GM was not inhibited by Antalarmin at 10 µg/ml (Fig. 2). Because 1 µg/ml Antalarmin was not inhibitory to CFU-GM from any species (Fig. 2), this concentration was defined as the highest non-toxic concentration.

In the definitive study, Antalarmin induced a concentration-related suppression of CFU-GM colony formation in 3 of 3 dog bone marrow specimens (Fig. 3). Individual dogs did demonstrate a range of sensitivity to Antalarmin myelotoxicity. CFU-GM from one dog donor exhibited sensitivity to Antalarmin that was quantitatively similar to that seen in the canine marrow donor used in the pilot study. The second dog demonstrated a similar response, but with a steeper concentration-response curve. Bone marrow CFU-GM from the third donor dog demonstrated less sensitivity to Antalarmin toxicity at low concentrations, but exhibited an IC₉₀ that was similar to the first two donor dogs. Overall, the IC₉₀ values (120 ± 25 µg/ml) calculated from CFU-GM assays with three different canine bone marrow donors displayed a reasonable degree of inter-animal consistency, and demonstrated that canine bone marrow is highly sensitive to Antalarmin toxicity.

By contrast to the results seen with CFU-GM in dog bone marrow, Antalarmin concentrations of up to 100 µg/ml had no effect on colony formation in rat CFU-GM or human CFU-GM (Fig.

3). In fact, bone marrow cells from the least sensitive dog in the definitive myelotoxicity study demonstrated a significant inhibition of colony formation at Antalarmin concentrations that had no effect on either rat or human bone marrow cells. These data clearly demonstrate that Antalarmin concentrations that would be predicted to induce severe neutropenia in the dog (IC₉₀ levels) have essentially no effect on either rat or human CFU-GM. The similar concentration-response curves for Antalarmin cytotoxicity in rat and human bone marrow cells *in vitro*, when compared to the much different cytotoxicity curves seen for dogs, suggest that the rat is more likely to be an effective predictor of Antalarmin toxicity in human bone marrow than is the dog.

4. Discussion

Preclinical toxicology studies of Antalarmin were performed to (i) identify any mutagenic or clastogenic activity of this agent; (ii) characterize the subchronic toxicity of Antalarmin in rats and dogs, with the goals of identifying both a NOAEL and target organs for agent toxicity in each species; (iii) determine the reversibility of Antalarmin toxicity following cessation of exposure; and (iv) compare the myelotoxic effects of Antalarmin across species, in order to identify the experimental model that is likely to be the most useful predictor of human response.

Antalarmin was not genotoxic in a standard battery of genetic toxicology bioassays that included the *Salmonella* reverse mutation assay (Ames assay), mouse lymphoma assay, and *in vivo* rat bone marrow micronucleus assay. The compound was well tolerated in both rats and dogs at the doses evaluated in the definitive toxicity study. When administered orally for a minimum of 90 days to rats (at doses of up to 300 mg/kg/day [1800 mg/m²/day]) or to dogs (at doses of up to 16 mg/kg/day [320 mg/m²/day]), Antalarmin had no effects on survival, body weight, food consumption, ophthalmology, ECGs (dogs only) or neurologic responses (evaluated by FOB). The doses of Antalarmin selected for use in the definitive 90-day studies were considered to be the highest doses that could be administered in a subchronic protocol; in a 14-day range-finding study, mortality was seen in rats exposed to Antalarmin at doses of 500 and 1000 mg/kg/day, while repeated emesis resulting in body weight loss was seen in dogs exposed to Antalarmin at doses of 50, 100, and 200 mg/kg/day.

Histopathologic evaluation of tissues identified the kidney and liver as primary targets of Antalarmin toxicity in rats. The kidney appears to be the most sensitive target tissue, as microscopic alterations were observed in the renal cortex of rats receiving 30, 100 or 300 mg/kg/day of Antalarmin for 90 days. Interestingly, serum chemistry did not identify any significant alterations in renal function in rats exposed to Antalarmin; however, increases in urine volume observed in the high dose group may be associated with alterations in renal concentrating function.

Microscopic liver pathology, increased GGT levels, mild anemia, and modest increases in coagulation time were seen in rats exposed to the high dose of Antalarmin. Because these changes were not observed in the low or middle dose group, the liver and hematopoietic system appear to be less sensitive to Antalarmin toxicity than is the kidney.

