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Oral repeated-dose toxicity studies of BIA 10–2474 in CD-1 mice

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ABSTRACT

We independently and retrospectively reviewed three studies that evaluated the toxicity of BIA 10–2474 (3-(1-(cyclohexyl(methyl)carbamoyl)-lH-imidazol-4-yl)pyridine 1-oxide), a novel fatty acid amide hydrolase (FAAH) inhibitor in male and female CD-1 mice based upon raw data obtained from Bial Portela & Companhia S.A. (São Mamede do Coronado, Portugal). These studies were carried out prior to the clinical trial with BIA 10–2474 and formed part of the regulatory submission. An initial oral dose range-finding study with BIA 10–2474 showed that doses from 600 mg/kg/day were poorly tolerated with a high mortality rate and signs of weakness, prostration, labored breathing, clear lacrimation, tachypnea/bradypnea and decreased activity. At lower doses (100 and 300 mg/kg/day) there were few signs but post-mortem analysis showed increased liver weight. In a 28-day study a third of the animals receiving 500 mg/kg/day died or required euthanasia, with similar signs to those seen in the dose-range finding study. At lower doses (i.e. 100 and 300 mg/kg/day) there were few clinical signs although there were dose-related decreases in erythrocyte count and hemoglobin. Histopathology was seen in the 300 and 500 mg/kg/day groups and included hepatocellular hypertrophy (with increased liver weight), nephropathy and enterocyte vacuolation. Finally, in the 13-week oral gavage study, BIA 10–2474 was administered to CD-1 mice of both sexes at dose levels of 25, 75 and 150 mg/kg/day. Under these conditions, there were almost no clinical signs apart from a tendency to increase body-weight. Cholesterol was increased at 75 and 150 mg/kg and remained high after recovery. Liver and spleen weights increased at 75 and 150 mg/kg/day. Histopathologically, there was a dose-dependent increase in sciatic nerve and myofiber degeneration, hepatocellular hypertrophy, nephropathy and inflammatory loci in the bladder. The nerve damage and nephropathy seen at 150 mg/kg/day persisted after a 4-week recovery period. Toxicokinetic analysis in the 4- and 13-week studies showed that exposure was broadly dose-proportional with no evidence of accumulation. On the basis of the changes seen during the 13-week study, the NOAEL was established at 75 mg/kg/day.

1. Introduction

Fatty acid amide hydrolase (FAAH) is an integral membrane enzyme that hydrolyzes the endocannabinoid anandamide and related amidated signaling lipids. Inhibition of FAAH increases the concentration of endocannabinoids which act on the cannabinoid receptors CB1 and CB2, as well as other receptors such as cannabinoid-sensitive G-protein coupled receptors, peroxisome proliferator-activated receptors and vanilloid receptors (Toczek and Malinowska, 2018). This approach to increasing endocannabinoid tone is expected to reduce the probability of cannabinoid-like adverse events compared to administration of exogenous agonists (Panlilio et al., 2015). Rodent studies indicate these receptors are expressed in a variety of tissues, such as brain, gastrointestinal tract, and bone (Ryberg et al., 2007) and FAAH inhibitors are considered potential therapeutic agents for a range of indications including chronic pain, metabolic disorders, psychoses, nausea and vomiting, depression, and anxiety disorders (Petrosino and Di Marzo, 2010; Toczek and Malinowska, 2018).

Bial Portela & Companhia S.A. (São Mamede do Coronado, Portugal) initiated a project in 2005 to develop FAAH inhibitors, resulting in the identification of BIA 10–2474 (Kiss et al., 2018). Preclinical studies with this compound started in 2009 with *in vitro* and *in vivo* pharmacological and toxicological evaluations. In 2015, a first-in-human trial with BIA 10–2474 started in healthy volunteers and during the multiple ascending dose phase one subject died and four were hospitalized with neurological symptoms (Gama et al., 2016). The

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subject who died, after receiving the 50 mg dose for 5 days, showed evidence of severe brain microhemorrhage and several of the surviving subjects also showed evidence of mild to moderate microhemorrhage as well as headache, cerebellar syndrome, memory impairment, and altered consciousness (Kerbrat et al., 2016). Magnetic resonance imaging showed bilateral and symmetric cerebral lesions, including microhemorrhages and hyperintensities on fluid-attenuated inversion recovery and diffusion-weighted imaging sequences predominantly involving the pons and hippocampi. The condition of two patients subsequently improved, but one patient had residual memory impairment, and the other patient had a residual cerebellar syndrome. One patient remained asymptomatic (Kerbrat et al., 2016). The investigations by the French authorities concluded that it was an unexpected effect of the test item, having ruled out other extraneous causes (CSST and Santé, 2016). To date, the action of BIA 10–2474 responsible for the death of the volunteer has not been determined.

The present paper describes the study design and results of the toxicity studies performed in CD-1 mice that formed part of the documentation supporting the clinical trial, including the dose-range finding (DRF), 4-, and 13-week toxicity studies. The information was drawn from the original study reports. All facts are listed and reported. No result or conclusion has been changed.

2. Materials and methods

2.1. Overall design of toxicology program

These studies were conducted at Harlan Laboratories Ltd (Itingen, Switzerland) and the histopathology evaluation was conducted by AnaPath GmbH (Switzerland). All studies were conducted in compliance with applicable national and international standards including European Directives and Guidelines 2001/83/EC, 2003/63/EEC, 91/507/EEC, 75/318/EEC, 83/571/EEC and European guidance documents CPMP/SWP/1042/99, CPMP/SWP/1094/04, ICH S3A (CPMP/ICH/384/95) and EC Document No. III/5380/96 as well as applicable FDA guidelines and the Swiss national ordonnance for Good Laboratory Practice (SR 813.112.1).

The studies were performed in an AAALAC-accredited laboratory in accordance with the Swiss Animal Protection Law under license nos. 25 and 36. The study procedures were reviewed and approved by the Animal Experimentation Ethics Committee at Harlan Laboratories S.A.

3. Materials

BIA 10–2474 (3-(1-(cyclohexyl(methyl)carbamoyl)-1H-imidazol-4-yl)pyridine 1-oxide) was used in all of the studies described below, and was both manufactured and supplied by Bial Portela & Companhia S.A. (São Mamede do Coronado, Portugal). In all three studies the purity of BIA 10–2474 was 99.8%.

3.1. Animals and maintenance

3.1.1. Animals

Crl:CD-1 (ICR) BR mice from Charles River Germany, Sandhoferweg 7, 97633 Sulzfeld/Germany were used in all studies.

The DRF study comprised three animals per sex and per group weighing 31.6–34.6 g (males) and 26.6–28.1 g (females) on the first treatment day. For the 28-day oral toxicity study, 100 animals per sex were used (n = 16 for vehicle groups and 28 for drug-treated groups), aged 7 weeks at delivery. At the start of acclimatization, the body weight ranged from 24.2 to 29.4 g (males) and 20.0–26.2 g (females). There were separate cohorts within each group for clinical (10 mice per sex) and toxicokinetic evaluations (6 for control groups and 18 for BIA 10–2474 treated groups).

For the 13-week oral toxicity study, 110 animals per sex were used (n = 21 for vehicle groups, 28 for the 25 and 75 mg/kg groups and 33 for the 150 mg/kg group), aged 7 weeks at delivery. At the start of acclimatization, the body weight ranged from 25.7 g to 32.0 g (males) and 20.5 g–25.3 g (females). There were separate cohorts within each group for clinical (10 mice per sex) and toxicokinetic evaluations (6 for control groups and 18 for BIA 10–2474 treated groups) and in addition 5 animals in the control and high dose groups to evaluate recovery.

