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Original Research

Toxicological assessment of anandamide

Gloria Yi[1], George Gao[1], Wayne Yi[1], Long Jiang[1,*]

[1]Nanjing Nutrabuilding Bio-tech Co., Ltd, Nanjing, China

*Author for correspondence: Email: ljjiang@nnbnutrition.com

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Abstract

Anandamide (AEA), an endogenous cannabinoid, has been widely studied for its physiological roles and potential therapeutic applications. However, concerns remain regarding its safety profile, particularly at higher doses. Therefore, this study aimed to investigate the acute, subchronic and genetic toxicological effects of AEA. In the bacterial reverse mutagenesis assay (Ames test), AEA did not exhibit any mutagenic activity. An *in vivo* mammalian erythrocyte micronucleus test showed no differences in micronucleus frequency between the negative control group and any of the AEA test groups. In addition, there was no evidence of mutagenicity or clastogenicity in an *in vitro* cell gene mutation assay on L5178Y $TK^{+/-}$ mouse lymphoma cells, and an *in vitro* mammalian chromosomal aberration test using CHO-K1 cells. An acute oral toxicity study reported a NOEL of 2,000 mg/kg bw. Based on the 90-day repeated dose oral toxicology study, the NOAEL is considered to be 2,000 mg/kg bw/day.

Keywords: Anandamide, Acute toxicity, Subchronic toxicity, Genotoxicity, Ames test, Micronucleus test, Chromosomal aberration test, Gene mutation assay

Abbreviations: bw: Body Weight; mg: Milligram; kg: kilogram; VC: Vehicle Control; LD: Low Dose; ILD: Intermediate Low Dose; IHD: Intermediate High Dose; HD: High Dose; NOEL: No Observed Effect Level; NOAEL: No Observed Adverse Effect Level; OECD: Organisation of Economic Co-operation and Development; AEA: Anandamide

Introduction

Anandamide (AEA), also known as N-arachidonoylethanolamine belongs to a class of fatty acyl lipids known as N-acylethanolamines (NAEs). Anandamide binds types 1 and 2 cannabinoid receptors (CB1 and CB2) with ow efficacy agonist at CB1 receptors and very low efficacy agonist at CB2 receptors [1,2], and non-cannabinoid receptors such as vanilloid (TRPV1) receptors and along with 2-arachidonoylglycerol (2AG) is a component of the endocannabinoid system [3]. Anandamide was the first endocannabinoid identified [4] and is the most investigated [5]. In addition to the porcine brain [4], anandamide has been detected in the human hippocampus [6], rat and mouse brain, mouse spinal cord, and other mammalian and invertebrate tissues.

Anandamide has been found in food such as chocolate [7]. It is also present in human milk at low levels [8]. When orally administered, 1.6 to 5 percent of anandamide enters the bloodstream. This has been attributed to the high amount of fatty acid amide hydrolase, which

catalyzes anandamide in the gastrointestinal tract [9]. The amount of anandamide present in these foods is thought to be adequate to induce psychotropic effects [8]. Oral consumption of anandamide has been reported to increase food ingestion in rats [10,11], mice [12], and in satiated fish [13]. No toxicological studies have previously been conducted on anandamide. This study reports on the first published toxicology studies on anandamide.

Materials and Methods

Test article

The test article was ≥ 60% anandamide (FastBliss®, Nanjing Nutrabuilding Bio-tech Co., Ltd).

In vitro studies on anandamide

Bacterial reverse mutation test: Ames was conducted with AEA using *Salmonella typhimurium* strains TA 98, TA100, TA102, TA1535, and TA1537 following OECD Test No. 471 (OECD, 1997). Based on the results of a precipitation test, the maximum concentration for the preliminary toxicity assessment was set at 5,000 µg/plate. The preliminary toxicity determination was conducted with 50, 100, 200, 400, 800, 1,600, 3,200, and 5,000 µg of anandamide/plate in triplicate in the presence and absence of metabolic activation. Using the preincubation method, concentrations of 50, 158, 500, 1,581, and 5,000 µg of anandamide were tested. The solvent control was dimethylsulfoxide (DMSO) and the positive control in the presence of metabolic activation was 2-aminoanthracene. The positive controls in the absence of metabolic activation were nitrofluorene (TA98), sodium azide (TA100 and TA1535), 9- aminoacridine (TA 1537) and TA 102 (mitomycin C).

Using the preincubation test the bacterial cells, AEA, S9, or control (sodium phosphate buffer) were incubated for 30 minutes in an incubator for 37 ± 1°C. Soft agar was then added and then plated on minimal glucose agar plates. The plates were incubated for 72 hours.

For the plate incorporation method, the bacterial cells, AEA, the solvent or positive control, and either S9 or sodium phosphate buffer control and soft agar containing histidine biotin were combined with an overlay agar and plated on soft agar containing histidine biotin for *Salmonella typhimurium* strains. The concentrations of anandamide that were tested were 100, 266, 707, 1,880, and 5,000 ug/plate. Following the 72-hour incubation period, the number of revertant colonies was determined and compared to the number of spontaneous revertants in plates containing the solvent control.

At the end of the incubation period, the numbers of colonies were counted and expressed as the number of colony-forming units per mL of suspension. For strains TA 98 and TA 100, a positive result was one in which the number of revertants in the treatment groups was at least two times the number in the solvent control group for at least three concentrations tested. For tester strains TA 1535, TA 1537, and TA 102, a minimum of a three-fold increase in the number of revertants compared with the solvent control was considered a positive result.

Mammalian cell gene mutation assay in L5178Y mouse lymphoma cells $TK^{+/-}$: The ability of anandamide to induce gene mutations in mammalian cells was assessed using the cultured mouse lymphoma cell line L5178Y $TK^{+/-}$ -3.7.2. The study adhered to OECD Test No. 490 (OECD, 2016c). The cells were tested to ensure they were free of mycoplasma, bacteria, and fungi and were cleaned by growing in CM10 which contained thymidine, hypoxanthine, methotrexate, and glycine (THG) for 24 hours, centrifuged, and grown in CM10 that contained thymidine, hypoxanthine, and glycine (THG) for an additional 24 hours. Centrifugation was performed to remove THG and the cells were then grown in CM10.

DMSO (1% v/v) was selected as the vehicle for the anandamide and the negative control. Initial cytotoxicity studies of anandamide were conducted in the presence and absence of metabolic activation for 3 hours (short-term study) and in the absence of metabolic activation for 24 hours (long-term study) at concentrations of 10, 20, 40, and 80 µg/mL of media.