Based on the lack of gross clinical toxicity in the highest dose group (300 mg/kg/day) in the definitive study, and the mortality seen in rats exposed to Antalarmin at doses of ≥ 500 mg/kg/day in the range-finding study, the MTD for subchronic oral administration of Antalarmin to rats is considered to fall within the range of 300 mg/kg/day (1800 mg/m²/day) < MTD < 500 mg/kg/day (3000 mg/m²/day). On the basis of the renal cortical pathology identified in all treated dose groups in the definitive study, the NOAEL for subchronic administration of Antalarmin to rats could not be determined; however, the NOAEL for the 14-day range-finding study was 50 mg/kg/day (300 mg/m²/day).

Histopathologic evaluations identified the bone marrow and lymphoid system as primary targets for Antalarmin toxicity in dogs. Bone marrow and lymphoid depletion were identified in dogs exposed to the high dose of Antalarmin (16 mg/kg/day; 320 mg/m²/day), but were not seen in dogs in either the middle or low dose groups; these changes were reversed during the one-month recovery period. Unlike the rat, the kidney was apparently not a target for Antalarmin toxicity in dogs, and dogs exposed to Antalarmin demonstrated only minimal evidence of hepatic toxicity.

Interestingly, changes in bone marrow and lymphoid tissue histology in the high dose group (16 mg/kg/day) in the definitive study were not accompanied by alterations in hematology parameters; in this study, hematology evaluations were performed at the end of the 90-day dosing period. By contrast, hematology assays performed at the end of the 14-day dosing period in the range-finding study did identify hematologic changes in dogs exposed to Antalarmin at 10 mg/kg/day. These data suggest that any effects of Antalarmin on hematologic parameters in dogs may be transient, and are reversible even upon continuation of drug exposure.

On the basis of the lack of limiting clinical toxicity observed in dogs exposed to Antalarmin at doses of up to 16 mg/kg/day in the definitive study, and the severe emesis seen in dogs administered Antalarmin at doses of ≥ 50 mg/kg/day in the range-finding study, the MTD for subchronic oral administration of Antalarmin in dogs falls within the range of 16 to 50 mg/kg/day (320 to 1000 mg/m²/day). On the basis of the bone marrow and lymphoid depletion observed in female dogs dosed with Antalarmin at 16 mg/kg/day and emesis in dogs dosed with Antalarmin at 8 or 16 mg/kg/day, the NOAEL for subchronic oral administration of Antalarmin in dogs is defined as 4 mg/kg/day (80 mg/m²/day).

The target tissues for Antalarmin toxicity in rats and dogs demonstrate substantial differences. Whereas the kidney and liver were the most sensitive target organs in rats, the bone marrow and lymphoid system appear to be the most sensitive sites of Antalarmin toxicity in dogs. Essentially no myelotoxicity was observed in rats, and dogs demonstrated renal toxicity only at the highest dose. In consideration of these differences in species sensitivity, it was of interest to determine whether the rat or the dog provides the better model for human responses to the Antalarmin myelotoxicity. Comparisons of Antalarmin myelotoxicity in bone marrow cells harvested from human, canine, and rat donors clearly demonstrated that human and rat bone marrow demonstrate similar responses to Antalarmin; by contrast, canine bone marrow cells are substantially more sensitive to Antalarmin toxicity than are bone marrow cells from the other two species. On this basis, it is concluded that the rat provides a better model of human bone marrow sensitivity to Antalarmin than does the dog, and that the dog is likely to over-predict Antalarmin myelotoxicity in humans.

Toxicokinetic data in dogs and rats suggest that the lower MTD for Antalarmin in dogs does not result from its greater bioavailability or plasma drug levels in that species. In the rat dose range-finding study, Antalarmin was detectable in the plasma at 30 minutes post-dosing in all groups receiving a single dose of drug (150 to 3000 mg/m²; 25 to 500 mg/kg). In higher dose groups, plasma levels remained above the limit of detection (20 ng/ml) for 24 hours. In the dog dose range-finding study, however, plasma drug levels were below the limit of detection at all time points in animals receiving a single oral dose of 200 mg/m² (10 mg/kg), and were only sporadically above the limit of detection in dogs receiving a single oral dose of 1000 to 4000 mg/m² (50 to 200 mg/kg). After 14 days of exposure, plasma Antalarmin levels in rats were consistently above the limit of detection in all dose groups (150 to 3000 mg/m²/day), and were above the limit of detection in dogs exposed to Antalarmin doses ≥ 200 mg/m²/day.