3.2. Diet/water

Pelleted standard Harlan Teklad 2914C (4-week study: batch nos. 82/09 and 20/10) rat, 13-week study: (batch nos. 30/10, 39/10 and 68/10)/mouse maintenance diet (Provimi Kliba AG, 4303 Kaiseraugst/Switzerland) was available ad libitum. The feed batch was analysed for contaminants. Community tap-water from Itingen, Switzerland was available ad libitum in water bottles.

3.3. Housing

The animals were individually housed in Makrolon type-2 cages with wire mesh tops and standardized softwood bedding ('Lignocel' J. Rettenmaier & Söhne GmbH & Co KG, 73494 Rosenberg/Germany, imported by Provimi Kliba SA, 4303 Kaiseraugst/Switzerland) including paper enrichment (ISO-BLOX from Harlan Laboratories B.V., Netherlands). The animals were housed under standard laboratory conditions with 10–15 air changes per hour, temperature of 22 ± 3 °C and relative humidity of 30–70%. There was a 12-h fluorescent light/12-h dark cycle with music during the light period.

Shortly after arrival, the animals were allocated to groups by a computerized randomising algorithm. The animals were acclimatized for 7 days under test conditions after health examination. Only animals without any visible signs of illness were used for the study.

4. Dose preparation

Dose calculations in all studies were based upon the most recent body weights of each animal. 0.2% hydroxypropylmethylcellulose (HPMC) solution, prepared with bi-distilled water (Fluka, Sigma-Aldrich Chemie GmbH) was used as vehicle to prepare the dose formulations which were prepared weekly. BIA 10–2474 was weighed into a tared glass beaker on a suitable precision balance and the vehicle added. The mixtures were prepared using a magnetic stirrer and stored at room temperature (20 ± 5 °C). Homogeneity of the test item in the vehicle was maintained during the daily administration period using a magnetic stirrer. The stability of the dose formulations was evaluated at least once per week by HPLC. They were stored at room temperature (20 ± 5 °C), protected from light.

5. Dose administration

In the dose range-finding toxicity study, BIA 10–2474 was administered daily by oral gavage to CD-1 mice of both sexes at dose levels of 100 or 300 mg/kg body weight for a period of 14 days. A high-dose group was started with 1000 mg/kg/day, but due to severe clinical signs and unscheduled deaths, the animals in this group were not dosed on day 4 of treatment and dosed with 600 mg/kg/day for 10 days afterwards. In the subsequent 4-week study, BIA 10–2474 was administered at doses of 100, 300 and 500 mg/kg/day and in the 13-week study, the animals received doses of 25, 75 and 150 mg/kg/day.

6. Clinical observation and examination

6.1. Clinical observations

The animals were observed twice daily for viability/mortality and for any change in behavior, reaction to treatment or ill-health. All animals were observed for clinical signs once daily during the

acclimatization period, twice daily on days 1–3 of the treatment period and once daily thereafter. The food consumption was recorded once during the acclimatization period and weekly thereafter. Body weights were recorded weekly during acclimatization and the treatment period and before necropsy.

6.2. Blood and urine sampling

After 28 days (4-week study), and 13 and 17 weeks (13-week study plus 4-weeks recovery), blood samples were drawn from the retro-orbital plexus from all main study animals using a micro-hematocrit glass capillary tube under light isoflurane anesthesia. The animals were fasted in metabolism cages for approximately 18 h before blood sampling. Prior to fasting, each mouse was administered 30 mL/kg body weight of tap water by gavage. The samples were always collected early in the working day to reduce biological variation caused by circadian rhythms. Urine was collected during the 18 h fasting period.

6.3. Toxicokinetic analysis

Blood samples (0.28 mL) were collected from the retro orbital plexus from all animals allocated for toxicokinetics under light isoflurane anesthesia according to the following schedule: day 1/2, day 28/29 (4-week study) or 90/91 (13-week study) at 0, 0.5. 1, 2, 6, and 24 h after administration (samples were only taken at 1 and 24 h from control animals). Three separate animals were used for each sampling time, with the same animals being used for the sampling at the start and end of the treatment phases. All surviving animals were euthanized after the last blood sample. Necropsy was only performed in case of unscheduled death during the study.

Blood samples were collected in lithium heparinized blood collection tubes and plasma prepared. Samples were kept on ice until centrifugation which was carried out within approximately 45 min of sampling. Following centrifugation plasma was transferred into plastic (polypropylene) tubes and stored frozen at -80 ± 10 °C in the dark. Plasma samples were shipped within 8 days after completion of each sampling event on dry ice for determination of BIA 10–2474 plasma concentrations by Swiss BioAnalytics AG (Birsfelden, Switzerland). Determination of BIA 10–2474 was made by LC-MS/MS according to a validated method. Toxicokinetic evaluations were performed and reported using WinNonLin Version 5.2.1 (Pharsight Corporation, Mountain View, California 94040/USA). Toxicokinetic and statistical analysis appropriate to the data was performed and include determination of maximum plasma concentration (C_{max}), time of maximum plasma concentration (t_{max}), and area under the plasma concentration time-curve (AUC_{0-t}) where t is the time point of the last quantifiable concentration.

Additionally, AUC0-t, C_{max} and, when possible, $t_{½}$,z were compared by calculating ratios in order to evaluate dose-proportionality, sex-related differences, and differences between single and repeated dosing (time dependency). Where relevant, AUC_{0-t} after repeated administration was compared to AUC_{0-inf} after single administration in order to evaluate steady-state conditions and accumulation after multiple dosing.

7. Clinical laboratory investigations

Hematology and clinical biochemistry analysis was performed in the 4- and 13-week studies, but not in the 14-day DRF study. Blood from 5 animals per treatment group were analysed at the end of the treatment phase for hematology (5 lowest identification numbers per group) and biochemistry parameters (the next 5 lowest identification numbers per group). In addition, the 3 lowest numbered mice in the recovery cohorts were analysed at the end of the treatment phase and the end of the recovery period for hematology and the next 2 lowest numbers analysed at the same time points for biochemistry parameters. In the 4-week study some groups had fewer animals than this due to unscheduled deaths or insufficient sample volume.

7.1. Hematology and clinical biochemistry

The hematology parameters measured were: erythrocyte count, hemoglobin, hemoglobin concentration distribution width, hematocrit, red cell volume distribution width, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet (thrombocyte) count, reticulocyte maturity index, reticulocyte count, total leukocyte count, differential leukocyte count (neutrophils, lymphocytes, monocytes, eosinophils, basophils, large unstained cells), prothrombin time, activated partial thromboplastin time. The biochemistry parameters measured were: glucose, urea, bilirubin, cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine kinase, gamma-glutamyltransferase, calcium, inorganic phosphorus, sodium, potassium, chloride, total protein, albumin, globulin.

7.2. Urinalysis

The parameters measured were: specific gravity (relative density), color, appearance, pH, protein, glucose, ketone, urobilinogen, bilirubin, erythrocytes, leukocytes, sediment (microscopic examination).

8. Anatomical pathology

At the end of the 14-day DRF study, all surviving animals were euthanized by anesthesia followed by exsanguination, and necropsied. None of the organs were analysed histopathologically, although the following organ weights were recorded: brain, heart, liver, thymus, kidneys, adrenals, spleen, testes, and ovaries.

