BIOMARKER FOR MITOCHONDRIAL TOXICITY ASSOCIATED WITH PHOSPHOLIPIDOSIS

For metabolic disorders such as drug induced phospholipidosis or phenylketonuria means for specific diagnosis are known.

However, the potential risk of these disorders for associated toxicities such as mitochondrial toxicity in humans is difficult to predict and the significance is unknown.

Mitochondria play a critical role in generating most of the cell's energy as ATP. They are also involved in other metabolic processes such as urea generation, haem synthesis and fatty acid beta oxidation.

Disruption of mitochondrial functions by drugs can result in cell death by apoptosis (K Chan et all, *Drug-induced mitochondrial toxicity*. Expert Opin. Drug Metab. Toxicol (2005) 1: (4):655-669).

Thus, there is a strong interest in a method for predicting mitochondrial toxicity associated with a metabolic disorder.

Therefore, the present invention provides a method for determining a risk of a phospholipidotic compound for inducing mitochondrial toxicity which is associated with a metabolic disorder, comprising

a) measuring the level of PAG in a body fluid sample,

b) comparing the level of determined PAG with the level of PAG of a control

wherein an increased level of PAG in comparison with said control is indicative that said compound induces mitochondrial toxicity which is associated with a metabolic disorder.

Preferably, the metabolic disorders is a drug-induced phospholipidosis or a metabolic disorder caused by inborn errors such as e.g. inborn error of ureagenesis, or an inherited metabolic disorders such as e.g. phenylketonuria.

A preferred embodiment of the invention is therefore a method for determining a risk of a phospholipidotic compound for inducing mitochondrial toxicity which is associated with drug-induced phospholipidosis, comprising

a) measuring the level of PAG in a body fluid sample,
b) comparing the level of determined PAG with the level of PAG of a control
wherein an increased level of PAG in comparison with said control is indicative that said compound induces mitochondrial toxicity which is associated with drug-induced phospholipidosis.

Drug-induced phospholipidosis is a storage disorder characterized by accumulation of phospholipids within cells, i.e., in the lysosomes. Compounds inducing phospholipidosis are cationic, generally amphiphilic molecules which interfere with the phospholipid metabolism and turnover. Few drugs have been reported to cause phospholipidosis in humans.

The onset and the severity of phospholipidosis depend on cumulative exposure and administration regimen (continuous versus intermittent).

The presence of foamy macrophages at light microscopic level is indicative of phospholipidosis. However, the final diagnosis of phospholipidosis is based on ultrastructural changes (membranous lamellar inclusions bodies) in the lysosomes of various cell types, especially in lymphocytes, macrophages, and parenchymal cells.

Phospholipidosis is a term for several of the lysosomal storage diseases in which there is an abnormal accumulation of lipids in the reticuloendothelial cells. The term "drug-induced phospholipidosis" means a phospholipidosis attributed to the presence of a drug in the body. Such a drug is called a phospholipidotic compound. The term "phospholipidotic compound" refers to a compound that is able to induce phospholipidosis (see for example Reasor and Kacew, "Drug-induced Phospholipidosis: Are there functional consequences?" Exp Biol Med, 2001, 226: 825-30).

A control may be an animal not treated with a compound or an animal treated with another compound whereby this other compound is not toxic for mitochondria, or the treated animal before treatment with a phospholipidotic compound (pre-dose values within the same individual).

The term "PAG" as used herein refers herein to phenylacetylglycine in rodents and to any molecule equivalent to phenylacetylglycine in species other than rodents such as for example phenylacetylglutamine in human. The term "PAG" also includes salts of phenylacetylglycine and of molecule equivalents of phenylacetylglycine.

This method may be used for testing of the toxicity of therapeutic compounds. Therapeutic compounds are compounds which may be used for treatment or prevention of diseases and disorders. Preferably, such a test may be done with a rat or a mouse or human body fluid samples. The test may be done with body fluid samples of any animal if said animal has a phenylacetylglycine equivalent.

Preferably, the body fluid sample is blood or urine. More preferably, the body fluid sample is urine. The methods for obtaining samples of body fluids are known to the skilled in the art.

The person skilled in the art is familiar with different methods of measuring the level of PAG, in particular of phenylacetylglycine and phenylacetyglutamine. The term "level" relates to amount or concentration of PAG in an individual or a sample taken from an individual.

In the context of the present invention, amount also relates to concentration. It is evident, that from the total amount of a substance of interest in a sample of known size, the concentration of the substance can be calculated, and vice versa.

The term "measuring" according to the present invention relates to determining the amount or concentration, preferably semi-quantitatively or quantitatively. Measuring can be done directly.

Methods for detecting PAG are well known to skilled in the art. Preferred methods comprise NMR (i.e. single pulse NMR as described in Keun, H.C et al., (2002) Physiological variation and analytical reproducibility in metabonomic urinalysis (Chem. Res. Tox. 15, 1380-1386), Mass Spectrometry (MS), MS combined with chromatographic techniques, liquid chromatography-ultraviolet detection (LC-UV), Liquid chromatography with photodiode array detection (LC-DAD), Gas Chromatography (GC).