Efficacy studies performed in the rhesus monkey demonstrate that desirable pharmacologic activity (inhibition of fear and anxiety) can be achieved with a single oral dose of Antalarmin

at 240 mg/m² (Habib et al., 2000). Although this pharmacologically effective dose approximates or exceeds the NOAEL identified in both rats (300 mg/m²/day on a 14-day schedule) and dogs (80 mg/m²/day on a 90-day schedule) in the present studies, it is below the MTD for Antalarmin in both species. As such, Antalarmin appears to have sufficient margin of safety for advancement into clinical trials. Based on these studies, a recommended starting dose of Antalarmin for use in Phase I clinical trials is 13 mg/m²/day (1/6th the NOAEL in the dog), which translates to a dose of approximately 25 mg for a 70 kg man.

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Fig. 1.
Chemical structure of Antalarmin.

Fig. 2. Pilot *in vitro* myelotoxicity evaluation of Antalarmin in rat, dog, and human bone marrow neutrophil progenitors (CFU-GM) harvested from a single marrow donor from each species. Note close comparability of cytotoxicity curves in bone marrow progenitors of rat and human origin, and much greater sensitivity of canine CFU-GM to an Antalarmin concentration of 10 µg/ml.

Fig. 3.

Definitive *in vitro* myelotoxicity evaluation of Antalarmin in rat, dog, and human bone marrow neutrophil progenitors (CFU-GM) ($N = 3$ marrow donors per species; data are presented as mean $\pm$ S.D.). Note similarity of dose-response curves for rat and human CFU-GM, and greater sensitivity of canine CFU-GM to Antalarmin cytotoxicity.

Table 1
Mutagenicity of Antalarmin in *Salmonella* tester strains with and without metabolic activation

Dose (µg/ plate)	Salmonella Tester Strains With S9					Salmonella Tester Strains Without S9				
	TA98	TA100	TA102	TA1535	TA1537	TA98	TA100	TA102	TA1535	TA1537
0	22 ± 5	115 ± 9	220 ± 38	15 ± 3	12 ± 8	20 ± 2	109 ± 8	197 ± 36	16 ± 3	13 ± 3
5	31 ± 11	106 ± 26	261 ± 17	14 ± 4	15 ± 4	16 ± 3	85 ± 8	121 ± 29	13 ± 2	8 ± 4
50	28 ± 7	107 ± 11	257 ± 13	21 ± 3	16 ± 1	23 ± 3	80 ± 13	174 ± 6	11 ± 1	10 ± 4
150	31 ± 13	120 ± 9	226 ± 19	17 ± 1	13 ± 4	17 ± 6	96 ± 11	78 ± 52	15 ± 3	7 ± 2
625	33 ± 8	112 ± 1	270 ± 16	11 ± 3	13 ± 3	16 ± 5	90 ± 8	172 ± 23	10 ± 4	8 ± 1
1250	25 ± 7	115 ± 16	280 ± 26	17 ± 8	17 ± 3	16 ± 4	76 ± 12	178 ± 21	15 ± 2	8 ± 2

Number of revertant colonies per plate; data are presented as mean ± S.D. Each bacterial strain and metabolic activation condition (± S9) for the vehicle control (DMSO) and Antalarmin dose were cultured in triplicate.

Table 2
Mutagenicity of Antalarmin in mouse lymphoma assay with and without metabolic activation

With S9				Without S9			
Conc. (µg/ml)	RCE Mean ^d	RTG Mean ^b	MF Mean ^c	Conc. (µg/ml)	RCE Mean	RTG Mean	MF Mean
MC ^d	100	100	90	MC	100	100	113
1% DMSO	36	33	148	0.5% DMSO	100	75	107
250	Toxic	Toxic	Toxic	125	Toxic	Toxic	Toxic
125	Toxic	Toxic	Toxic	62.5	Toxic	Toxic	Toxic
62.5	60	37	77	31.3	94	48	170
31.3	94	51	98	15.6	104	176	95
15.6	88	72	Contaminated	7.8	97	176	100
7.8	57	44	88	3.9	99	205	153
3.9	77	77	87	1.9	90	130	85
1.9	82	55	87	0.9	80	120	96
B[a]P (2) ^e	31	11	878	MMS (20) ^f	74	47	312