The positive control in the absence of metabolic activation was 12 µg/mL cyclophosphamide monohydrate and the positive control in the presence of metabolic activation was 10 µg/mL methyl methanesulfonate. After the cells were treated, the suspensions were centrifuged, and the supernatant was removed. The cells were washed with complete media two times, resuspended in fresh medium and incubated for 24 hours. The cells were counted and then split to a density of 3×10^5 cells/mL. Suspension growth was determined for the first 24 hours and the second 24 hours to determine the relative suspension growth (RSG). Samples from each replicate culture were plated to estimate cell survival. A sample of the suspensions was incubated in a CO_2 chamber for 12 days and the cloning efficiency (CE) and relative cloning efficiency (RCE) were determined. The RCS and RCE were used to calculate the relative total growth (RTG). Cytotoxicity was the relative total growth (RTG) that included the RSG for the 2-day expression period and the RCE and the end of the expression period in the preliminary cytotoxicity test and the time of mutant selection in the gene mutation test [14].

RTG = RSG x RCE x 100.

For mutant selection, samples of cell suspensions were taken, replicates were placed in medium in microtiter plates and incubated in a CO_2 incubator for 12 days, and colonies were counted. The plates were scored using a microscope and the number of total wells (T) and number of positive wells (P) was counted. The number of negative or empty wells or E was (T - P). An inverted microscope was used to assess the size of the mutant colonies. Small colonies covered less than 25% of the well diameter and large colonies covered more than 25% of the well diameter.

Mutant frequency (MF) was defined as the cloning efficiency of the mutants in selective medium (CE_M) adjusted by the cloning efficiency in non-selective medium when mutants were selected (CE_V) or MF = CE_M/CE_V.

***In vitro* mammalian chromosomal aberration test using chinese hamster ovary (CHO)-K1 cells:** A chromosomal aberration study on anandamide was conducted with Chinese hamster ovary (CHO-K1) cells in the presence and absence of metabolic activation with short-term and long- term treatment under OECD Test No. 473 (OECD, 2016a). The study was conducted in accordance with Good Laboratory Practice (OECD, 1998). The cell line was obtained from the American Type Culture Collection (ATCC). The cells were incubated in a CO_2 incubator at 37 degrees Celsius and 5% CO_2. DMSO was used as the solvent for anandamide because anandamide was not soluble in reverse osmosis water. In the short-term treatment studies, cells were exposed to anandamide for 4 hours in the presence and absence of metabolic activation. Cells were arrested in the metaphase state using colcemid two hours before the end of the incubation period. In the long-term treatment studies, cells were exposed to anandamide for 20 hours and 30 minutes in the absence of metabolic activation. Colcemid was added two hours before the end of the incubation period so that the cells were arrested in metaphase.

A pretest using doses of 15.625, 31.25, 62.5, 125, and 250 µg/mL anandamide was conducted for the short-term study in the presence and absence of metabolic activation and with continuous treatment for 1.5 normal cell cycle lengths (20 hours and 30 minutes). Cytotoxicity was calculated using the formula (% Cytotoxicity = 100 - Relative Increase in Cell Counts (RICC)), where RICC was determined using the following formula [15]:

$$\frac{\text{Increase in No. of cells in treated cultures (final-starting)}}{\text{Increase in No. of cells in control cultures (final-starting)}} \times 100$$

Doses of 3.906, 7.812, and 15.625 µg/mL were selected for short-term treatment in the presence and long-term treatment in the absence of metabolic activation. No precipitation or significant pH change was observed at concentrations of 15.625, 31.25, 62.5, 125, and 250 µg/mL.

In the main study, doses of 3.906, 7.812, and 15.625 µg/mL of anandamide were used in the presence and absence of metabolic activation for the short-term treatment and in the absence of metabolic activation for the long-term treatment. Flasks were observed to ensure that the cells were healthy and free of contaminants. One hundred fifty well-spread metaphase cultures (300 metaphases per concentration) were scored under the 100 × oil immersion objective to observe structural and numerical changes.

***In vivo* mammalian erythrocyte micronucleus assay of anandamide**

***In vivo* mammalian erythrocyte micronucleus assay in mice:** The ability of anandamide to induce the formation of micronuclei in polychromatic erythrocytes was investigated in 6- to 10-week-old Swiss albino mice. The study was conducted in accordance with OECD Test No. 474: Mammalian Erythrocyte Micronucleus Test (OECD, 2016a) and in accordance with Good Laboratory Practice (OECD, 1998). To determine the maximum tolerable dose, a dose range-finding study was conducted. Two mice per sex per dose group were administered, via gavage, 0 (normal control/vehicle, corn oil), 250, 500, 1,000, or 2,000 mg/kg bw of anandamide 24 hours apart. The animals were observed for clinical signs and mortality and were then asphyxiated using CO_2 twenty-four hours after receiving the last dose of anandamide. Samples of bone marrow were obtained.

Based on the dose range-finding study, it was determined that the highest dose for the limit test would be 2,000 mg/kg bw.

Limit test: In the limit test, mice were assigned to one of three groups, each including five males and five females. Mice in the negative control group were administered corn oil and another group of mice in the 2,000 mg/kg bw anandamide via gavage. The treatments were administered for two days 24 hours apart. A third group of mice was administered the positive control (mitomycin-C) as a single intraperitoneal dose of 1.0 mg/kg bw on day two only. The mice were observed for clinical signs and mortality 30 min and 1, 2, 3, and 4 hours after administration of the last dose. Mice that were treated with the negative control and anandamide were killed via carbon dioxide asphyxiation within 22 hours and 50 minutes of receiving the final dose and mice that were treated with the positive control were killed within 24 hours of receiving the final dose.

Bone marrow cells were removed from the femur using fetal bovine serum. The cell suspension was centrifuged at 1,500 rpm for 10 minutes. After the supernatant was removed, at least two slides, each containing a smear, were created for each mouse and allowed to air dry. The slides were fixed in methanol and then stained with Giemsa. The cells were coded prior to scoring by individuals not involved in scoring and then decoded. At least 500 normochromatic erythrocytes to the corresponding polychromatic erythrocytes were examined. The ratio of polychromatic erythrocytes to normochromatic erythrocytes was determined. At least 4,000 polychromatic erythrocytes per mouse were evaluated and the percentage frequency of micronucleated polychromatic erythrocytes was determined.

Target tissue exposure (Plasma Concentration Assessment): During the limit test, rats were divided into two groups, each containing 3 mice per sex. They were administered either test item or vehicle and blood samples were obtained from the retroorbital sinus of three mice per sex at two hours after administration of the vehicle control group and the dose group on day 2. The unpaired t-test was used to compare the differences between the control group and the test group. The significant difference between positive and negative control was determined using the GraphPad Prism software.