The present invention also provides a use of PAG as marker for mitochondrial toxicity. Preferred is the use of PAG as marker for mitochondrial toxicity associated with a metabolic disorder.

Preferably, the metabolic disorders is a drug-induced phospholipidosis or a metabolic disorder caused by inborn errors such as e.g. inborn error of ureagenesis, or an inherited metabolic disorders such as e.g. phenylketonuria. More preferably, the metabolic disorder is drug-induced phospholipidosis.

PAG may be used as marker for determining mitochondrial toxicity in body fluid samples of any animal if said animal has endogenous phenylacetylglycine or an equivalent thereof. Preferably, PAG is used as marker determining mitochondrial toxicity in body fluid samples of human or rodent, whereby the rodent is preferably a rat or a mouse.

The term "biomarker" or "marker" as used herein refers to molecules in an individual which are differentially present (i.e. present in increased or decreased levels) depending on presence or absence of a certain condition, disease, or complication. In particular, biochemical markers are gene expression products which are differentially present (e.g. through increased or decreased level of expression or turnover) in presence or absence of a certain condition, disease, or complication. The level of a suitable biomarker can indicate the presence or absence of a particular condition, disease, or risk, and thus allow diagnosis or determination of the condition, disease or risk.

The present invention also relates to a kit comprising a means or an agent for measuring PAG.

Such a means or agent may be any suitable means or agent known to the person skilled in the art. For example, a suitable agent may be any kind of ligand or antibody specific for measuring said biomarkers. The kit may also comprise any other components deemed appropriate in the context of measuring the level(s) of the respective biomarkers, such as suitable buffers, filters, etc.

Optionally, the kit may additionally comprise a user's manual for interpreting the results of any measurement(s) with respect to determining whether an individual suffers from mitochondrial toxicity associated a metabolic disorder wherein the metabolic disorders is preferably drug-induced phospholipidosis or a metabolic disorder caused by inborn errors such as inborn error of ureagenesis or an inherited metabolic disorders such as e.g. phenylketonuria. Particularly, such manual may include information about what measured level corresponds to an increased level.

The present invention also relates to the use of said kit for assessing mitochondrial toxicity associated with a metabolic disorder in an individual. Furthermore, the invention relates to the use of said kit for determining the risk of a phospholipidotic compound for inducing mitochondrial toxicity which is associated with a metabolic disorder. Preferably, the metabolic disorder is a drug-induced phospholipidosis or a metabolic disorder caused by inborn errors such as e.g. inborn error of ureagenesis, or an inherited metabolic disorder such as e.g. phenylketonuria.

The present invention also relates to the use of said kit in any of the methods according to the present invention for determining the risk of a phospholipidotic compound for inducing mitochondrial toxicity which is associated with a metabolic disorder or for assessing mitochondrial toxicity associated with a metabolic disorder in an individual. Preferably, the metabolic disorder is a drug-induced phospholipidosis or a metabolic disorder caused by inborn errors such as e.g. inborn error of ureagenesis, or an inherited metabolic disorder such as e.g. phenylketonuria.

The term "normal level" as used herein refers to the range of the level of PAG in a body fluid sample of a control. A control is one or more individuals not suffering from mitochondrial toxicity associated with phospholipidosis or the treated animal before treatment (pre-dose values within the same individual). For rats, the number of individuals is preferably higher than 100, more preferably more than 500, most preferably more than 1000. The normal range is determined by methods well known to the skilled person in the art. A preferred method is for example to determine the range of the values between quantile 2.5 and quantile 97.5, which leaves 5% of "normal" values outside the normal range or in other words, it covers 95% of all values of the control.

The pathological status is defined as deviation from the normal status. According to the invention this pathological status is indicated by an increased level of a biomarker. The term "increased level" as used herein refers to the level of PAG in a body fluid sample which is significantly higher than the normal level. Significantly higher means that the level is higher and that the difference to the normal level is statistically relevant ($p \leq 0.05$, preferably, $p \leq 0.01$).

PAG may also be used as target. Therefore, the present invention provides a method of screening for a compound which interacts with PAG. Such methods are well known in the art.

A suitable method is for example the method of screening for a phospholipidotic compound which interacts with PAG, comprising a) contacting PAG with a compound or a plurality of compounds under conditions which allow interaction of said compound or a plurality of compounds with PAG; and b) detecting the interaction between said compound or plurality of compounds with PAG.

PAG may be immobilized prior step a) or between step a) and step b).

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures.

Figures

Figure 1 shows the chemical structure of phenylacetylglycine (A); phenylacetylglutamine (B); Compound 1: 2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide (C); Compound 2: 2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide (D).

Figure 2 shows a graphical representation of a summary of spectral data: The top panel shows one example of a ^{1}H NMR urine spectrum taken on a control rat. The aromatic region boxed in light grey and the aliphatic region boxed in black contains signals of PAG. This is shown in more detail in the expansion panel (bottom), where 15 spectra of the time point +144 h are shown as a stacked plot.