At the end of the 4- and 13-week studies, all surviving animals were fasted for at least 18 h prior to euthanasia and necropsy. All animals were anesthetized by intraperitoneal injection of pentobarbitone and killed by exsanguination. Necropsy was performed as soon as possible after death and descriptions of all macroscopic abnormalities were recorded. Decedents were necropsied as soon as possible. The tissues and organs listed in Table S1 were collected from all animals from each study at necropsy and weighed before samples were fixed in neutral phosphate-buffered 4% formaldehyde solution (10% formalin), except for eyes with optic nerve and Harderian glands which were fixed in Davidson's solution and epididymides and testes which were fixed in Bouin's solution. Paired organs were weighed separately.

All organ and tissue samples from the 4- and 13-week studies collected at necropsy were trimmed, processed, embedded, cut at a nominal thickness of 4 μm and stained with hematoxylin and eosin (H&E). Histopathologic examination of slides from all tissues, except for the bone marrow smears, were performed. Histological examinations were performed on organs and tissues from all control and high dose animals, and on all target tissues and gross lesions from animals of intermediate groups. No histopathology evaluation was performed in the 14-day DRF study.

9. Data compilation and statistical analyses

9.1. Statistical analyses

For the preliminary 14-day, 4-week and 13-week oral toxicity studies, the following statistical methods were used to analyze body weight, body temperature, clinical laboratory data, ECGs and organ weights: if the variables could be assumed to follow a normal distribution, the Dunnett-test (Dunnett, 1955), many-to-one *t*-test, based on a pooled variance estimate was applied for the comparison of the treated groups with the control groups for each sex. The Steel-test (Miller, 1981), many-one rank test, was applied instead of the Dunnett-

Table 1
Summary of clinical and pathological signs seen in the 14-day toxicity study with BIA 10–2474. The values shown are the number affected/number evaluated. Arrows show direction of change for statistically significant differences compared to vehicle treated animals that occurred regularly during the study. Only significant tissue weight changes are shown. Note that at 1000/600 mg the clinical signs are those recorded prior to euthanasia on the 4th or 6th day. Three mice per sex were studied at each dose level.

Observation	100 mg/kg/day		300 mg/kg/day		1000/600 mg/kg/day	
	M	F	M	F	M	F
Clinical signs						
Found dead or required euthanasia					2/3	3/3
Weakened condition					2/3	3/3
Hypothermia						1/3
Ataxia						1/3
Shivering						1/3
Decreased activity/sedation						1/3
Pale appearance						1/3
Prostrate					2/3	2/3
Hunched posture					2/3	3/3
Visible weight loss					2/3	3/3
Ruffled fur					3/3	3/3
Laboured breathing/bradypnea					2/3	3/3
Ptosis					1/3	1/3
Food intake			↑		↓	↓
Food intake per body weight			↑		↑	↓
Body weight gain					↓	↓
Body weight						↓
Pathology						
Liver weight			+17.0%			
Testes weight					−28.3%	
Heart weight				+7.5%		

test when the data could not be assumed to follow a normal distribution.

10. Results

10.1. 14-Day dose range finding study

10.1.1. Mortality/viability

BIA 10–2474 was administered daily at dose levels of 100 or 300 mg/kg body weight for a period of 14 days. In the 1000/600 mg/kg/day dose group two of the females and one male were euthanized in extremis on day 4 of treatment after three applications with 1000 mg/kg/day. One male was euthanized in extremis on day 6 of treatment after reduction of the dose level and one female died spontaneously on day 13 of treatment (Table 1).

10.1.2. Clinical signs

One female treated with 300 mg/kg/day showed ruffled fur on days 3–6 of treatment. The surviving male treated with 1000/600 mg/kg/day showed ruffled fur from day 3 to the end of treatment and hunched posture on days 6–11. The other two males and three females treated with 1000/600 mg/kg/day showed ruffled fur, hunched posture and visible weight loss for different duration, depending on the time of survival. On the day before death or the day of euthanasia, further clinical signs such as weakness, prostration, labored breathing, clear lacrimation, tachypnea/bradypnea, decreased activity etc. were noted. These clinical signs were considered to be test item-related.

In animals treated with 1000/600 mg/kg/day, the absolute and relative food consumption from day 1–4 of treatment was reduced and was also reduced compared to control animals on days 4–11. In contrast, the relative food consumption of the surviving male from day 11–14 was higher than that of the control animals and the mean relative food consumption of males at 300 mg/kg/day was also generally slightly higher than that of the control animals, especially towards the end of treatment.

The mean body weight and body weight gain of mice treated with 100 or 300 mg/kg/day and control animals were comparable, but most animals treated with 1000/600 mg/kg/day lost body weight during treatment (up to 25% in 4 days).

11. Pathology

11.1. Macroscopic findings

No test item-related macroscopic findings were noted.

11.2. Organ weights

Increased mean absolute and relative liver weights in males at 300 mg/kg/day and in females at 100 or 300 mg/kg/day, as well as decreased absolute and relative thymus weights in females at 100 or 300 mg/kg/day were considered to be test item-related (Table 1).

12. 4-Week oral toxicity study

12.1. Viability/mortality

About a third of the animals treated with 500 mg/kg/day were found dead or required euthanasia (Table 2). Most of these deaths occurred towards the end of the treatment period and were considered to be related to treatment with the test item. There were three additional deaths that were not clearly treatment related: one male and one female treated with 100 mg/kg/day were found dead on day 2 and 9 respectively and one female treated with 300 mg/kg/day was euthanized in extremis on day 8 due to misgavage.

12.2. Clinical signs

Clinical signs such as ruffled fur, visible weight loss, hunched posture, weakened condition, stiff gait, decreased activity, prostration, labored breathing, tilted head and an enlarged abdomen were noted in several animals treated with 500 mg/kg/day starting at the end of the

Table 2
Summary of clinical and pathological signs seen in the 4-week-day toxicity study with BIA 10–2474. The values shown are the number affected/number evaluated. Arrows show direction of change for frequently occurring statistically significant differences compared to vehicle treated animals. 16 mice per sex were evaluated in the vehicle-treated group and 28 per sex in the other groups.

Observation	100 mg/kg/day		300 mg/kg/day		500 mg/kg/day	
	M	F	M	F	M	F
Clinical signs						
Found dead or required euthanasia	1/28	1/28		1/28[a]	8/28	11/28
Weakened condition				1/28	5/28	7/28
Stiff gait				1/28	3/28	4/28
Decreased activity				1/28	12/28	11/28
Hunched posture				1/28	9/28	7/28
Prostration					1/28	1/28
Shivering						1/28
Visible weight loss				1/28	2/28	
Ruffled fur				1/28	21/28	14/28
Labored breathing				1/28	4/28	5/28
Tilted head					1/28	
Enlarged abdomen					7/28	5/28
Food consumption			↑			
Body weight gain			↓		↓	↑
Body weight					↓	

[a] Not considered treatment related.

first week of treatment in the first animals. These clinical signs were not noted continuously but as they increased in both frequency and/or severity towards the end of the study they were considered to be test item-related (Table 2).

There were no test item-related differences in absolute or relative food consumption and no test item-related effect was noted on the body weight development of females. The mean body weight and body weight gain of males treated with 500 mg/kg/day were lower than in controls throughout treatment with the difference attaining statistical significance from day 8 onwards in body weight and on days 8 and 15 in body weight gain.