Animal studies on anandamide

Acute oral toxicity study: A study was conducted in accordance with OECD Guideline 425 (OECD, 2022) to investigate the acute oral toxicity of anandamide in 8- to 10-week-old female Sprague Dawley rats. The rats were randomly assigned based on body weight to be administered 175, 550, 1750, 2,000, or 5,000 mg/kg bw or anandamide via gavage with a dose volume of 10 mL/kg/day based on body weight. A limit test was also conducted with 2,000 mg/kg bw of anandamide. Cage-side observations were performed to assess mortality and morbidity a minimum of two times per day during the study. The animals were assessed for detailed clinical observations, including changes in skin, fur, eyes, and mucous membranes and respiratory, circulatory, and behavior patterns. The animals were observed for tremors, convulsions, salivation, diarrhea, lethargy, and coma before the initiation of dosing during the acclimatization period and again following randomization. The rats were weighed on receipt, the day that they were randomized, before dosing on the first day of treatment (day 0), and on days 3, 7, and 14. Change in body weight was assessed for animals. The rats were killed on day 15 using isoflurane and gross pathological examinations were performed. Statistical analysis was performed using Graph-Pad Prism Software version 5.01. The mean ± S.D. was calculated.

Ninety-day repeated dose oral toxicity study: A 90-day repeated dose oral toxicity study was conducted in accordance with OECD 408 (OECD, 2018) to assess the toxicological potential of anandamide.

Male and female Sprague Dawley rats, aged 8 to 10 weeks, were randomly assigned based on body weight to be administered via gavage 0 (G1, control group, corn oil vehicle), 500 (G2), 1,000 (G3), 1,500 (G4), or 2,000 mg/kg bw/day (G5) of anandamide. The animals in the main study group (n=10 rats/ sex/dose group) were administered anandamide for 90 consecutive days and were killed on day 91. In addition, two satellite groups of animals were either untreated (control recovery, G6) or administered 2,000 mg/kg bw/day (G7) of anandamide) for an additional 28 consecutive days.

Detailed clinical examinations were conducted prior to the first administrations during acclimatization and following randomization

and a minimum of once weekly afterward. These include changes in skin, fur, eyes, mucous membranes, respiratory, circulatory, and behavior patterns were assessed once before the start of administration and a minimum of once weekly (on the day of body weight measurement) thereafter. Cage-side observations were conducted for mortality and morbidity twice daily. The rats were assessed for tremors, convulsions, salivation, diarrhea, lethargy, and coma. Body weights were measured when they were received by the facility, on the day of randomization, once per week during the administration period, and on the final day of dosing. The amount of feed consumed was recorded weekly on the day the rats were weighed and feed input was also measured weekly. Ophthalmological examinations were performed on the last day of treatment for the main study groups and at the end of the recovery period for the recovery group. Grip strength measurements were performed on day 90 and day 118 for the main and recovery groups, respectively.

At the end of the 90-day administration period for the main study and on day 119 for the recovery groups the rats were fasted overnight but were given access to water, and blood samples were collected for hematology and biochemistry analyses via the retro-orbital sinus. Hematology analyses included hemoglobin, hematocrit, red blood corpuscles, platelet count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, mid-cell leukocytes, white blood cells, lymphocytes, and granulocytes. Clinical chemistry analyses included fasting glucose, urea, creatinine, cholesterol, triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, bilirubin (total), serum glutamic-pyruvic transaminase (SGPT) or alanine aminotransferase, serum glutamic-oxaloacetic transaminase (SGOT) or aspartate aminotransferase, alkaline phosphate, calcium, protein, albumin, and potassium. On the day after the end of the administration period for the main study animals and on day 119 for the recovery group animals, blood was collected, and the concentrations of thyroxine (T4), triiodothyronine (T3), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2) and testosterone in serum were determined.

Urine samples were collected after the overnight fast and the volume, pH, specific gravity, color, urine albumin, and appearance were determined. Urine concentration of glucose, urobilinogen, ketone bodies, and blood in urine were measured.

On the day of necropsy, the rats were killed using isoflurane, and the adrenals, brain, epididymides, esophagus, eyes with optic nerve, femur bone with joint, gross lesions, heart, kidneys, liver, lungs, lymph nodes, ovaries, pancreas, seminal vesicles, skin, spinal cord, spleen, sternum with marrow, stomach, testes, thymus, trachea, thyroid and parathyroid, urinary bladder, and uterus with cervix were collected. Blood smears were performed. All gross lesions were examined. Histopathological analyses were performed on all animals in the control and anandamide treatment groups on brain, liver, kidneys, lungs, adrenals, ovaries, spleen, heart, testis, uterus, and epididymides. The following were weighed with paired organs being weighed together: adrenal, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, and uterus with cervix.

Statistical analyses

Statistical analyses for the bacterial reverse mutation test included the mean and standard deviation and differences among solvent control and positive control groups using ANOVA. Statistical analysis was carried out using the software Graph pad prism. Dunnett's 't' test at a 5% level ($p<0.05$) of significance was used to study the differences between the solvent control and treatment groups. Data were represented as Mean ± SD revertant colonies.

In the *in vitro* Mammalian Cell Gene Mutation Test with Anandamide using L5178Y $TK^{+/-}$ cells, it is assumed that the number of negative control values follow a Poisson Distribution. A test of the significance of the difference between negative control values over different dose groups and the positive control was conducted using a method of analysis suggested by M. R. Thomas (Cole and Arlett, 1984), based on χ^2, with a 5% level of significance ($p<0.05$).

For the CHO-K1 assay, a Chi-square test for trend was conducted to determine if there was a statistically significant (p-value <0.05) between negative control and all treatment groups. A Fisher's exact test was performed to investigate the statistically significant (p-value <0.05) difference between negative and positive control.

For the *in vivo* mammalian erythrocyte micronucleus assay in mice, an unpaired 't'-test was performed to compare the difference between vehicle control and anandamide-dosed groups and the significant difference between negative control versus positive control using statistical software GraphPad Prism.

For the acute oral toxicity study, the difference between the treatment and control groups was determined using one-way ANOVA with Dunnett's test. Values with $P<0.05$ were concluded to be statistically significant.

For the 90-day toxicity assay, Graph-Prism software version 5.01 was used for statistical analysis of body weight, feed consumption, clinical chemistry, hematology, electrolytes, and organ weights. Means ± SD were calculated and one-way ANOVA with Dunnett's test was used to determine the significant difference between control and treatment groups. P-values of less than 0.05 were considered significant.