Figure 3 shows a graphical representation of the relative mean PAG concentration levels in samples derived from animals treated with compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide) related to time matched control samples. Control animals were indicated by a square, low-dosed animals (300mg/kg) by a full circle and high-dosed animals (1000mg/kg) were depicted in a triangle.

Figure 4 shows a graphical representation of the relative mean PAG concentration levels of samples derived from animals treated with compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) related to time matched control samples. Control animals were indicated by a square, low-dosed animals (300mg/kg) by a full circle and high-dosed animals (1500mg/kg) were depicted in a triangle. High standard deviations visible for high-dosed animals can be attributed to differences of individual response kinetics and response intensities.

Examples

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

Example 1: Animals, Doses and Sampling

Animals: Male Wistar Rats (RCC, Inc., Füllinsdorf)

All animals received (human) care as specified by Swiss law and in accordance with the "Guide for the care and use of laboratory animals" published by the NIH. Male Wistar rats (5 animals/dose-group) were purchased from RCC (Füllingsdorf, Switzerland) and housed individually. Treated animals were dosed orally by gavage with several doses of test compounds (Table 1). Control animals received the same volume of vehicle as placebo. Necropsy was performed 48 (subgroup A) or 168 hours (subgroup B) after a single administration (= Day 1) and urine samples were collected in metabolism cages at 0 to 4°C (automatically refrigerated by a Tecniplast sampling/cooling unit (RACK B940, Tecniplast, USA)) at the intervals given in the sampling schedule in Table 2 into labelled sample tubes containing 1 ml of an aqueous Na-azide (1%) solution. Before aliquoting, urine volumes were determined.

Compounds:

Compound 1 = 2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide;

Compound 2 = 2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide,

Vehicle/control: Thixtrope vehicle

The structural difference between both molecules can be mainly characterized by the exchange of the piperazine against a morpholine moiety in compound 2. This leads to a down shift of the basic pKa value from 7.67 to 4.07. Besides the reduced amphiphilicity the lower basic pKa value of compound 2 is the most important reason why compound 2 has a low potential and compound 1 a high potential to induce phospholipidosis.

The above described clear differences in the physico-chemical properties of the two compounds allow despite the high structural similarity of both compounds (differences are depicted in red in Figure 1) a clear distinction of the compounds.

Dose Groups	Compound	Dose level (mg/kg/day)	Number of rats Subgroup A (necropsy 48 hours after application)	Number of rats Subgroup B (necropsy 168 hours after application)
1	Vehicle	0	5 (1-5)	5 (6-10)
2	Compound 1	300	5 (11-15)	5 (16-20)
3	Compound 1	1500	5 (21-25)	5 (26-30)
4	Compound 2	300	5 (31-35)	5 (36-40)
5	Compound 2	1000	5 (41-45)	5 (46-50)

Table 1: Group Design. **Compound 1** = 2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide; **Compound 2** = 2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide, Vehicle/control: Thixtrope vehicle

Example 2: PAG Measurement by NMR

Urine samples were taken on day -7, -2, -1, 1, 2, 3, 4, 5, 6 and 7, whereby Day 1 was the Day of dosing.

Days	Absolute day of urine sample collection	Time	Groups 1 - 5		
			Subgr. A	**Subgr. B**	**Label**
-7	-7 to -6 (24h)	Arriv. to -144h	X	X	-144h
-2	- 2 (0-8h)	-48h to -40 h	X	X	-40h
-1	-1 (0-8h)	-24h to -16h	X	X	-16h
-1	-1 to 1 (8-24h)	-16h to 0h	X	X	0h

1	1 (0-8)	0h to 8h	X	X	8h
1	1 to 2 (8-24h)	8h to 24h	X	X	24h
2	2 to 3 (0-24h)	24h to 48h	X	X	48h
3	3 to 4 (0-24h)	48h to 72h		X	72h
4	4 to 5 (0-24h)	72h to 96h		X	96h
5	5 to 6 (0-24h)	96h to 120h		X	120h
6	6 to 7 (0-24h)	120h to 144h		X	144h
7	7 to 8 (0-24h)	144h to 168h		X	168h

Table 2. Urine sampling for analysis. Time = 0h corresponds to the time of dosing in the morning of day 1

Immediately after reaching the end of the sampling period, the volume was determined and the samples were centrifuged at 3000u/min (500g) for 10 minutes.

Aliquot: Approximately 2ml was transferred in 1.8ml cryvials, round bottom, freestanding, with screw caps and septum for NMR-spectroscopic examinations (Bruker Analytik GmbH, Rheinstetten, Germany, Bruker part No. 85372). The vials were stored at -80°C.