^aRelative cloning efficiency (%)
^bRelative total growth
^cMutant frequency (mutant colonies/10⁶ survivors)
^dMedia control
^eBenzo[α]pyrene (2 mg/ml); used as a positive control in the +S9 test system
^fMethyl methanesulfonate (20 mg/ml); used as a positive control in the -S9 test system

Table 3

Micronuclei frequency of Antalarmin in rat bone marrow

Dose (mg/kg/day)	Males		Females	
	PCE/TE ^a	%MPCE ^b	PCE/TE	%MPCE
0	0.570 ± 0.104	0.11 ± 0.05	0.550 ± 0.199	0.07 ± 0.04
30	0.501 ± 0.256	0.08 ± 0.03	0.703 ± 0.282	0.04 ± 0.06
100	0.741 ± 0.223	0.04 ± 0.05*	0.567 ± 0.056	0.02 ± 0.03*
300	0.545 ± 0.220	0.03 ± 0.04*	0.633 ± 0.098	0.07 ± 0.04
Cyclophosphamide ^c	0.601 ± 0.093	1.50 ± 0.48*	0.621 ± 0.264	0.23 ± 0.10*

Data are presented as mean ± S.D. *N* = 6 rats/sex/dose. Significant difference from sex-matched vehicle control group:

* $p \leq 0.05$.

^a Polychromatic erythrocytes (PCE)/Total erythrocytes (TE)

^b Percent of micronucleated polychromatic erythrocytes (MPCE)

^c Cyclophosphamide (30 mg/kg) was used as a positive control and was injected iv 24 h before sacrifice

Table 4
Selected clinical pathology data in male and female rats exposed to Antalarmin for 90 days