Results

In vitro studies

Reverse mutagenesis (Ames) test: The study conducted using the direct plate incorporation method showed that the mean number of revertant colonies per plate for the solvent control was within the range of spontaneous revertants for each strain. For both the plate incorporation and preincubation methods, the numbers of revertants were close to those for the solvent control in the presence or absence of metabolic activation. Negative and positive controls produced results that supported the validity of the test. Anandamide was considered to be non-mutagenic under the conditions of the study.

***In vitro* mammalian chromosomal aberration test using chinese hamster ovary (CHO)-K1 cells:** In the short-term and long-term treatment studies, cytotoxicity as evidenced by a selective reduction in RICC occurred only at the highest dose of anandamide used (15.625 µg/mL). There were no significant increases in the number of aberrant cells in the presence and absence of metabolic activation in the anandamide treatment group compared with the control group. Anandamide had negative results in the presence and absence of metabolic activation in the short-term study and in the absence of metabolic activation in the long-term study.

Mammalian cell gene mutation assay on L5178Y mouse lymphoma TK$^{+/-}$ cells: Cytotoxicity was not observed at any of the test concentrations either in the presence or absence of metabolic activation in the short-term (3-hour) study. There was no significant increase in the frequency of mutations compared with negative control in the absence and presence of metabolic activation. In cultures exposed to anandamide for 24 hours, in the long- term study, there was no significant increase in mutation frequency at any test dose in the absence of metabolic activation compared with the negative control. There were significant increases in the mutation frequency in cultures treated with positive control both in the absence and presence of metabolic activation. The results of this study indicate that anandamide is non-mutagenic in a cell gene mutation test using L5178Y TK$^{+/-}$ cells in both the absence and presence of metabolic activation (1% v/v S9 mix) for short and long-term treatment duration.

***In vivo* studies**

***In vivo* mammalian erythrocyte micronucleus assay in mice:** The animals were found to be normal and there were no mortalities or clinical signs in any dose group in the dose range-finding study. There were also no clinical signs or mortality in the limit test. There was no percentage reduction in the P/E ratio for the mice that received 2,000 mg/kg bw anandamide from which it can be inferred that anandamide was not toxic to bone marrow cells. No toxic response was observed for mice administered the negative control; whereas mice treated with the positive control showed a significant increase in percent MNPCE compared with the negative control. The authors concluded that the test was valid because the incidence of MNPCE for mice treated with the vehicle control was comparable to the historical in-house control data range, the animals treated with the positive control showed an increase in MNPCE compared with the control group, and the adequate number of doses and cells were evaluated. It was concluded that anandamide was non-mutagenic under the conditions of this study. Anandamide was present in the plasma at the second hour after treatment with anandamide and was not present in the group treated with vehicle control.

Animal study

Acute oral toxicity study: No unscheduled mortality or morbidity was observed in the study at doses of 175, 555, 1750, or 2,000 mg/kg bw; however, unscheduled mortality was observed at 5,000 mg/kg bw of anandamide on day 1 for two animals and on day 4 for the remaining animal. No clinical signs were observed at any dose except the 5,000 mg/kg bw dose. Significant increases in body weight were observed compared with day zero for doses of 175, 555, 1750, and 2,000 mg/kg bw anandamide; however, reductions in body weight were observed for animals administered 5,000 mg/kg bw of anandamide. Gross pathological examinations did not reveal any abnormalities at any dose of anandamide except the 5,000 mg/kg bw dose. These included severe lung congestion, increased lung volume, inflammatory lesions on the cranial region of the lungs, suspected pulmonary edema, localized gross lesions in the lungs, and multifocal interstitial pneumonitis and peribronchial fibrosis in the left lung. The lethal dose was concluded to be 2,000 mg/kg bw and anandamide was categorized as Category 5 based on the Globally Harmonized System of Classification and Labelling of Chemicals.

Ninety-day repeated dose oral toxicity study: No unscheduled deaths of animals occurred during the study and no clinical signs were observed. No significant reduction in body weight was observed for animals treated with anandamide compared with animals treated with control for the main and recovery study groups.

There was no significant difference in feed consumption for males and females in the anandamide groups compared with the control group for the main or recovery study group. There were no differences between groups in ophthalmological examinations or grip strength. There was no significant difference in organ weights between anandamide and control groups for male or female rats. Gross pathological examinations did not show any unusual results. Histopathological analyses of organs did not reveal inflammatory or structural abnormalities.

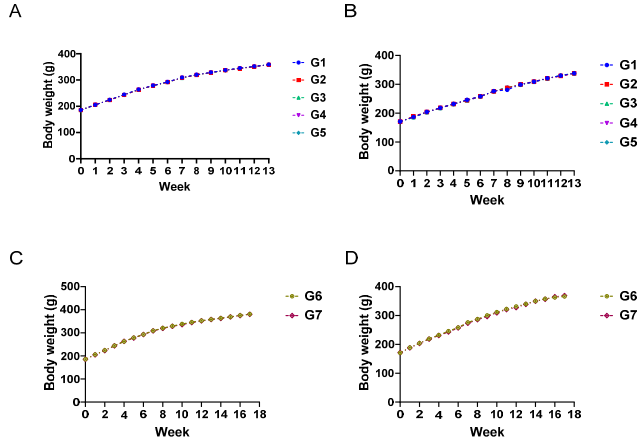

Figure 1. Anandamide Combined Body Weight Males and Females. **A:** Males main study, **B:** Females main study, **C:** Males recovery study, **D:** Females recovery study. G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1,000 mg/kg) main study; G4: High Dose (1,500 mg/kg) main study; G5: Exploratory High Dose (2,000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2,000 mg/kg) Recovery study. Values were expressed as mean ± SD (n = 10), Statistical significance are compared between Normal Control (Group 1) versus other treatment groups (* P Value<0.05; ** P Value<0.001; *** P Value<0.0001).

Analyses of hematological parameters showed no difference for male or female rats treated with anandamide compared with control groups (**Table 1**– Hematology Males, **Table 2** - Hematology Females).

No significant differences between control and anandamide-treated groups were noted in clinical chemistry parameters including total cholesterol, triglycerides, LDL-C, HDL-C, sodium, potassium, calcium, or phosphorus or for liver function measurements for male or female rats (**Table 3** - Anandamide Clinical Chemistry Males) and (**Table 4** - Anandamide Clinical Chemistry Females).

No significant differences in absolute organ weights were reported for the male or female rats treated with anandamide compared with the control group (**Table 5** – Anandamide Absolute Organ Weights Males) and (**Table 6** – Anandamide Absolute Organ Weights Females).

Table 1. Anandamide Hematology Males.