Urine samples were prepared and measured on a Bruker 500MHz NMR instrument according to the COMET 1H-NMR protocol (Keun, H.C et al., (2002) Physiological variation and analytical reproducibility in metabonomic urinalysis. Chem. Res. Tox. 15, 1380-1386) and as described above. In total 467 urine samples were analyzed. After measurement, all data were processed by XWINMR 3.5.6 (Bruker Biospin AG, Fällanden). Representative spectra are depicted in Figure 2. Phase correction and baseline correction were performed with NMRPROC 0.3 (T. Ebbels, H. Keun; Imperial College). Spectral data were binned using AMIX 2.6 (Bruker Biospin AG, Fällanden) following the COMET Data Processing & Pattern Recognition protocol (Keun, H.C., et al., (2002) Physiological variation and analytical reproducibility in metabonomic urinalysis. Chem. Res. Tox. 15, 1380-1386). QC check procedures (Dieterle F., Ross A., Schlotterbeck G., Senn H. (2006) Probabilistic Quotient Normalization as Robust Method to Account for Dilution of Complex Biological Mixtures. Application in 1H NMR Metabonomics. *Anal.*

Chem. 78, 4281-4290) were applied on binned data to detect outliers. In total 7 outliers were detected and removed from further analysis. All data were mean-centered and normalized using the probabilistic quotient normalization (Dieterle F., Ross A., Schlotterbeck G., Senn H. (2006) Probabilistic Quotient Normalization as Robust Method to Account for Dilution of Complex Biological Mixtures. Application in 1H NMR Metabonomics. *Anal. Chem.* 78, 4281-4290.). All binned data were annotated and transferred to the ANT (ANT: Affymetrix NMR Toxicology Data System) database.

The exact PAG quantification was performed according to following procedure:

- Baseline subtraction of spectra
- Integration of PAG peaks ((=7.47ppm, (=7.39ppm, (=3.79ppm, and (=3.7ppm respectively) and Citrate region ((=2.66-2.74ppm and 2.50-2.58ppm).
- Quotient normalization (Dieterle F., Ross A., Schlotterbeck G., Senn H. (2006) Probabilistic Quotient Normalization as Robust Method to Account for Dilution of Complex Biological Mixtures. Application in 1H NMR Metabonomics. Anal. Chem. 78, 4281-4290.)
- Divide each of the PAG bins by its average (all spectra) to correct for different relaxation times and number of protons
- Calculate median out of the PAG bins
- Calculate ratio versus time-matched controls

Compound 2: 2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide

PAG levels were normalized to time matched control animals for compound 2 as described above. No significant dose dependant change of PAG was detected for individual animals dosed with Compound 2 (see Figure 3 and Table 3). The PAG levels of both dose groups were comparable to their time matched controls.

Compound 1: 2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide

Mean PAG levels were determined (as described above) relatively to time matched controls for all animals dosed with Compound 1. A significant dose dependant elevation

of PAG levels was found starting at 24h after dosing for both dose groups. High-dosed animals show 4-fold increased PAG levels, whereas in low-dosed animals an increase of a factor of two was found compared to time matched controls (see Figure 4 and Table 3). The levels of low-dosed animals decrease with time and at time points later than 72h, mean levels of PAG fall below control samples. Mean PAG levels of high-dosed animals remain elevated (3-4 fold increased) until the end of the study.

High standard deviations visible for high-dosed animals can be attributed to differences of individual response kinetics and response intensities.

Table 3A: PAG levels in animals 7 days (-114h) before treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118317_10_1	Control	1	-144	1.1031
R118318_10_1	Control	2	-144	0.7560
R118319_10_1	Control	3	-144	0.8519
R118320_10_1	Control	4	-144	0.7022
R118321_10_1	Control	5	-144	1.1145
R118322_10_1	Control	6	-144	0.8381
R118323_10_1	Control	7	-144	0.9943
R118324_10_1	Control	8	-144	1.2731
R118325_10_1	Control	9	-144	0.6968
R118326_10_1	Control	10	-144	1.0355
R118327_10_1	Compound 1_Low_Dose	11	-144	0.8210
R118328_10_1	Compound 1_Low_Dose	12	-144	1.1559
R118329_10_1	Compound 1_Low_Dose	13	-144	0.9260
R118330_10_1	Compound 1_Low_Dose	14	-144	0.7456
R118331_10_1	Compound 1_Low_Dose	15	-144	0.8081
R118332_10_1	Compound 1_Low_Dose	16	-144	0.6038
R118333_10_1	Compound 1_Low_Dose	17	-144	1.5903
R118334_10_1	Compound 1_Low_Dose	18	-144	1.1241
R118335_10_1	Compound 1_Low_Dose	19	-144	0.9275
R118336_10_1	Compound 1_Low_Dose	20	-144	0.8848
R118337_10_1	Compound 1_High_Dose	21	-144	0.7666
R118338_10_1	Compound 1_High_Dose	22	-144	0.8823
R118339_10_1	Compound 1_High_Dose	23	-144	1.0974
R118340_10_1	Compound 1_High_Dose	24	-144	0.6021
R118341_10_1	Compound 1_High_Dose	25	-144	1.2709
R118342_10_1	Compound 1_High_Dose	26	-144	1.0355