Analyte	Time Period	Dose (mg/kg/day)							
		Males				Females			
		0	30	100	300	0	30	100	300
GGT	week 5	3 ± 1	3 ± 1	2 ± 1	5 ± 1 ^{**}	3 ± 1	3 ± 1	4 ± 1	16 ± 16 ^{**}
	week 9	3 ± 1	4 ± 1	3 ± 1	8 ± 5 ^{**}	3 ± 2	3 ± 1	4 ± 1	9 ± 4 ^{**}
	week 13	4 ± 2	3 ± 1	3 ± 1	7 ± 2 ^{**}	4 ± 1	4 ± 1	5 ± 2	14 ± 6 ^{**}
	recovery	3 ± 1	4 ± 1	4 ± 1	4 ± 1	4 ± 1	5 ± 2	5 ± 2	4 ± 1
CHOL	week 5	46 ± 5	49 ± 8	45 ± 6	49 ± 8	52 ± 8	51 ± 5	59 ± 7	60 ± 9 [*]
	week 9	45 ± 4	50 ± 9	48 ± 9	49 ± 10	53 ± 9	51 ± 3	61 ± 5	68 ± 13 ^{**}
	week 13	34 ± 10	39 ± 10	39 ± 8	35 ± 7	40 ± 8	34 ± 6	53 ± 12 ^{**}	58 ± 10 ^{**}
	recovery	39 ± 13	46 ± 19	33 ± 10	50 ± 12	42 ± 11	50 ± 9	51 ± 12	51 ± 7 ^{**}
HGB	week 5	15.1 ± 0.5	14.9 ± 0.6	14.7 ± 0.5	14.1 ± 0.5 ^{**}	14.7 ± 0.5	14.9 ± 0.3	14.2 ± 0.3	14.0 ± 0.6 ^{**}
	week 9	14.8 ± 0.6	14.4 ± 0.4	14.2 ± 0.4 [*]	13.2 ± 0.5 ^{**}	14.3 ± 0.5	14.3 ± 0.5	13.6 ± 0.4 [*]	13.0 ± 0.6 ^{**}
	week 13	15.9 ± 0.7	15.3 ± 0.6 [*]	15.1 ± 0.4 ^{**}	14.4 ± 0.7 ^{**}	15.5 ± 0.4	15.2 ± 0.5	14.5 ± 0.6 ^{**}	14.1 ± 0.7 ^{**}
	recovery	14.7 ± 0.9	14.7 ± 0.8	14.6 ± 0.6	14.4 ± 0.5	14.4 ± 0.4	14.4 ± 0.3	14.3 ± 0.2	13.6 ± 0.6 [*]
HCT	week 5	47.1 ± 1.7	46.6 ± 1.4	46.4 ± 1.5	43.5 ± 1.4 ^{**}	44.5 ± 1.4	44.9 ± 1.6	43.7 ± 1.3	43.1 ± 1.8
	week 9	46.8 ± 1.3	45.9 ± 1.6	45.7 ± 1.6	42.2 ± 1.5 ^{**}	44.6 ± 1.4	44.1 ± 0.9	42.8 ± 1.4 [*]	41.3 ± 2.0 ^{**}
	week 13	48.1 ± 1.9	46.3 ± 2.0 [*]	45.9 ± 1.3	43.6 ± 2.2 ^{**}	47.6 ± 1.4	46.1 ± 1.9	44.6 ± 1.5 ^{**}	43.2 ± 2.3 ^{**}
	recovery	46.6 ± 2.9	46.3 ± 1.6	45.9 ± 2.0	46.3 ± 2.9	44.1 ± 0.9	44.5 ± 0.8	44.0 ± 0.7	42.9 ± 2.0
MCV	week 5	61.4 ± 1.7	60.8 ± 1.5	61.0 ± 2.1	58.7 ± 0.9 ^{**}	59.8 ± 1.4	58.6 ± 2.2	59.5 ± 1.7	58.2 ± 1.3
	week 9	57.9 ± 1.3	56.8 ± 1.5	57.2 ± 1.9	55.3 ± 1.0 ^{**}	58.1 ± 1.3	57.2 ± 2.3	57.5 ± 1.2	55.8 ± 1.4 ^{**}
	week 13	55.4 ± 1.3	54.3 ± 1.4	54.6 ± 1.8	53.2 ± 1.7 ^{**}	57.6 ± 1.3	57.4 ± 0.7	57.1 ± 1.0	55.5 ± 1.1 ^{**}
	recovery	53.9 ± 2.6	54.8 ± 1.0	54.0 ± 2.5	54.0 ± 1.6 ^{**}	56.3 ± 1.6	56.1 ± 3.5	56.4 ± 1.5	55.1 ± 1.9 ^{**}
MCH	week 5	19.7 ± 0.4	19.4 ± 0.5	19.4 ± 0.7	19.0 ± 0.3 ^{**}	19.7 ± 0.3	19.4 ± 0.8	19.3 ± 0.3	18.8 ± 0.5 ^{**}
	week 9	18.3 ± 0.6	17.9 ± 0.5	17.8 ± 0.4	17.3 ± 0.4 ^{**}	18.6 ± 0.5	18.6 ± 0.6	18.3 ± 0.4	17.6 ± 0.5 ^{**}
	week 13	18.3 ± 0.5	17.9 ± 0.6	17.9 ± 0.5	17.5 ± 0.6 ^{**}	18.8 ± 0.6	19.0 ± 0.4	18.5 ± 0.4	18.1 ± 0.4 ^{**}
	recovery	17.1 ± 0.7	17.4 ± 0.2	17.2 ± 0.7	16.9 ± 0.3	18.4 ± 0.5	18.2 ± 1.1	18.3 ± 0.5	17.4 ± 0.5 ^{**}
APTT	week 13	19.7 ± 0.9	19.8 ± 1.3	21.3 ± 1.8	23.8 ± 2.7 ^{**}	16.4 ± 0.7	17.4 ± 1.0	18.7 ± 1.6 ^{**}	17.9 ± 1.6 ^{**}
	recovery	19.4 ± 2.4	19.7 ± 0.9	20.5 ± 1.1	21.3 ± 0.8	16.9 ± 0.2	17.4 ± 0.9	16.8 ± 0.7	18.7 ± 0.9 ^{**}

Data are presented as mean ± S.D. $N = 9-10$ rats/sex/dose during weeks 5 and 9; 15 rats/sex/dose during week 13 (terminal necropsy); and 5 rats/sex/dose at recovery necropsy. Abbreviations used in table: GGT, γ -glutamyl transferase (international units/l); CHOL, cholesterol (mg/dl); HGB, hemoglobin (g/dl); HCT, hematocrit (%); MCV, mean cell volume (10^{-15} l); MCH, mean cellular hemoglobin (10^{-9} g); and APTT, activated partial thromboplastin time (s). Significant difference from sex- and time-matched vehicle control group:

* $p \leq 0.05$;
 ** $p \leq 0.01$.