12.3. Clinical laboratory investigations

12.3.1. Hematology

There were several changes which were predominantly dose-dependent and for which there was a statistically significant difference between test item treated animals and controls after 4 weeks of treatment, usually at the highest dose tested. These included decreased erythrocyte count, decreased hemoglobin and decreased mean corpuscular hemoglobin concentration in both males and females at 500 mg/kg/day, as well as increased hemoglobin concentration distribution width and increased red cell volume distribution width in both males and females at 300 and 500 mg/kg/day. Increased absolute number of reticulocytes were also seen in males treated with 500 mg/kg/day.

Other findings, which were not clearly dose-dependent and which were only seen in males consisted of decreased white blood cell count and absolute number of lymphocytes in males treated with 100 and 500 mg/kg/day together with decreased absolute number of monocytes in test item-treated males at all dose levels. Although only seen in males, a relationship to the test item cannot be excluded for these additional findings as the effects were quite marked (Table 3).

Several blood biochemistry parameters increased dose-dependently and reached statistical significance at 500 mg/kg/day, predominantly in females (Table 3) and were considered treatment-related.

12.3.2. Urinalysis

Although not statistically significant compared to controls, several changes in urinary parameters were considered to be test item-related because they were consistently noted in both sexes. These included increased protein content and increased urinary erythrocytes in males and females treated with 500 mg/kg/day as well as increased leukocytes in males and females treated with 300 and 500 mg/kg/day.

12.4. Pathology

12.4.1. Macroscopic findings

Four males and one female treated with 500 mg/kg/day had macroscopic alterations of the liver, consisting of accentuated lobular pattern and isolated light red foci. Microscopically, these findings correlated with hepatocellular hypertrophy.

Two males treated with 500 mg/kg/day showed reduced size of testes which microscopically correlated with tubular degeneration. Reduced size of the seminal vesicles noted in two males, treated with 300 and 500 mg/kg/day, may be related to this finding even though no histological correlate was found.

Six females treated with 500 mg/kg/day had findings in the intestines, such as fluid or liquid contents in the cecum or/and jejunum and ileum, a thickened mucosa of duodenum and jejunum, discoloration of the jejunum or discoloration of jejunum and a gelatinous pancreas and cecum. In view of the histological alterations found (see below), these findings are considered to be test item-related.

12.4.2. Organ weights

After 4 weeks of treatment, there were several statistically significant differences in organ weights of test item-treated animals compared to controls. These are shown in Table 4.

12.4.3. Microscopic evaluation

BIA 10–2474 caused changes in the intestinal system, kidneys, reproductive organs, lymphoid organs and the nervous system, mainly of animals treated with 500 mg/kg/day (summarized in Table 5). Therefore, the liver, duodenum, jejunum, ileum, kidneys, testes, epididymides, ovaries, vagina, bone marrow, spleen, thymus, sciatic nerve and brain from the mid- and low-dose group animals were examined to establish a no-effect level.

Single fiber degeneration was found in the sciatic nerve of animals of both sexes treated with 500 mg/kg/day at elevated incidences and severity degrees (Fig. 1). In the brain of one male and one female treated with 500 mg/kg/day, bilateral neuronal degeneration of the hippocampus, partially with infiltration of inflammatory cells was found (Figs. 2 and 3).

In the liver, centrilobular to diffuse hepatocellular hypertrophy was recorded at elevated incidences at minimal to moderate severity in test item-treated animals at all dose levels. In some instances, hypertrophic hepatocytes showed a pale coarse granular cytoplasm. The finding was not paralleled by inflammatory or degenerative changes.

In the duodenum and jejunum, villus hypertrophy was found in one female at 500 mg/kg/day at a moderate severity. In addition, in the duodenum, jejunum and ileum of test item-treated animals at all dose levels and of both sexes, there was enterocyte vacuolation. There were no further morphological indicators of toxicity in the intestinal segments.

Nephropathy was recorded in both sexes treated with 300 or 500 mg/kg/day. This condition was characterized by hyaline tubular casts in the cortical-corticomedullary region, tubular necrosis/degeneration, tubular basophilia, tubular mineralization (in females at 500 mg/kg/day and in both sexes at 300 mg/kg/day) and increased severity of interstitial inflammatory infiltrate. Tubular casts in females at 300 mg/kg/day were mostly recorded in the papillary region.

Testicular tubular degeneration (including increased giant cell spermatids) was recorded in males treated with 500 mg/kg/day at elevated incidences and/or severity degrees. A single male with tubular degeneration treated with 300 mg/kg/day was considered to be of

Table 3
Significant changes in clinical chemistry parameters after 4-weeks treatment with BIA 10–2474. Values shown are percent change for statistically significant differences compared to vehicle treated animals. n = 5 per sex and per group except where uncheduled deaths occurred (†) or there was insufficient sample volume (*).

Observation	100 mg/kg/day		300 mg/kg/day		500 mg/kg/day	
	M	F	M	F	M	F
Hematology and urinalysis	n = 5	n = 4 †	n = 5	n = 4 †	n = 4 †	n = 2 †,*
Erythrocyte count					−12%	−14%
Erythrocyte volume distribution width			+24%	+21%	+52%	+30%
Mean corpuscular hemoglobin concentration					−7%	−10%
Hemoglobin					−15%	−14%
Hemoglobin concentration distribution width			+15%	+19%	+33%	+42%
White blood cell count	−45%				−57%	
Lymphocytes	−54%				−60%	
Monocytes	−67%		−50%		−67%	
Reticulocytes					+78%	
Biochemistry	n = 5	n = 5	n = 5	n = 5	n = 5	n = 4 *
Urea					+58%	
Alanine aminotransferase						+106%
Sodium						+4%
Potassium				+25%		+35%
Chloride						+6%
Protein						+15%
Glucose			+90%			
Globulin						+38%

Table 4
Summary of pathological signs seen in the 4-week toxicity study with BIA 10–2474. Ten mice per sex and per treatment (including vehicle treatment) were necropsied.

Observation	100 mg/kg/day		300 mg/kg/day		500 mg/kg/day	
	M	F	M	F	M	F
Pathology						
Lung weight					+20%	
Liver weight			+21%	+24%		+21%
Testes weight					−50%	
Epididymides weight					−19%	
Prostate weight					−32%	
Spleen weight			+24%			
Pancreas weight				+37%		
Uterus/body weight ratio						−34%
Liver/body weight ratio	+9%		+16%	+16%	+22%	+20%
Brain/body weight ratio				−10%		
Heart/body weight ratio				−12%		−10%
Kidneys/body weight ratio				−11%		

Values shown are percent change for statistically significant differences compared to vehicle treated animals.

spontaneous nature. It is however noted that this finding was reflected by epididymal oligozoospermia. In females, maturation arrest in the ovaries was observed after treatment with 500 mg/kg/day. This finding was characterized by an absence of antral follicles and corpora lutea. The finding in the ovaries was associated by signs of anestrus in the vaginal mucosa that was found in two females at 500 mg/kg/day.

In the bone marrow, granulopoiesis was recorded at an increased incidence in three females treated with 500 mg/kg/day. Thymic atrophy was found at slight to marked severity degrees in animals treated with 500 mg/kg/day. In the spleen, extramedullary hemopoiesis increased in mean severity in animals at 500 mg/kg/day (more pronounced in females).

12.5. Toxicokinetic evaluation

All animals in the treated dose groups were consistently exposed to BIA 10–2474 and maximum plasma concentrations were reached rapidly, between 0.5 and 2 h. The terminal half-life ranged between 1.3 and 5.0 h.