Parameters	Groups						
	G1	G2	G3	G4	G5	G6	G7
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
WBC (cells/cu mm)	8.41 ± 0.79	8.12 ± 0.46	8.36 ± 0.70	8.38 ± 0.75	8.22 ± 0.58	8.73 ± 0.39	8.79 ± 0.41
RBC (mill/cu mm)	6.38 ± 0.19	6.30 ± 0.21	6.24 ± 0.23	6.28 ± 0.25	6.36 ± 0.23	6.24 ± 0.10	6.19 ± 0.17
HGB (gm/dL)	16.41 ± 0.38	16.25 ± 0.20	16.38 ± 0.29	16.31 ± 0.25	16.41 ± 0.32	16.11 ± 0.34	16.09 ± 0.20
HCT (%)	51.20 ± 1.03	51.00 ± 0.82	51.70 ± 2.41	50.70 ± 1.06	51.30 ± 2.11	50.20 ± 1.23	50.10 ± 0.74
MCV (fL)	61.49 ± 1.01	60.90 ± 1.59	60.57 ± 1.61	60.67 ± 1.69	60.59 ± 1.50	61.04 ± 1.59	60.72 ± 0.89
MCH (pg)	17.60 ± 0.62	17.32 ± 0.71	17.59 ± 0.74	17.56 ± 0.64	17.73 ± 0.41	17.07 ± 0.46	16.85 ± 0.95
MCHC (g/dL)	31.30 ± 1.11	30.81 ± 0.87	30.60 ± 1.20	31.17 ± 1.11	30.70 ± 0.98	30.43 ± 1.06	29.89 ± 0.75
PLT (Lakh/cu mm)	6.19 ± 0.25	6.28 ± 0.21	6.06 ± 0.88	6.48 ± 0.32	6.27 ± 0.27	6.27 ± 0.18	6.24 ± 0.17
LY (%)	60.80 ± 2.57	60.20 ± 3.26	59.90 ± 2.77	60.70 ± 1.70	59.70 ± 2.50	59.00 ± 2.87	59.20 ± 2.78
MI (%)	9.00 ± 1.89	9.40 ± 1.79	9.40 ± 2.12	9.20 ± 2.30	9.90 ± 1.79	9.60 ± 2.46	9.10 ± 1.60
GR (%)	30.20 ± 1.32	30.40 ± 2.91	30.70 ± 1.42	30.10 ± 1.97	30.40 ± 2.17	31.40 ± 1.65	31.70 ± 2.87

GR: Granulocytes; HCT: Hematocrit; HGB: Hemoglobin; LY: Lymphocytes; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; MI: Mid Cell Leukocytes; PLT: Platelet; RBC: Red Blood Corpuscle; WBC: White Blood Cells.

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1000 mg/kg) main study; G4: High Dose (1500 mg/kg) main study; G5: Exploratory High Dose (2000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (* P Value<0.05; ** P Value<0.001; *** P Value<0.0001).

Table 2. Anandamide Hematology Females.

Parameters	Groups						
	G1	G2	G3	G4	G5	G6	G7
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
WBC (cells/cu mm)	8.86 ± 0.54	8.66 ± 0.43	8.92 ± 0.43	8.73 ± 0.61	8.78 ± 0.55	9.16 ± 0.41	8.94 ± 0.41
RBC (mill/cu mm)	5.53 ± 0.25	5.50 ± 0.21	5.44 ± 0.11	5.50 ± 0.16	5.39 ± 0.14	5.48 ± 0.23	5.38 ± 0.15
HGB (gm/dL)	15.65 ± 0.30	15.63 ± 0.30	15.64 ± 0.26	15.76 ± 0.33	15.59 ± 0.23	15.69 ± 0.27	15.60 ± 0.26
HCT (%)	50.40 ± 1.43	50.20 ± 1.69	50.50 ± 1.65	51.50 ± 1.96	50.50 ± 1.51	50.20 ± 1.40	49.60 ± 1.18
MCV (fL)	62.05 ± 1.71	62.13 ± 1.36	61.60 ± 1.54	61.59 ± 1.16	61.75 ± 2.27	61.55 ± 1.05	61.37 ± 1.30
MCH (pg)	17.57 ± 0.83	17.66 ± 0.45	17.59 ± 0.61	17.62 ± 0.57	17.66 ± 0.52	17.12 ± 0.34	17.26 ± 0.28
MCHC (g/dL)	30.38 ± 1.01	30.40 ± 0.96	30.23 ± 1.03	30.34 ± 1.02	30.22 ± 0.74	30.06 ± 0.81	30.00 ± 0.97
PLT(Lakh/cu mm)	6.31 ± 0.48	6.20 ± 0.34	6.38 ± 0.65	6.41 ± 0.41	6.15 ± 0.51	6.23 ± 0.25	6.25 ± 0.28
LY (%)	60.00 ± 2.98	59.60 ± 2.37	59.50 ± 2.22	59.40 ± 2.72	59.70 ± 2.60	59.50 ± 2.64	59.00 ± 3.13
MI (%)	9.80 ± 2.15	9.80 ± 1.62	10.50 ± 2.51	10.10 ± 2.13	10.20 ± 2.78	10.30 ± 2.21	10.50 ± 2.12
GR (%)	30.20 ± 1.75	30.60 ± 1.90	30.00 ± 1.94	30.50 ± 2.80	29.70 ± 1.89	30.20 ± 1.30	30.50 ± 1.08

GR: Granulocytes; HCT: Hematocrit; HGB: Hemoglobin; LY: Lymphocytes; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; MI: Mid Cell Leukocytes; PLT: Platelet; RBC: Red Blood Corpuscle; WBC: White Blood Cells

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1000 mg/kg) main study; G4: High Dose (1,500 mg/kg) main study; G5: Exploratory High Dose (2,000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2,000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (* P Value<0.05; ** P Value<0.001; *** P Value<0.0001).

Table 3. Anandamide Clinical Chemistry Males.