Table 5
Absolute organ weights in male and female rats exposed to Antalarmin for 90 days

Dose (mg/ kg/day)	Absolute Weight at Terminal Necropsy (g)								
	Adrenals	Brain	Heart	Kidneys	Liver	Spleen	Testes	Thymus	Thyroids
Males									
0	0.066 (0.014)	2.16 (0.10)	1.68 (0.23)	3.36 (0.41)	13.26 (2.11)	0.89 (0.16)	3.53 (0.36)	0.37 (0.10)	0.035 (0.006)
30	0.062 (0.010)	2.16 (0.12)	1.73 (0.19)	3.59 (0.40)	16.42 (2.58) *	0.95 (0.17)	3.49 (0.34)	0.46 (0.09)	0.035 (0.008)
100	0.072 (0.014)	2.18 (0.13)	1.78 (0.30)	3.72 (0.42) *	17.92 (2.56) *	0.93 (0.16)	3.67 (0.22)	0.40 (0.08)	0.031 (0.008)
300	0.066 (0.011)	2.19 (0.11)	1.64 (0.23)	3.27 (0.31)	20.51 (2.61) *	0.98 (0.15)	3.64 (0.35)	0.33 (0.12)	0.039 (0.015)
Females									
0	0.070 (0.010)	1.91 (0.06)	0.98 (0.08)	1.80 (0.11)	7.12 (0.47)	Spleen 0.54 (0.07)	0.114 (0.030)	0.34 (0.06)	0.032 (0.006)
30	0.075 (0.007)	1.93 (0.09)	1.03 (0.11)	1.85 (0.17)	8.17 (0.79)	0.56 (0.08)	0.127 (0.041)	0.31 (0.06)	0.031 (0.007)
100	0.078 (0.014)	1.94 (0.08)	1.04 (0.07)	1.88 (0.15)	10.63 (1.58) *	0.58 (0.06)	0.150 (0.047)	0.32 (0.06)	0.031 (0.006)
300	0.082 (0.011) *	1.96 (0.11)	1.01 (0.08)	2.00 (0.20) *	14.31 (1.54) *	0.67 (0.09) *	0.140 (0.040)	0.34 (0.05)	0.035 (0.007)
Dose (mg/ kg/day)	Absolute Weight at Recovery Necropsy (g)								
Males									
0	0.069 (0.004)	2.29 (0.06)	1.93 (0.16)	3.64 (0.10)	16.11 (1.84)	Spleen 0.94 (0.19)	3.72 (0.16)	0.41 (0.06)	0.028 (0.006)
30	0.062 (0.009)	2.16 (0.08)	1.72 (0.22)	3.61 (0.47)	15.70 (2.06)	0.93 (0.22)	3.36 (0.38)	0.37 (0.08)	0.032 (0.004)
100	0.073 (0.010)	2.21 (0.10)	1.70 (0.17)	3.70 (0.46)	14.53 (1.18)	0.87 (0.12)	3.54 (0.24)	0.32 (0.08)	0.033 (0.004)
300	0.073 (0.015)	2.10 (0.12)	1.82 (0.21)	3.88 (0.34)	18.70 (2.50)	0.97 (0.15)	3.53 (0.39)	0.26 (0.02)	0.036 (0.003)
Females									
0	0.070 (0.012)	2.01 (0.11)	1.14 (0.08)	1.94 (0.26)	8.25 (1.16)	Spleen 0.57 (0.10)	0.151 (0.027)	0.27 (0.10)	0.028 (0.008)
30	0.077 (0.016)	2.06 (0.10)	1.11 (0.22)	2.04 (0.26)	8.72 (1.11)	0.63 (0.16)	0.127 (0.020)	0.30 (0.04)	0.026 (0.004)
100	0.073 (0.010)	1.96 (0.06)	1.02 (0.11)	2.03 (0.24)	8.43 (1.39)	0.56 (0.03)	0.102 (0.008) *	0.28 (0.04)	0.032 (0.007)
300	0.078 (0.018)	2.02 (0.16)	1.13 (0.13)	2.01 (0.25)	9.52 (1.33)	0.63 (0.06)	0.155 (0.025)	0.27 (0.03)	0.027 (0.006)