On day 1, BIA 10–2474 exposure tended to be less than dose-proportionally increased at incrementing doses: between the low and high doses, separated by a 5-fold dose increment, the $AUC_{0\text{-}t}$ ratios were 1.3 for both males and females. On day 28, BIA 10–2474 exposure tended to be dose-proportionally increased: the $AUC_{0\text{-}t}$ ratios were 5.0 and 6.4, for males and females, respectively. No sex difference was identified. The $AUC_{0\text{-}t}$ female/male ratios ranged from 0.91 to 1.3.

After 28-day repeated administration, BIA 10–2474 exposure tended to slightly increase at low and mid doses: the AUC0-t day 28/1 ratios ranged between 1.7 and 2.1. However, at the high dose, the exposure increase was notable: the $AUC_{0\text{-}t}$ day 28/1 ratios were 6.7 for males and 8.8 for females. Under a chronic dosing regimen at 500 mg/kg/day, accumulation of BIA 10–2474 has to be anticipated (Table 6).

13. 13-Week oral toxicity study followed by a 2-week recovery period

13.1. Viability/mortality

All animals survived their scheduled study periods.

13.2. Clinical signs

No test item-related clinical signs were noted.

In males, no test item-related effects on food consumption were noted. The mean body weight and body weight gain of test item-treated males at all dose levels were slightly higher than that of the control males from week 2 of treatment (week 3 at 150 mg/kg/day) onwards. The difference frequently attained statistical significance in mean body weight at all dose levels and in body weight gain in males treated with 25 mg/kg/day. At the end of treatment, males treated with 25, 75 and 150 mg/kg/day weighed 9.6%, 11.6% and 11.4% more than control males respectively. In test item-treated females, no statistically significant differences to controls in body weight were noted. However, the mean body weight gain was higher than in controls from week 3 of

Table 5
Incidence and mean severity of lesions in nervous system, liver, small intestine, kidneys, testes and epididymides, ovaries and vagina, lymphatic organs of mice at the end of the 4-weeks treatment with BIA 10–2474. Ten mice per sex and per group were evaluated.

Treatment	Vehicle		100 mg/kg/day		300 mg/kg/day		500 mg/kg/day	
Sex	M	F	M	F	M	F	M	F
Sciatic Nerve: Single fiber degeneration	3/1.0	0	0	1/1.0	1/1.0	2/1.5	9/2.0	7/2.3
Hippocampus: Neuronal degeneration	0	0	0	0	0	0	1/2.0	1/1.0
Hepatocellular hypertrophy	1/1.0	0	7/1.4	5/1.2	8/2.3	9/1.7	8/1.8	8/1.9
Duodenum: Villus hypertrophy	0	0	0	0	0	0	0	1/3.0
Duodenum: Enterocyte vacuolation	0	0	0	0	3/1.7	4/1.8	2/3.0	5/1.8
Jejunum: Villus hypertrophy	0	0	0	0	0	0	0	1/3.0
Jejunum: Enterocyte vacuolation	0	0	0	1/1.0	3/2.0	4/1.5	3/2.3	4/2.5
Ileum: Enterocyte vacuolation	0	0	1/1.0	1/1.0	4/1.3	2/1.0	0	3/2.3
Tubular mineralization	1/1.0	2/1.0	3/1.0	0	6/1.0	7/1.0	1/1.0	5/1.6
Tubular casts	1/1.0	1/1.0	0	1/1.0	3/1.0	8/1.3	9/2.7	8/3.0
Tubular basophilia	7/1.0	4/1.0	6/1.0	5/1.0	7/1.6	9/1.9	10/2.9	10/2.9
Tubular necrosis/degeneration	0	0	0	0	0	0	6/1.3	5/1.8
Interstitial inflammatory infiltrate	3/1.0	1/1.0	3/1.0	3/1.0	4/1.3	6/1.5	8/1.3	6/1.7
Tubular degeneration	2/1.0		0		1/4.0		8/3.3	
Oligozoospermia	0		0		0		5/2.4	
Maturation arrest		0		0		0		0
Anestrus		0		0		0		0
Bone marrow: Increased granulopoiesis	0	0	0	0	0	0	0	3/2.7
Spleen: Extramedullary hemopoiesis	8/1.3	9/1.6	9/2.0	10/1.4	10/2.5	10/2.6	7/2.3	9/2.4
Thymus: Atrophy	0	2/2.0	0	0	0	1/2.0	4/2.5	7/2.6

The values are the number of affected animals/mean severity (calculated by sum of severity in affected animals divided by number affected). Severity degrees are out of a scale of 0–5.

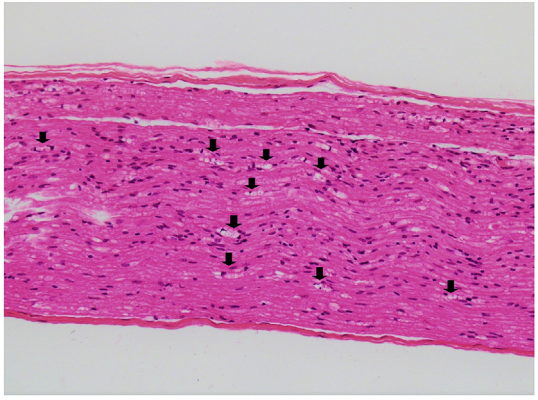

Fig. 1. Female mouse (4-week, end of treatment, 500 mg/kg/day). Sciatic nerve with single nerve fiber degeneration characterized by multifocal vacuolation ('digestion chambers'). H&E, lens x20.

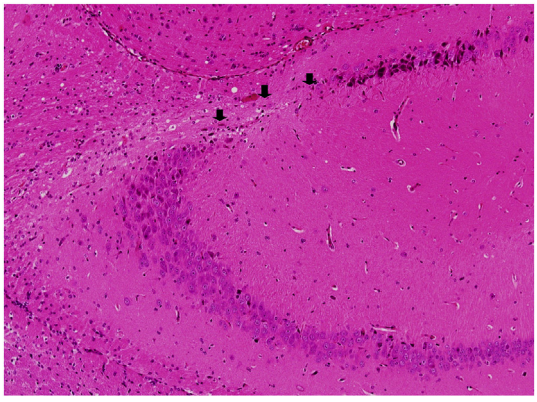

Fig. 2. Female mouse (4-week, end of treatment, 500 mg/kg/day). Hippocampus degeneration. CA1 and Ca3 is affected. H&E, lens x10.

treatment onwards at all dose levels. The difference attained statistical significance in weeks 4 and 5 of treatment in females treated with 150 mg/kg/day only. Generally, the observed differences were slight even if attaining statistical significance and did not show a clear dose-relationship. However, an effect of the test item towards higher body weights and/or body weight gain could not be excluded (Table 7).

13.3. Clinical laboratory investigations

13.3.1. Hematology

The statistically significant differences in hematology parameters between test item-treated animals and controls after 13 weeks of treatment are shown in Table 8). No test item-related findings were present after the recovery period.

13.3.2. Clinical biochemistry

After 13 weeks of treatment, increased cholesterol concentration in males treated with 75 mg/kg/day and in both sexes treated with 150 mg/kg/day was the only finding which was considered to be test item-related, although the effect was not dose-related in males (Table 8). After the recovery period, cholesterol levels were still increased in both sexes treated with 150 mg/kg/day. However, the difference to control animals was smaller than at the end of treatment indicating reversibility.