Parameters	Groups						
	G1	G2	G3	G4	G5	G6	G7
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Bilirubin (mg/dL)	0.19 ± 0.03	0.19 ± 0.03	0.18 ± 0.03	0.18 ± 0.03	0.20 ± 0.03	0.21 ± 0.02	0.21 ± 0.01
ALP (IU/L)	191.02 ± 7.48	193.73 ± 6.35	192.64 ± 6.72	193.86 ± 7.63	190.86 ± 6.41	196.57 ± 10.13	197.03 ± 3.82
Protein (g/dL)	7.81 ± 0.52	7.79 ± 0.29	7.51 ± 0.64	7.78 ± 0.37	7.69 ± 0.61	7.45 ± 0.31	7.47 ± 0.29
Albumin (g/dL)	3.58 ± 0.20	3.62 ± 0.16	3.68 ± 0.27	3.60 ± 0.22	3.66 ± 0.22	3.39 ± 0.25	3.36 ± 0.14
SGOT (IU/L)	110.75 ± 4.35	112.45 ± 5.17	111.44 ± 3.59	112.36 ± 4.36	110.27 ± 4.85	107.06 ± 3.52	106.68 ± 3.99
SGPT (IU/L)	34.29 ± 2.85	34.48 ± 2.41	35.39 ± 2.05	34.87 ± 2.26	35.22 ± 1.18	34.40 ± 2.02	34.18 ± 1.11
B.UREA (mg/dL)	38.47 ± 2.03	38.51 ± 2.12	38.12 ± 1.06	38.23 ± 2.48	38.10 ± 1.70	37.69 ± 1.55	37.48 ± 1.37
S.Creatinine (mg/dL)	0.90 ± 0.09	0.88 ± 0.09	0.87 ± 0.04	0.88 ± 0.07	0.90 ± 0.06	0.89 ± 0.06	0.90 ± 0.04
Total Cholesterol (mg/dL)	68.69 ± 3.14	69.85 ± 3.78	70.45 ± 2.59	69.86 ± 2.83	70.62 ± 3.00	72.15 ± 2.92	71.58 ± 2.94
Triglycerides (mg/dL)	75.79 ± 5.78	76.71 ± 3.53	78.03 ± 2.95	76.47 ± 3.98	76.59 ± 3.78	79.18 ± 4.58	79.54 ± 2.54
HDL (mg/dL)	38.27 ± 1.61	37.42 ± 2.03	37.19 ± 0.97	38.24 ± 1.89	38.19 ± 1.74	37.01 ± 1.51	36.86 ± 1.33
LDL (mg/dL)	8.60 ± 0.77	8.85 ± 0.66	8.92 ± 0.62	8.57 ± 0.47	8.54 ± 0.430	9.04 ± 0.56	9.13 ± 0.52
Sodium (mmol/L)	141.30 ± 1.49	141.50 ± 2.64	140.90 ± 3.18	142.20 ± 2.15	140.90 ± 2.13	142.30 ± 2.31	143.40 ± 1.65
Potassium(mmol/L)	4.62 ± 0.23	4.61 ± 0.19	4.61 ± 0.15	4.72 ± 0.20	4.65 ± 0.32	4.62 ± 0.14	4.58 ± 0.09
Calcium (mg/dL)	9.52 ± 0.57	9.66 ± 0.47	9.65 ± 0.42	9.58 ± 0.36	9.66 ± 0.46	9.26 ± 0.28	9.30 ± 0.28
Fasting Glucose (mg/dL)	97.34 ± 4.30	98.97 ± 2.70	97.90 ± 2.59	100.03 ± 4.60	99.12 ± 4.40	100.58 ± 3.17	100.91 ± 2.82

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1000 mg/kg) main study; G4: High Dose (1500 mg/kg) main study; G5: Exploratory High Dose (2000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (* P Value < 0.05;** P Value < 0.001; *** P Value < 0.0001).

Table 4. Anandamide Clinical Chemistry Females.

Parameters	Groups						
	G1	G2	G3	G4	G5	G6	G7
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Bilirubin (mg/dL)	0.13 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.15 ± 0.05	0.14 ± 0.02	0.14 ± 0.02	0.14 ± 0.01
ALP (IU/L)	212.49 ± 6.62	212.96 ± 5.32	214.36 ± 5.49	213.08 ± 4.72	213.88 ± 4.39	213.58 ± 2.25	213.42 ± 5.07
Protein (g/dL)	7.85 ± 0.42	7.82 ± 0.41	7.81 ± 0.36	7.79 ± 0.41	7.82 ± 0.65	7.59 ± 0.31	7.57 ± 0.23
Albumin (g/dL)	3.62 ± 0.24	3.60 ± 0.21	3.65 ± 0.22	3.62 ± 0.27	3.67 ± 0.25	3.47 ± 0.18	3.51 ± 0.14
SGOT (IU/L)	95.19 ± 4.01	97.45 ± 5.17	96.44 ± 3.50	95.99 ± 4.26	100.68 ± 5.42	91.46 ± 8.39	92.97 ± 2.50
SGPT (IU/L)	31.29 ± 2.89	31.48 ± 2.41	32.39 ± 2.05	32.30 ± 2.16	34.22 ± 1.48	33.12 ± 1.52	33.74 ± 1.24
B.UREA (mg/dL)	41.73 ± 2.04	42.72 ± 3.57	42.96 ± .2.71	42.45 ± 1.86	42.02 ± 2.23	42.61 ± 1.52	42.51 ± 3.23
S.Creatinine (mg/dL)	0.92 ± 0.09	0.93 ± 0.11	0.92 ± 0.05	0.93 ± 0.07	0.95 ± 0.08	0.91 ± 0.03	0.92 ± 0.03
Total Cholesterol (mg/dL)	68.09 ± 2.45	69.02 ± 5.15	70.12 ± 3.22	69.83 ± 3.15	69.49 ± 2.55	72.33 ± 3.44	72.63 ± 2.66

Triglycerides (mg/dL)	78.16 ± 4.06	80.07 ± 4.14	78.86 ± 3.91	78.53 ± 5.17	78.58 ± 3.78	78.69 ± 3.66	79.22 ± 3.34
HDL (mg/dL)	49.23 ± 2.11	49.08 ± 2.08	48.62 ± 2.45	48.65 ± 1.33	48.36 ± 1.90	46.49 ± 1.59	46.75 ± 0.90
LDL (mg/dL)	8.15 ± 0.79	8.29 ± 0.74	8.18 ± 0.48	8.33 ± 0.63	8.40 ± 0.72	8.57 ± 0.41	8.39 ± 0.50
Sodium (mmol/L)	140.10 ± 2.60	140.80 ± .2.04	141.50 ± 3.54	141.80 ± 3.05	142.30 ± 2.36	144.00 ± 1.83	143.90 ± 1.85
Potassium(mmol/L)	4.62 ± 0.19	4.59 ± 0.15	4.65 ± 0.17	4.55 ± 0.16	4.59 ± 0.16	4.59 ± 0.17	4.51 ± 0.17
Calcium (mg/dL)	11.15 ± 0.47	11.12 ± 0.48	11.03 ± 0.35	10.96 ± 0.34	10.84 ± 0.25	10.64 ± 0.27	10.62 ± 0.28
Fasting Glucose (mg/dL)	101.17 ± 5.59	102.61 ± 3.84	101.36 ± 4.61	102.45 ± 5.62	102.89 ± 6.98	104.59 ± 5.45	104.19 ± 2.02

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1,000 mg/kg) main study; G4: High Dose (1,500 mg/kg) main study; G5: Exploratory High Dose (2,000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2,000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (* P Value<0.05; ** P Value<0.001; *** P Value<0.0001).