Data are presented as mean ($\pm$ S.D. in parentheses). $N = 15$ rats/sex/dose at terminal necropsy; and 5 rats/sex/dose at recovery necropsy. Significant difference from sex-matched vehicle control group:

* $p \leq 0.05$.

Table 6
Relative organ weights (normalized to body weights) in male and female rats exposed to Aantalarmin for 90 days

Dose (mg/ kg/day)	Relative Weight at Terminal Necropsy (%)						
	Adrenals	Brain	Heart	Kidneys	Liver	Spleen	Thyroids
Males							
0	0.012 (0.002)	0.40 (0.04)	0.31 (0.03)	0.62 (0.04)	2.45 (0.23)	0.17 (0.03)	0.006 (0.001)
30	0.011 (0.002)	0.37 (0.04)	0.30 (0.04)	0.62 (0.04)	2.81 (0.24)*	0.16 (0.02)	0.006 (0.001)
100	0.013 (0.003)	0.39 (0.03)	0.32 (0.03)	0.67 (0.06)*	3.20 (0.26)*	0.17 (0.03)	0.006 (0.002)
300	0.013 (0.002)	0.44 (0.04)	0.33 (0.05)	0.65 (0.05)	4.07 (0.37)*	0.20 (0.03)*	0.008 (0.003)
Females							
0	0.025 (0.004)	0.68 (0.05)	0.35 (0.03)	0.64 (0.05)	2.53 (0.14)	0.19 (0.02)	0.011 (0.002)
30	0.026 (0.003)	0.66 (0.06)	0.35 (0.03)	0.63 (0.05)	2.79 (0.23)*	0.19 (0.02)	0.011 (0.002)
100	0.027 (0.005)	0.67 (0.06)	0.36 (0.03)	0.65 (0.05)	3.67 (0.39)*	0.20 (0.02)	0.011 (0.002)
300	0.029 (0.004)*	0.70 (0.06)	0.36 (0.04)	0.71 (0.06)*	5.08 (0.34)*	0.24 (0.03)*	0.012 (0.002)
	Relative Weight at Recovery Necropsy (%)						
Males							
0	0.011 (0.001)	0.37 (0.03)	0.31 (0.03)	0.59 (0.04)	2.59 (0.27)	0.15 (0.03)	0.005 (0.001)
30	0.010 (0.002)	0.36 (0.02)	0.29 (0.04)	0.60 (0.04)	2.63 (0.13)	0.15 (0.02)	0.005 (0.001)
100	0.013 (0.002)	0.40 (0.04)	0.31 (0.01)	0.67 (0.03)*	2.63 (0.16)	0.16 (0.02)	0.006 (0.001)
300	0.013 (0.001)	0.36 (0.03)	0.31 (0.01)	0.67 (0.04)*	3.20 (0.17)*	0.17 (0.01)	0.006 (0.001)
Females							
0	0.021 (0.004)	0.62 (0.06)	0.36 (0.04)	0.60 (0.03)	2.53 (0.22)	0.17 (0.02)	0.008 (0.002)
30	0.024 (0.003)	0.66 (0.07)	0.35 (0.03)	0.65 (0.06)	2.76 (0.15)	0.20 (0.03)	0.008 (0.002)
100	0.024 (0.003)	0.65 (0.07)	0.33 (0.02)	0.67 (0.05)	2.75 (0.21)	0.19 (0.01)	0.011 (0.002)
300	0.028 (0.005)	0.72 (0.03)	0.40 (0.04)	0.72 (0.05)*	3.39 (0.29)*	0.22 (0.01)*	0.010 (0.002)

Data are presented as mean (± S.D. in parentheses). N = 15 rats/sex/dose at terminal necropsy, and 5 rats/sex/dose at recovery necropsy. Significant difference from sex-matched vehicle control group:

* $p \leq 0.05$.