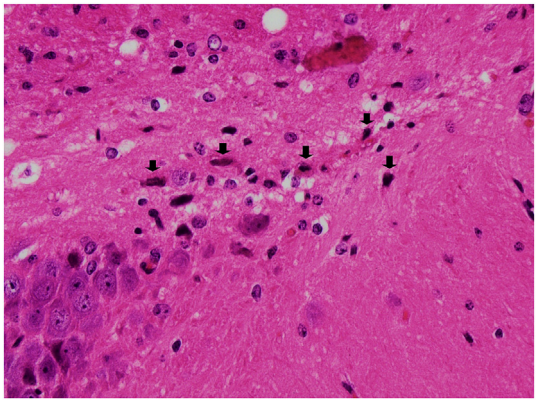

Fig. 3. Female mouse (4-week, end of treatment, 500 mg/kg/day). Hippocampus degeneration. Loss of neurons, neuronal necrosis (dark red staining neurons) and glial cell infiltration (dark small round nuclei). Vacuoles are likely representing swollen astrocyte feet. H&E, lens x40.

13.3.3. Urinalysis

No statistically significant differences between test item-treated animals and control animals in urinalysis parameters were observed.

13.4. Pathology

13.4.1. Macroscopic findings

All macroscopic findings noted at the end of treatment or the recovery period were within the range of background lesions observed in animals of this strain and age and were considered to be incidental.

13.4.2. Organ weights

After 13 weeks of treatment, several differences between test item-treated animals and control animals in organ weights were considered to be test item-related because they were dose-related and/or present in the high-dose group (Table 9).

After the recovery period, only the mean liver to body weight ratio of females treated with 150 mg/kg/day was still significantly increased (+8.6% compared to controls). However, the difference was smaller than at the end of treatment (+16%) indicating reversibility.

13.4.3. Microscopic evaluation

BIA 10–2474 caused changes in the sciatic nerve, skeletal muscle, kidneys, urinary bladder, and spleen mainly of animals treated with 150 mg/kg/day. Therefore, these organs were also examined from the mid- and low-dose group animals to establish a no-effect level (Table 10). Of note is the single fiber degeneration seen in the sciatic nerve, which increased in incidence and mean severity in animals treated with 150 mg/kg/day. The finding persisted after the recovery period.

Apart from this, most other findings at the end of the 13-week treatment period were either absent after recovery or present at a lower level, indicating reversibility. These included an increased incidence and severity of myofiber degeneration/regeneration in the skeletal muscle of animals treated with 150 mg/kg/day which was characterized by degeneration of individual myofibers, various degrees of inflammatory cell foci, infiltrates and various degrees of regeneration and myoblast proliferation. In the liver, centrilobular to diffuse hepatocellular hypertrophy was recorded at minimal to moderate severity in animals treated with 75 or 150 mg/kg/day. There was no evidence for an additional increased cellular turnover, inflammatory changes or degeneration. In kidneys, nephropathy was recorded in animals of both sexes treated with 150 mg/kg/day, consisting of tubular necrosis/degeneration and/or tubular basophilia associated with tubular casts. The mean severity of renal tubular basophilia increased clearly in animals at 150 mg/kg/day and was associated by tubular necrosis/degeneration in individual males and females of this group. A pronounced increased incidence and severity of tubular casts were recorded in animals at 150 mg/kg/day. In the urinary bladder, the mean incidence and/or severity of submucosal inflammatory cell foci increased in test item-treated males and females at all dose levels. Often, these findings were in perivascular regions. After the treatment-free recovery period, the finding partially regressed in females but not males.

13.5. Toxicokinetics

All animals in the treated dose groups were consistently exposed to BIA 10–2474. Maximum plasma concentrations were reached rapidly between 0.50 and 1.0 h. The toxicokinetic profiles displayed parallel terminal phases with terminal half-life ranging from 0.90 to 1.4 h. BIA 10–2474 exposure tended to be dose-proportionally increased at incrementing doses: between the low and high doses, separated by a 6.0-fold dose increment, the AUC_{o-t} ratios ranged from 6.0 to 8.6. No sex difference was identified as the AUC_{o-t} female/male ratios ranged from 0.73 to 1.5.

After 13 weeks of repeated administration, BIA 10–2474 exposure tended to be stable or to slightly increase: the $AUC0_{-t}$ day 90/day 1 ratios ranged between I.I and 1.3, with an exception for males of the mid dose group with 2.1. Therefore, no notable accumulation of BIA 10–2474 was observed (Table 11).

14. Discussion

The studies reported here were carried out as part of the regulatory package to obtain approval for clinical trials with BIA 10–2474. The mouse was used for these studies in order to provide not only an additional species for the toxicology package but more importantly to provide information allowing the correct choice of doses for the long-

Table 6
Toxicokinetics data obtained in the 4-week study. Blood samples were taken from 3 separate mice at 0, 0.5, 1, 2, 6 and 24 h after drug treatment for the analysis of plasma concentrations of BIA 10–2474 and calculation of toxicokinetic parameters.

Males		Day 1			Day 28		
		100 mg/kg/day	300 mg/kg/day	500 mg/kg/day	100 mg/kg/day	300 mg/kg/day	500 mg/kg/day
C_{max}	ng/ml (mean)	31000	51700	25500	46200	92100	11700
t_{max}	h	1.0	1.0	0.5	1.0	1.0	1.0
AUC_{0-t}	ng.h/ml (mean)	92100	203000	120000	160000	420000	805000
$t_{½}$	h (mean)	1.3	2.6	2.6	1.7	2.2	3.7
Females							
C_{max}	ng/ml (mean)	30000	65800	41100	53200	98500	93400
t_{max}	h	1.0	1.0	1.0	1.0	1.0	2.0
AUC_{0-t}	ng.h/ml (mean)	86200	257000	109000	149000	475000	960000
$t_{½}$	h (mean)	1.4	3.1	1.3	1.7	3.7	5.0

Table 7
Summary of clinical signs seen during the 13-week-day toxicity study with BIA 10–2474.

Observation	25 mg/kg/day		75 mg/kg/day		150 mg/kg/day	
	M	F	M	F	M	F
Clinical signs						
Decreased activity	1/28					
Abnormal gait						1/33
Hunched posture	1/28					
Ruffled fur	1/28					1/33
Visible weight loss	1/28					
Food intake	↓	↑	↓	↑		↑
Food intake per body weight	↓	↑	↓	↑	↓	↑
Body weight gain	↑				↑	↑
Body weight	↑		↑		↑	

The values shown are the number affected/number evaluated. Groups of 21, 28, 28 and 33 mice per sex were evaluated for the vehicle, 25, 75 and 150 mg/kg groups respectively. Arrows show direction of change for statistically significant differences compared to vehicle treated animals and indicate changes that were seen frequently throughout the 13-week study.
Arrows indicate significant changes relative to controls (Dunnett or Steel test as appropriate).

Table 9
Significant changes in pathology parameters after 13-weeks treatment with BIA 10–2474. 15, 10, 10 and 15 mice per sex were evaluated for vehicle, 25, 75 and 150 mg/kg groups.

Observation	25 mg/kg/day		75 mg/kg/day		150 mg/kg/day	
	M	F	M	F	M	F
Pathology						
Lung weight					+10%	
Liver weight			+15%		+24%	+21%
Spleen weight				+25%	+28%	+30%
Brain/body weight ratio	−8%		−9%		−7%	
Liver/body weight ratio					+12%	+16%
Thymus/body weight ratio						−26%
Spleen/body weight ratio				+22%		+26%

Only the statistically significant differences compared to vehicle treated animals are shown.

term carcinogenicity testing. This manuscript is part of a series describing the toxicology studies in different species (Hardisty et al., 2019; Harris et al., 2019; Hayes et al., 2019a, 2019b; Weber et al., 2019).