Table 5. Anandamide Absolute Organ Weights Males.

Parameters	**Groups**						
	G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Liver	12.23 ± 0.62	12.32 ± 0.44	12.34 ± 0.58	12.41 ± 0.50	12.35 ± 0.60	12.54 ± 0.49	12.58 ± 0.53
Heart	1.11 ± 0.09	1.15 ± 0.05	1.17 ± 0.08	1.16 ± 0.07	1.18 ± 0.05	1.31 ± 0.05	1.32 ± 0.06
Kidney	2.94 ± 0.25	2.96 ± 0.23	2.17 ± 0.19	2.99 ± 0.24	2.99 ± 0.19	3.01 ± 0.20	3.04 ± 0.21
Brain	1.96 ± 0.16	1.98 ± 0.12	2.00 ± 0.14	2.03 ± 0.11	2.01 ± 0.13	2.09 ± 0.12	2.11 ± 0.12
Spleen	1.30 ± 0.06	1.32 ± 0.04	1.35 ± 0.04	1.36 ± 0.05	1.35 ± 0.06	1.39 ± 0.05	1.40 ± 0.04
Adrenal (mg)	99.17 ± 4.51	100.86 ± 7.58	102.22 ± 9.27	103.30 ± 8.47	103.15 ± 7.88	102.01 ± 5.24	102.15 ± 4.79
Testes	3.19 ± 0.18	3.14 ± 0.22	3.16 ± 0.18	3.10 ± 0.15	3.08 ± 0.12	3.15 ± 0.12	3.19 ± 0.15
Lungs	2.87 ± 0.19	2.89 ± 0.15	2.92 ± 0.22	2.95 ± 0.24	2.96 ± 0.17	3.07 ± 0.16	3.06 ± 0.11
Epididymis	1.31 ± 0.03	1.28 ± 0.03	1.29 ± 0.03	1.31 ± 0.04	1.29 ± 0.05	1.32 ± 0.05	1.34 ± 0.03

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1,000 mg/kg) main study; G4: High Dose (1,500 mg/kg) main study; G5: Exploratory High Dose (2,000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2,000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (G2, G3, G4, G5, G6 & G7) (* P Value<0.05; ** P Value<0.001; *** P Value<0.0001).

Table 6. Anandamide Absolute Organ Weights Females.

Parameters	**Groups**						
	G1	**G2**	**G3**	**G4**	**G5**	**G6**	**G7**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Liver	9.99 ± 0.36	10.08 ± 0.24	10.09 ± 0.18	10.18 ± 0.16	10.16 ± 0.25	10.46 ± 0.23	10.44 ± 0.32
Heart	0.91 ± 0.04	0.93 ± 0.04	0.94 ± 0.03	0.95 ± 0.02	0.94 ± 0.03	0.97 ± 0.03	0.97 ± 0.04
Kidney	2.38 ± 0.06	2.40 ± 0.04	2.39 ± 0.04	2.45 ± 0.06	2.41 ± 0.06	2.47 ± 0.09	2.48 ± 0.09
Brain	1.69 ± 0.08	1.72 ± 0.07	1.70 ± 0.03	1.73 ± 0.05	1.74 ± 0.06	1.76 ± 0.06	1.77 ± 0.06
Spleen	1.21 ± 0.05	1.28 ± 0.04	1.26 ± 0.03	1.27 ± 0.02	1.26 ± 0.05	1.31 ± 0.02	1.31 ± 0.05
Adrenal (mg)	88.34 ± 4.18	90.12 ± 2.71	89.55 ± 1.61	89.88 ± 2.04	88.88 ± 2.63	90.54 ± 2.31	90.32 ± 1.28
Ovaries	0.158 ± 0.01	0.154 ± 0.0	0.153 ± 0.0	0.157 ± 0.01	0.155 ± 0.0	0.155 ± 0.0	0.156 ± 0.01
Uterus	0.694 ± 0.02	0.689 ± 0.02	0.691 ± 0.02	0.693 ± 0.03	0.689 ± 0.03	0.693 ± 0.02	0.694 ± 0.02
Lungs	2.39 ± 0.07	2.42 ± 0.06	2.39 ± 0.06	2.41 ± 0.07	2.41 ± 0.04	2.43 ± 0.06	2.38 ± 0.04

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1000 mg/kg) main study; G4: High Dose (1500 mg/kg) main study; G5: Exploratory High Dose (2000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (G2, G3, G4, G5, G6 & G7) (* P Value<0.05; ** P Value<0.001; *** P Value<0.0001).

Normal levels of T4, T3, TSH, FSH, E2, LH, prolactin, estradiol, and testosterone were reported for all animals treated with anandamide. No differences were observed between control and anandamide-treated animals.

Urinalysis showed no change related to the administration of anandamide compared with the control group. No significant differences in absolute organ weights were reported for the male or female rats treated with anandamide compared with the control group (**Table 7** – Urinalysis Males and **Table 8** – Urinalysis Females).

In summary, the 90-day oral toxicology study reported that anandamide resulted in significant toxicity at the highest dose, 5,000 mg AEA/kg bw, but no mortality or morbidity, or clinical signs at doses of 175, 555, 1,750, or 2,000 mg/kg bw. Accordingly, the NOEL was determined to be 2,000 mg AEA/kg bw. The 90-day subchronic repeated dose oral toxicity study of AEA with a 28- day recovery period reported no differences in any of the experimental groups in body weight, feed consumption, observations or behavior, organ weight, pathology or histopathology, hematology, urinalysis, or clinical chemistry. There were no indications of lung or kidney damage at any dose. The NOAEL for AEA was determined to be 2,000 mg/kg bw.

Table 7. Anandamide Urinalysis Males.

Parameters		Groups						
		G1	G2	G3	G4	G5	G6	G7
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Volume		6.54 ± 0.25	6.63 ± 0.20	6.64 ± 0.24	6.59 ± 0.21	6.57 ± 0.24	6.47 ± 0.25	6.43 ± 0.34
Colour	Normal	02	03	03	04	03	03	04
	Yellow	08	07	07	06	07	07	06
Appearance		Clear	Clear	Clear	Clear	Clear	Clear	Clear
Specific gravity		1.02 ± 0.00	1.02 ± 0.01	1.02 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	1.04 ± 0.03
pH		6.93 ± 0.28	6.70 ± 0.39	6.55 ± 0.28	6.72 ± 0.35	6.71 ± 0.31	6.79 ± 0.48	6.61 ± 0.34
Urine glucose		Nil	Nil	Nil	Nil	Nil	Nil	Nil
Urobilinogen		Absent	Absent	Absent	Absent	Absent	Absent	Absent
Ketone bodies		Absent	Absent	Absent	Absent	Absent	Absent	Absent
Blood in Urine		Absent	Absent	Absent	Absent	Absent	Absent	Absent

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1,000 mg/kg) main study; G4: High Dose (1,500 mg/kg) main study; G5: Exploratory High Dose (2,000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2,000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (G2, G3, G4, G5, G6 & G7) (*P Value<0.05; **P Value<0.001; *** P Value<0.0001).