R118343_10_1	Compound 1_High_Dose	27	-144	0.6455
R118344_10_1	Compound 1_High_Dose	28	-144	0.7716
R118345_10_1	Compound 1_High_Dose	29	-144	0.9808
R118346_10_1	Compound 1_High_Dose	30	-144	0.7150
R118347_10_1	Compound 2_Low_Dose	31	-144	0.8959
R118348_10_1	Compound 2_Low_Dose	32	-144	0.9866
R118349_10_1	Compound 2_Low_Dose	33	-144	0.6113
R118350_10_1	Compound 2_Low_Dose	34	-144	1.0040
R118351_10_1	Compound 2_Low_Dose	35	-144	0.8700
R118352_10_1	Compound 2_Low_Dose	36	-144	1.0067
R118353_10_1	Compound 2_Low_Dose	37	-144	0.8499
R118354_10_1	Compound 2_Low_Dose	38	-144	0.9771
R118355_10_1	Compound 2_Low_Dose	39	-144	1.1025
R118356_10_1	Compound 2_Low_Dose	40	-144	1.5236
R118357_10_1	Compound 2_High_Dose	41	-144	1.1113
R118358_10_1	Compound 2_High_Dose	42	-144	0.6810
R118359_10_1	Compound 2_High_Dose	43	-144	1.0115
R118360_10_1	Compound 2_High_Dose	44	-144	0.6066
R118361_10_1	Compound 2_High_Dose	45	-144	0.6015
R118362_10_1	Compound 2_High_Dose	46	-144	2.0608
R118363_10_1	Compound 2_High_Dose	47	-144	2.0477
R118364_10_1	Compound 2_High_Dose	48	-144	2.2465
R118365_10_1	Compound 2_High_Dose	49	-144	2.5194
R118366_10_1	Compound 2_High_Dose	50	-144	2.3206

Table 3B: PAG levels in animals 2 days (-40h) before treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118368_10_1	Control	1	-40	0.4574
R118369_10_1	Control	2	-40	0.5685
R118370_10_1	Control	3	-40	1.1028
R118371_10_1	Control	4	-40	0.2144
R118372_10_1	Control	5	-40	0.6290
R118373_10_1	Control	6	-40	0.3959
R118374_10_1	Control	7	-40	0.4078
R118375_10_1	Control	8	-40	0.5413
R118376_10_1	Control	9	-40	0.6181
R118377_10_1	Control	10	-40	0.7609
R118378_10_1	Compound 1_Low_Dose	11	-40	0.2510
R118379_10_1	Compound 1_Low_Dose	12	-40	0.3534
R118380_10_1	Compound 1_Low_Dose	13	-40	0.5756
R118381_10_1	Compound 1_Low_Dose	14	-40	0.4157

R118382_10_1	Compound 1_Low_Dose	15	-40	1.0106
R118383_10_1	Compound 1_Low_Dose	16	-40	0.3133
R118384_10_1	Compound 1_Low_Dose	17	-40	0.6161
R118385_10_1	Compound 1_Low_Dose	18	-40	0.5487
R118386_10_1	Compound 1_Low_Dose	19	-40	0.7364
R118387_10_1	Compound 1_Low_Dose	20	-40	0.2943
R118388_10_1	Compound 1_High_Dose	21	-40	0.6913
R118389_10_1	Compound 1_High_Dose	22	-40	0.5278
R118390_10_1	Compound 1_High_Dose	23	-40	0.8411
R118391_10_1	Compound 1_High_Dose	24	-40	0.6109
R118392_10_1	Compound 1_High_Dose	25	-40	0.9294
R118393_10_1	Compound 1_High_Dose	26	-40	0.6309
R118394_10_1	Compound 1_High_Dose	27	-40	0.2161
R118395_10_1	Compound 1_High_Dose	28	-40	0.3309
R118396_10_1	Compound 1_High_Dose	29	-40	0.3963
R118397_10_1	Compound 1_High_Dose	30	-40	0.6621
R118398_10_1	Compound 2_Low_Dose	31	-40	1.1566
R118399_10_1	Compound 2_Low_Dose	32	-40	1.2483
R118400_10_1	Compound 2_Low_Dose	33	-40	0.4473
R118401_10_1	Compound 2_Low_Dose	34	-40	1.2604
R118402_10_1	Compound 2_Low_Dose	35	-40	0.3418
R118403_10_1	Compound 2_Low_Dose	36	-40	0.5238
R118404_10_1	Compound 2_Low_Dose	37	-40	0.5336
R118405_10_1	Compound 2_Low_Dose	38	-40	0.7115
R118406_10_1	Compound 2_Low_Dose	39	-40	0.6105
R118407_10_1	Compound 2_Low_Dose	40	-40	0.5232
R118408_10_1	Compound 2_High_Dose	41	-40	0.5320
R118409_10_1	Compound 2_High_Dose	42	-40	0.2568
R118410_10_1	Compound 2_High_Dose	43	-40	0.4805
R118411_10_1	Compound 2_High_Dose	44	-40	0.4717
R118412_10_1	Compound 2_High_Dose	45	-40	0.4514
R118413_10_1	Compound 2_High_Dose	46	-40	0.4760
R118414_10_1	Compound 2_High_Dose	47	-40	0.6419
R118415_10_1	Compound 2_High_Dose	48	-40	0.7515
R118416_10_1	Compound 2_High_Dose	49	-40	0.6794
R118417_10_1	Compound 2_High_Dose	50	-40	0.7696