In the initial DRF study, several animals treated with 1000 mg/kg/day were found dead and even after reduction to 600 mg/kg/day, no animals survived the scheduled treatment period. Prior to death, these mice were in a clearly weakened condition and showed clinical signs which included ruffled fur, prostration and hunched posture, labored breathing and visible weight loss. These signs were seen in most of the animals. Food intake was reduced and body weight gain was reduced in both sexes. At lower doses, 100 and 300 mg/kg/day, none of these clinical signs were seen. Indeed, in contrast to the higher dose, food intake and body weight gain were higher than controls in males treated with 300 mg/kg.

Based on the results of the 14-day DRF study, dose levels of 100, 300 and 500 mg/kg/day were used for the 28-day study. At the highest dose about a third of the animals died before the end of the study and the clinical signs seen at that dose were consistent with the signs seen during the DRF study: ruffled fur, weakened condition, decreased activity, hunched posture and labored breathing. However, most of these signs were seen in fewer than half the animals, although their occurrence and severity tended to increase with treatment duration. At 100 and 300 mg/kg/day there were essentially no clinical signs, although one male and one female receiving the lowest dose were found dead early on. Although deemed unlikely, a role for BIA 10–2474 could not be excluded.

Consistent hematology findings were observed on erythrocyte parameters, with decreases in erythrocyte count and hemoglobin concentration at the highest dose and increases in the distribution parameters for both erythrocyte volume and hemoglobin concentration at both 300 and 500 mg/kg/day. Less consistent effects on white blood cell parameters were seen across all doses but only in males.

The increase in liver weight seen in the DRF study was also seen in the 4-week study, primarily at 300 and 500 mg/kg/day but also in males at 100 mg/kg/day. This was coupled with a hepatocellular hypertrophy in the majority of animals at all doses. In some instances, hypertrophic hepatocytes showed a pale, coarse granular cytoplasm. As this finding was not associated with inflammatory or degenerative changes it was deemed to represent an adaptive change. Although no

Table 8
Significant changes in hematology (absolute values) and clinical chemistry parameters after 13-weeks treatment with BIA 10–2474 compared to vehicle treated animals. Measurements were made using samples from 8, 5, 5 and 8 mice per sex for the hematology measures and 7, 5, 5 and 7 for the biochemisty measures (vehicle, 25, 75 and 150 mg/kg groups respectively).

Observation	25 mg/kg/day		75 mg/kg/day		150 mg/kg/day	
	M	F	M	F	M	F
Hematology						
Erythrocyte volume distribution width					+12.6%	+13.8%
Hemoglobin						−6.7%
Hemoglobin concentration distribution width			+9.4%		+18.0%	+16.9%
Hematocrit						−5.9%
Reticulocyte count			+26.9%		+45.8%	
Platelets			−17.5%	−23.1%	−16.1%	−19.0%
White blood cells		−56.0%				
Eosinophils		−50%				
Basophils		−100%		−50%		−50%
Lymphocytes		−62.5%				
Monocytes		−61.5%				−53.8%
Large unstained cells		−75.0%				−50%
Biochemistry						
Urea						−24.2%
Cholesterol			+43.1%		+30.4%	+64.3%
Aspartate aminotransferase						−36.2%
Globulin						+13.8%

Table 10
Incidence and Mean Severity of Lesions in Sciatic Nerve, Skeletal Muscle, Liver, Kidneys and Urinary Bladder, Spleen in animals treated for 13 weeks with BIA 10–2474.

Finding/Groups	Vehicle		25 mg/kg/day		75 mg/kg/day		150 mg/kg/day	
Main Test								
Sex (n = 10 per sex/group)	M	F	M	F	M	F	M	F
Sciatic Nerve: Single fiber degeneration	2/1.0	2/1.0	1/1.0	1/1.0	0	3/1.0	5/1.2	4/1.0
Myofiber degeneration/regeneration	3/1.0	3/1.0	1/1.0	2/1.0	5/1.0	4/1.0	9/1.3	9/1.1
Hepatocellular hypertrophy	0	0	0	0	8/1.4	4/1.5	7/1.4	5/1.0
Kidneys: Tubular basophilia	8/1.3	4/1.0	8/1.1	9/1.1	10/1.2	6/1.2	10/2.2	9/1.9
Kidneys: Tubular necrosis/degeneration	0	0	0	0	0	0	3/1.3	1/2.0
Kidneys: Tubular casts	1/1.0	3/1.0	4/1.0	3/1.3	4/1.0	5/1.0	6/2.0	7/1.3
Urinary bladder: Inflammatory foci	0	1/1.0	5/1.0	5/1.4	6/1.0	6/1.0	4/1.0	7/1.0
Spleen: Extramedullary hemopoiesis	10/2.0	10/2.5	10/1.8	0	10/1.8	0	10/2.5	10/2.4
Recovery								
Sex (n = 5 per sex/group)	M	F	M	F	M	F	M	F
Sciatic Nerve: Single fiber degeneration	1/1.0	1/1.0	–	–	–	–	5/1.2	3/1.3
Myofiber degeneration/regeneration	0	3/1.0	–	–	–	–	3/1.0	3/1.0
Hepatocellular hypertrophy	0	0	–	–	–	–	0	0
Kidneys: Tubular basophilia	5/1.0	5/1.2	–	–	–	–	5/1.8	4/1.0
Kidneys: Tubular necrosis/degeneration	0	0	–	–	–	–	0	0
Kidneys: Tubular casts	2/1.0	2/2.0	–	–	–	–	4/1.3	3/1.0
Urinary bladder: Inflammatory foci	1/1.0	1/1.0	–	–	–	–	4/1.0	2/1.0
Spleen: Extramedullary hemopoiesis	1/1.0	4/1.0	–	–	–	–	4/1.0	4/1.5

The values are the number of affected animals/mean severity (calculated by sum of severity in affected animals divided by number affected). Severity degrees are out of a scale of 0–5.

changes in thymus size were seen in the 4-week study, there was thymus atrophy at the highest dose and dose-related histopathological changes were seen in the nervous system, gastro-intestinal system and kidney. Both male and female sex organs were negatively affected in about half the animals at the highest dose. Based on the results of this study, especially in view of the microscopic findings, 100 mg/kg/day was considered the NOAEL for BIA 10–2474 after 4-weeks administration.

At the lower doses used for the 13-week study, there were no deaths and an almost complete absence of clinical signs. The significant erythrocyte and hemoglobin changes seen in the 4-week study were also seen in this longer study but at lower doses. Although this might suggest that these effects are occurring at lower doses with longer treatment durations, it should be noted that the 13-week study is much higher powered in this regard (n = 15 compared to 5) and the dose-responses for the actual changes are broadly consistent between the two studies. The other hematology changes seen in males in the 4-week study were absent in males in the 13-week study but were seen in females in the 13-week study despite being absent in females during the 4-week study. Despite this inconsistency, the repeat findings suggest that they were test-item related.

As in the 4-week study, hepatocellular hypertrophy was seen in the 13-week study at comparable doses whereas the tubular basophilia in the kidney seen at 300 mg/kg/day in the 4-week study was seen at 150 mg/kg/day in the longer study. Although the incidence of tubular casts and inflammatory foci in the kidney were slightly increased at

Table 11
Toxicokinetics data obtained in the 13-week study. Blood samples were taken from 3 separate mice at 0, 0.5, 1, 2, 6 and 24 h after drug treatment for the analysis of plasma concentrations of BIA 10–2474 and calculation of toxicokinetic parameters.