Table 8. Anandamide Urinalysis Females.

Parameters		Groups						
		G1	G2	G3	G4	G5	G6	G7
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Volume		6.51 ± 0.24	6.63 ± 0.26	6.46 ± 0.22	6.52 ± 0.22	6.49 ± 0.23	6.55 ± 0.25	6.69 ± 0.20
Colour	Normal	03	04	03	03	03	03	04
	Yellow	07	06	07	07	07	07	06
Appearance		Clear	Clear	Clear	Clear	Clear	Clear	Clear
Specific gravity		1.02 ± 0.01	1.02 ± 0.01	1.02 ± 0.01	1.09 ± 0.035	1.07 ± 0.33	1.12 ± 0.23	1.21 ± 0.30
pH		6.45 ± 0.44	6.35 ± 0.43	6.41 ± 0.29	6.29 ± 0.39	6.40 ± 0.24	6.49 ± 0.24	6.39 ± 0.32
Urine glucose		Nil	Nil	Nil	Nil	Nil	Nil	Nil
Urobilinogen		Absent	Absent	Absent	Absent	Absent	Absent	Absent
Ketone bodies		Absent	Absent	Absent	Absent	Absent	Absent	Absent
Blood in Urine		Absent	Absent	Absent	Absent	Absent	Absent	Absent

G1: Normal Control (Corn oil) main study; G2: Low Dose (500 mg/kg) main study; G3: Middle Dose (1000 mg/kg) main study; G4: High Dose (1500 mg/kg) main study; G5: Exploratory High Dose (2000 mg/kg) main study; G6: Vehicle Control (Corn oil) Recovery study; G7: High Dose (2000 mg/kg) Recovery study.

Values were expressed as mean ± SD (n = 10), Statistical significance was compared between Normal Control (Group 1) versus other treatment groups (G2, G3, G4, G5, G6 & G7) (* P Value<0.05; ** P Value<0.001; *** P Value<0.0001).

Discussion and Conclusion

A battery of *in vitro* and *in vivo* studies was conducted in anandamide including a bacterial reverse mutation assay, a mammalian cell gene mutation assay on L5178Y mouse lymphoma cells $TK^{+/-}$, an *in vitro* mammalian chromosomal aberration test using Chinese hamster ovary (CHO)-K1 cells, an in vivo mammalian erythrocyte micronucleus assay in mice, an acute oral toxicity study and a 90-day repeated dose toxicity study with recovery period.

In an acute oral toxicity test, significant mortality with lung congestion, inflammatory lesions, pneumonitis, and fibrosis was observed at the highest dose administered during the study, 5,000 mg/kg bw of AEA. There was no mortality or morbidity and no clinical signs when AEA was administered at doses of 175, 555, 1750, or 2,000 mg/kg bw. Accordingly, the NOEL was determined to be 2,000 mg AEA/kg bw.

In the 90-day subchronic repeated dose oral toxicity study with a 28-day recovery period, AEA was administered at doses of 0, 500, 1,000, 1,500 and 2,000 mg/kg bw/day on the main study and at 0 and 2,000 mg/kg bw/day in the recovery group. No differences were observed between the AEA and respective control groups with respect to body weight, feed consumption, observations or behavior, organ weight, gross pathology or histopathology, hematology, clinical chemistry, hormone analysis, or urinalysis. There were no indications of lung or kidney damage at any dose. The NOAEL for AEA was determined to be 2,000 mg/kg bw.

There were no differences from negative controls in a bacterial reverse mutation (Ames) test at doses up to 5,000 µg AEA/ plate. An *in vivo* mammalian erythrocyte micronucleus test in mice showed no negative clinical or behavior changes or gross pathological effects. There were no differences in micronucleus frequency between the negative control group and any of the AEA test groups. Moreover, there was no evidence of mutagenicity or clastogenicity in an *in vitro* cell gene mutation assay on L5178Y $TK^{+/-}$ mouse lymphoma cells, and an *in vitro* mammalian chromosomal aberration test using CHO-K1 cells.

There have been no published toxicological studies of AEA. Because the kidneys are enriched in AEA and other components of the endocannabinoid system (ECS). They have been suggested to be involved in regulation of renal blood flow and tubular reabsorption [16].

The effect of intravenous administration of up to 3 mg/kg AEA had no effects on blood pressure or kidney function in Dahl SS rats fed a normal salt diet, but AEA treatment aggravated hypertension and increased interstitial fibrosis and glomerular damage in late-stage hypertension in Dahl SS rats fed a high salt diet [17]. Treatment of male mice pups with AEA during the lactation period resulted in increased accumulated food intake, body weight, epididymal fat, and insulin resistance during adulthood [12]. In contrast, diets rich in arachidonic acid had no effect on appetite, feed intake, body weight or body weight gain in rats [18]. These potential areas of concern were not supported by the results under the conditions of repeated oral dose toxicity studies or at the non-lethal levels of acute toxicology study.

Despite the longstanding and widespread interest in the biological activities of the ECS and an increasingly large number of publications addressing the pharmacological properties, especially regarding tetrahydrocannabinol and cannabidiol, there is a surprisingly scant literature on the toxicology of endogenous ECS agonists [19,20]. In addition to AEA, other ECS agonists include 2-arachidonoylglycerol, oleoylethanolamide, and other long chain fatty acid conjugates of ethanolamine. The ECS has been considered primarily responsible for maintaining homeostasis of temperature control, mood, and immune functions and energy output/input, influencing a variety of physiological and pathophysiological processes [19]. This has resulted in an interest in developing therapeutics targeting the ECS metabolic pathway.

Fatty acid amide hydrolase (FAAH) is the first step in the catabolism of AEA, a key controller of AEA tone in vivo, and the target of several putative inhibitors [21]. Preclinical and clinical safety studies of FAAH inhibitors [22-26] have identified adverse consequences at high doses including high mortality rates, signs of weakness, prostration, labored breathing, clear lacrimation, tachypnea/bradypnea and decreased activity at high doses. However, FAAH is involved in the catabolism of most of the ECS agonists and other metabolic pathways and, therefore, its relevance to an understanding of the safety of AEA is limited.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and / or publication of this article.

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