Table 3C: PAG levels in animals 1 day (-16h) before treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118419_10_1	Control	1	-16	0.4757
R118420_10_1	Control	2	-16	0.4325

R118421_10_1	Control	3	-16	0.5395
R118422_10_1	Control	4	-16	0.2286
R118423_10_1	Control	5	-16	0.4863
R118424_10_1	Control	6	-16	0.5213
R118425_10_1	Control	7	-16	0.3688
R118426_10_1	Control	8	-16	0.9799
R118427_10_1	Control	9	-16	0.8335
R118428_10_1	Control	10	-16	0.8029
R118429_10_1	Compound 1_Low_Dose	11	-16	0.2393
R118430_10_1	Compound 1_Low_Dose	12	-16	0.4349
R118431_10_1	Compound 1_Low_Dose	13	-16	0.5528
R118432_10_1	Compound 1_Low_Dose	14	-16	0.5649
R118433_10_1	Compound 1_Low_Dose	15	-16	0.8171
R118434_10_1	Compound 1_Low_Dose	16	-16	0.3201
R118435_10_1	Compound 1_Low_Dose	17	-16	0.5236
R118436_10_1	Compound 1_Low_Dose	18	-16	0.6045
R118437_10_1	Compound 1_Low_Dose	19	-16	0.4646
R118438_10_1	Compound 1_Low_Dose	20	-16	0.2537
R118439_10_1	Compound 1_High_Dose	21	-16	0.7486
R118440_10_1	Compound 1_High_Dose	22	-16	0.5615
R118441_10_1	Compound 1_High_Dose	23	-16	0.6169
R118442_10_1	Compound 1_High_Dose	24	-16	0.5637
R118443_10_1	Compound 1_High_Dose	25	-16	0.4281
R118444_10_1	Compound 1_High_Dose	26	-16	0.4828
R118445_10_1	Compound 1_High_Dose	27	-16	0.2993
R118446_10_1	Compound 1_High_Dose	28	-16	0.4535
R118447_10_1	Compound 1_High_Dose	29	-16	0.5361
R118448_10_1	Compound 1_High_Dose	30	-16	0.5442
R118449_10_1	Compound 2_Low_Dose	31	-16	1.0275
R118450_10_1	Compound 2_Low_Dose	32	-16	1.1002
R118451_10_1	Compound 2_Low_Dose	33	-16	0.4610
R118452_10_1	Compound 2_Low_Dose	34	-16	1.3440
R118453_10_1	Compound 2_Low_Dose	35	-16	0.2541
R118454_10_1	Compound 2_Low_Dose	36	-16	0.7357
R118455_10_1	Compound 2_Low_Dose	37	-16	0.5260
R118456_10_1	Compound 2_Low_Dose	38	-16	0.6162
R118457_10_1	Compound 2_Low_Dose	39	-16	0.5218
R118458_10_1	Compound 2_Low_Dose	40	-16	0.4132
R118459_10_1	Compound 2_High_Dose	41	-16	0.6280
R118460_10_1	Compound 2_High_Dose	42	-16	0.3499
R118461_10_1	Compound 2_High_Dose	43	-16	outlier
R118462_10_1	Compound 2_High_Dose	44	-16	0.4531
R118463_10_1	Compound 2_High_Dose	45	-16	0.5078
R118464_10_1	Compound 2_High_Dose	46	-16	0.6008
R118465_10_1	Compound 2_High_Dose	47	-16	0.3961
R118466_10_1	Compound 2_High_Dose	48	-16	0.6035
R118467_10_1	Compound 2_High_Dose	49	-16	0.5396
R118468_10_1	Compound 2_High_Dose	50	-16	0.6968

Table 3D: PAG levels in animals within 8 hours (0h) before treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118470_10_1	Control	1	0	0.4509
R118471_10_1	Control	2	0	0.3356
R118472_10_1	Control	3	0	0.3812
R118473_10_1	Control	4	0	0.1276
R118474_10_1	Control	5	0	0.2457
R118475_10_1	Control	6	0	0.3824
R118476_10_1	Control	7	0	0.3107
R118477_10_1	Control	8	0	0.7300
R118478_10_1	Control	9	0	0.5202
R118479_10_1	Control	10	0	0.4288
R118480_10_1	Compound 1_Low_Dose	11	0	0.3142
R118481_10_1	Compound 1_Low_Dose	12	0	0.2440
R118482_10_1	Compound 1_Low_Dose	13	0	0.3422
R118483_10_1	Compound 1_Low_Dose	14	0	0.2530
R118484_10_1	Compound 1_Low_Dose	15	0	0.3700
R118485_10_1	Compound 1_Low_Dose	16	0	outlier
R118486_10_1	Compound 1_Low_Dose	17	0	0.6319
R118487_10_1	Compound 1_Low_Dose	18	0	0.3708
R118488_10_1	Compound 1_Low_Dose	19	0	0.3307
R118489_10_1	Compound 1_Low_Dose	20	0	0.2336
R118490_10_1	Compound 1_High_Dose	21	0	0.3099
R118491_10_1	Compound 1_High_Dose	22	0	0.4951
R118492_10_1	Compound 1_High_Dose	23	0	0.2661
R118493_10_1	Compound 1_High_Dose	24	0	0.1961
R118494_10_1	Compound 1_High_Dose	25	0	0.1580
R118495_10_1	Compound 1_High_Dose	26	0	0.3419
R118496_10_1	Compound 1_High_Dose	27	0	0.1895
R118497_10_1	Compound 1_High_Dose	28	0	0.2711
R118498_10_1	Compound 1_High_Dose	29	0	0.3305
R118499_10_1	Compound 1_High_Dose	30	0	0.4159
R118500_10_1	Compound 2_Low_Dose	31	0	0.6632
R118501_10_1	Compound 2_Low_Dose	32	0	0.5179
R118502_10_1	Compound 2_Low_Dose	33	0	0.2795
R118503_10_1	Compound 2_Low_Dose	34	0	0.6376
R118504_10_1	Compound 2_Low_Dose	35	0	0.3104
R118505_10_1	Compound 2_Low_Dose	36	0	0.3524
R118506_10_1	Compound 2_Low_Dose	37	0	0.4991
R118507_10_1	Compound 2_Low_Dose	38	0	0.5273