Males		Day 1			Day 90		
		25 mg/kg/day	75 mg/kg/day	150 mg/kg/day	25 mg/kg/day	75 mg/kg/day	150 mg/kg/day
C_{max}	ng/ml (mean)	10600	27200	58500	11500	36400	72300
t_{max}	h	1.0	1.0	1.0	0.5	1.0	1.0
AUC_{0-t}	ng.h/ml (mean)	26000	43700	156000	30700	90000	202000
$t_{½}$	h (mean)	1.4	1.6[a]	1.4	1.3	1.3	1.3
Females							
C_{max}	ng/ml (mean)	10300	22600	60600	12100	34800	70000
t_{max}	h	0.5	0.5	1.0	1.0	1.0	1.0
AUC_{0-t}	ng.h/ml (mean)	18900	65100	163000	24900	83800	178000
$t_{½}$	h (mean)	1.3	1.3	1.3	0.9	1.1	1.1

[a] Value considered unreliable as terminal phase insufficiently defined.

both 25 and 75 mg/kg/day, the severity scores were similar to those seen in control animals and so these changes were considered non-adverse. It should also be noted that whereas only 1 or 2 animals (out of 10) showed evidence of sciatic nerve degeneration at 300 mg/kg/day in the 4-week study, nearly half the animals (5 males and 4 females) showed a similar severity of sciatic nerve damage at the lower dose of 150 mg/kg/day in the 13-week study. This suggests that, unlike most of the other measured parameters, the sciatic nerve degeneration is aggravated by longer treatment duration.

Following the 4-week recovery period the changes seen in hematology and blood biochemistry at the end of the treatment period were either absent or reduced in incidence and/or severity, suggesting reversibility. The same was largely true for the changes seen in the liver which, as in the 4-week study and in the absence of inflammatory or degenerative changes were deemed to represent an adaptive change. Although the kidney changes were less marked in the recovery animals compared to end-of-treatment animals, hyaline casts were still present. In contrast, the sciatic nerve degeneration seen at the end of the 13-week treatment period was still present at the end of the recovery period suggesting that this damage was not reversible.

The pharmacokinetic parameters measured in the 4- and 13-week studies suggest some variability at the start of the treatment period but that after repeated treatment the exposure was broadly dose-proportional and similar between the sexes. Although at most doses there was no clear evidence of accumulation (i.e. comparison of day 1 and day 28 or 91 parameters) this was not the case for the 500 mg/kg/day dose used in the 4-week study.

The toxicity seen in the clinic was either neuronal or neurovascular, based on the findings presented by Kerbrat et al. (2016). The only comparable findings in the mouse toxicity studies is the sciatic nerve damage and hippocampus degeneration seen at 500 mg/kg/day in the 4-week study. Although the incidence and severity of the sciatic nerve changes were lower in the 13-week study, they were still present four weeks after stopping drug treatment. However, the Cmax at that dose (11.7–93.4 μg/ml, see Table 6), is much higher than the maximum values seen in humans where a C_{max} of 0.67 and 1.8 μg/ml was measured after a single dose of 50 and 100 mg respectively (Rocha et al., 2016).

These studies clearly demonstrate that adverse effects in the CD-1 mouse were dose-dependent and, based on the data from the 13-week study, the NOAEL could be established at 75 mg/kg/day, primarily due to the nephropathy and nerve degeneration observed at 150 mg/kg. There were some observations at 75 mg/kg, but they were considered non-adverse. This is largely consistent with the value of 100 mg/kg/day obtained in the 4-week study. Although this suggests that there is no marked increase in toxicity as treatment duration increases, some caution should be exercised in view of the increase sciatic nerve damage even though this occurred at doses above the NOAEL. In this context it is useful to examine the long-term development and reproductive toxicity testing data which also demonstrated the absence of concerning findings in the early evaluation of BIA 10–2474 (Harris et al., 2019). In summary, these mouse studies do not demonstrate any effects which could have predicted, or are related to, the death and neurological changes that occurred in humans.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. The study was funded by Bial Portela & Companhia S.A. (São Mamede do Coronado, Portugal). Stephen B. Harris, Jerold F. Hardisty, A. Wallace Hayes, and Klaus Weber were paid consultants of Bial. Furthermore, one of the authors (KW) was the study pathologist on all of the studies. The manuscript was written by the authors and reviewed by Bial; however, the work product and conclusions are those of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2019.104557.

References

CSST, 2016. Report by the temporary specialist scientific committee (TSSC): FAAH (fatty acid amide hydrolase),” on the causes of the accident during a phase 1 clinical trial in rennes in january 2016. In: Santé, A.N.d.S.d.M.e. d.P.d. (Ed.), Saint-Denis, France.

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. 50, 1096–1121.

Gama, H., et al., 2016. Preliminary safety evaluation in study BIA-10-2474-101. In: Proceedings of the British Pharmacological Society, at. http://www.pa2online.org/abstract/abstract.jsp?abid=33277&kw=2474&author=Rocha&cat=-1&period=-1. 16, 188P.

Hardisty, J.F., et al., 2019. Oral repeated-dose toxicity studies of BIA 10-2474 in beagle dogs. Regul. Toxicol. Pharmacol (in press).

Harris, S.B., et al., 2019. Developmental and Reproductive Toxicity Studies of BIA 10-2474. Regulatory Toxicology & Pharmacology. (in press).

Hayes, A.W., et al., 2019a. Oral repeated-dose toxicity studies of BIA 10-2474 in Wistar rat. Regul. Toxicol. Pharmacol (in press).

Hayes, A.W., et al., 2019b. The absence of genotoxicity of a novel fatty acid amide hydrolase inhibitor, BIA 10-2474. Regul. Toxicol. Pharmacol (in press).

Kerbrat, A., et al., 2016. Acute neurologic disorder from an inhibitor of fatty acid amide hydrolase. N. Engl. J. Med. 375, 1717–1725.

Kiss, L.E., et al., 2018. Discovery of a potent, long-acting, and CNS-active inhibitor (BIA 10-2474) of fatty acid amide hydrolase. ChemMedChem 13, 2177–2188.

Miller, R.G., 1981. Simultaneous Statistical Inferences. Springer Verlag, New York.

Panlilio, L.V., et al., 2015. Cannabinoid abuse and addiction: clinical and preclinical findings. Clin. Pharmacol. Ther. 97, 616–627.

Petrosino, S., Di Marzo, V., 2010. FAAH and MAGL inhibitors: therapeutic opportunities from regulating endocannabinoid levels. Curr. Opin. Investig. Drugs 11, 51–62.

Rocha, F., et al., 2016. Tolerability, pharmacokinetic and pharmacodynamic profile of BIA 10-2474 in healthy volunteers following multiple ascending doses. In: Proceedings of the British Pharmacological Society, at. http://www.pa2online.org/abstract/abstract.jsp?abid=33267&kw=2474&author=Rocha&cat=-1&period=-1.16,178P.

Ryberg, E., et al., 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. Br. J. Pharmacol. 152, 1092–1101.

Toczek, M., Malinowska, B., 2018. Enhanced endocannabinoid tone as a potential target of pharmacotherapy. Life Sci. 204, 20–45.

Weber, K., et al., 2019. Oral repeated-dose toxicity studies of BIA 10-2474 in Cynomolgus monkeys. Regul. Toxicol. Pharmacol (in press).