R118508_10_1	Compound 2_Low_Dose	39	0	0.2959
R118509_10_1	Compound 2_Low_Dose	40	0	0.3191
R118510_10_1	Compound 2_High_Dose	41	0	0.3191
R118511_10_1	Compound 2_High_Dose	42	0	0.4082
R118512_10_1	Compound 2_High_Dose	43	0	0.1757
R118513_10_1	Compound 2_High_Dose	44	0	0.4688
R118514_10_1	Compound 2_High_Dose	45	0	0.3904
R118515_10_1	Compound 2_High_Dose	46	0	0.3807
R118516_10_1	Compound 2_High_Dose	47	0	0.4568
R118517_10_1	Compound 2_High_Dose	48	0	0.5015
R118518_10_1	Compound 2_High_Dose	49	0	0.3805
R118519_10_1	Compound 2_High_Dose	50	0	0.4405

Table 3E: PAG levels in animals within 8 hours (8h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118521_10_1	Control	1	8	0.4611
R118522_10_1	Control	2	8	0.2946
R118523_10_1	Control	3	8	0.6547
R118524_10_1	Control	4	8	0.4422
R118525_10_1	Control	5	8	0.5698
R118526_10_1	Control	6	8	0.8907
R118527_10_1	Control	7	8	0.4853
R118528_10_1	Control	8	8	0.6687
R118529_10_1	Control	9	8	0.5258
R118530_10_1	Control	10	8	0.6474
R118531_10_1	Compound 1_Low_Dose	11	8	1.1423
R118532_10_1	Compound 1_Low_Dose	12	8	0.5270
R118533_10_1	Compound 1_Low_Dose	13	8	0.3918
R118534_10_1	Compound 1_Low_Dose	14	8	0.5331
R118535_10_1	Compound 1_Low_Dose	15	8	0.6815
R118536_10_1	Compound 1_Low_Dose	16	8	0.2977
R118537_10_1	Compound 1_Low_Dose	17	8	0.6756
R118538_10_1	Compound 1_Low_Dose	18	8	0.7527
R118539_10_1	Compound 1_Low_Dose	19	8	0.4111
R118540_10_1	Compound 1_Low_Dose	20	8	0.3688
R118541_10_1	Compound 1_High_Dose	21	8	0.8523
R118542_10_1	Compound 1_High_Dose	22	8	0.4093
R118543_10_1	Compound 1_High_Dose	23	8	0.7986
R118544_10_1	Compound 1_High_Dose	24	8	0.5172
R118545_10_1	Compound 1_High_Dose	25	8	0.2995
R118546_10_1	Compound 1_High_Dose	26	8	0.4361

R118547_10_1	Compound 1_High_Dose	27	8	0.5473
R118548_10_1	Compound 1_High_Dose	28	8	0.4307
R118549_10_1	Compound 1_High_Dose	29	8	1.0174
R118550_10_1	Compound 1_High_Dose	30	8	0.3275
R118551_10_1	Compound 2_Low_Dose	31	8	0.8897
R118552_10_1	Compound 2_Low_Dose	32	8	0.6310
R118553_10_1	Compound 2_Low_Dose	33	8	0.3915
R118554_10_1	Compound 2_Low_Dose	34	8	1.2919
R118555_10_1	Compound 2_Low_Dose	35	8	0.3580
R118556_10_1	Compound 2_Low_Dose	36	8	0.9744
R118557_10_1	Compound 2_Low_Dose	37	8	0.3039
R118558_10_1	Compound 2_Low_Dose	38	8	0.7208
R118559_10_1	Compound 2_Low_Dose	39	8	0.6353
R118560_10_1	Compound 2_Low_Dose	40	8	0.4304
R118561_10_1	Compound 2_High_Dose	41	8	0.6552
R118562_10_1	Compound 2_High_Dose	42	8	0.2654
R118563_10_1	Compound 2_High_Dose	43	8	0.3244
R118564_10_1	Compound 2_High_Dose	44	8	0.3661
R118565_10_1	Compound 2_High_Dose	45	8	0.5090
R118566_10_1	Compound 2_High_Dose	46	8	0.8253
R118567_10_1	Compound 2_High_Dose	47	8	0.6223
R118568_10_1	Compound 2_High_Dose	48	8	0.6794
R118569_10_1	Compound 2_High_Dose	49	8	0.5865
R118570_10_1	Compound 2_High_Dose	50	8	0.5553

Table 3F: PAG levels in animals within 8 to 24 hours (24h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118572_10_1	Control	1	24	0.3221
R118573_10_1	Control	2	24	0.1270
R118574_10_1	Control	3	24	0.3634
R118575_10_1	Control	4	24	0.1511
R118576_10_1	Control	5	24	0.2565
R118577_10_1	Control	6	24	0.3562
R118578_10_1	Control	7	24	0.2586
R118579_10_1	Control	8	24	0.5263
R118580_10_1	Control	9	24	0.4187
R118581_10_1	Control	10	24	0.4749
R118582_10_1	Compound 1_Low_Dose	11	24	outlier
R118583_10_1	Compound 1_Low_Dose	12	24	outlier
R118584_10_1	Compound 1_Low_Dose	13	24	outlier
R118585_10_1	Compound 1_Low_Dose	14	24	0.6300

R118586_10_1	Compound 1_Low_Dose	15	24	outlier
R118587_10_1	Compound 1_Low_Dose	16	24	0.8072
R118588_10_1	Compound 1_Low_Dose	17	24	1.0165
R118589_10_1	Compound 1_Low_Dose	18	24	0.7250
R118590_10_1	Compound 1_Low_Dose	19	24	0.5035
R118591_10_1	Compound 1_Low_Dose	20	24	0.6637
R118592_10_1	Compound 1_High_Dose	21	24	1.7579
R118593_10_1	Compound 1_High_Dose	22	24	1.3035
R118594_10_1	Compound 1_High_Dose	23	24	1.4190
R118595_10_1	Compound 1_High_Dose	24	24	0.4001
R118596_10_1	Compound 1_High_Dose	25	24	0.8788
R118597_10_1	Compound 1_High_Dose	26	24	1.2898
R118598_10_1	Compound 1_High_Dose	27	24	1.4210
R118599_10_1	Compound 1_High_Dose	28	24	1.9488
R118600_10_1	Compound 1_High_Dose	29	24	1.6305
R118601_10_1	Compound 1_High_Dose	30	24	1.2496
R118602_10_1	Compound 2_Low_Dose	31	24	0.6934
R118603_10_1	Compound 2_Low_Dose	32	24	0.3762
R118604_10_1	Compound 2_Low_Dose	33	24	0.4835
R118605_10_1	Compound 2_Low_Dose	34	24	0.8446
R118606_10_1	Compound 2_Low_Dose	35	24	0.3743
R118607_10_1	Compound 2_Low_Dose	36	24	0.5615
R118608_10_1	Compound 2_Low_Dose	37	24	0.3777
R118609_10_1	Compound 2_Low_Dose	38	24	0.4892
R118610_10_1	Compound 2_Low_Dose	39	24	0.3393
R118611_10_1	Compound 2_Low_Dose	40	24	0.4166
R118612_10_1	Compound 2_High_Dose	41	24	0.4048
R118613_10_1	Compound 2_High_Dose	42	24	0.3363
R118614_10_1	Compound 2_High_Dose	43	24	0.1408
R118615_10_1	Compound 2_High_Dose	44	24	0.3750
R118616_10_1	Compound 2_High_Dose	45	24	0.4284
R118617_10_1	Compound 2_High_Dose	46	24	0.6538
R118618_10_1	Compound 2_High_Dose	47	24	0.4408
R118619_10_1	Compound 2_High_Dose	48	24	0.2588
R118620_10_1	Compound 2_High_Dose	49	24	0.4111
R118621_10_1	Compound 2_High_Dose	50	24	0.3592

Table 3G: PAG levels in animals 2 days (48h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118623_10_1	Control	1	48	0.3650
R118625_10_1	Control	3	48	0.5825
R118626_10_1	Control	4	48	0.2236

R118627_10_1	Control	5	48	0.3294
R118628_10_1	Control	6	48	0.4203
R118629_10_1	Control	7	48	0.4495
R118630_10_1	Control	8	48	0.3544
R118631_10_1	Control	9	48	0.4253
R118632_10_1	Control	10	48	0.4665
R118633_10_1	Compound 1_Low_Dose	11	48	0.7584
R118634_10_1	Compound 1_Low_Dose	12	48	0.6368
R118635_10_1	Compound 1_Low_Dose	13	48	0.9878
R118636_10_1	Compound 1_Low_Dose	14	48	0.6461
R118637_10_1	Compound 1_Low_Dose	15	48	1.0386
R118638_10_1	Compound 1_Low_Dose	16	48	0.3452
R118639_10_1	Compound 1_Low_Dose	17	48	0.5698
R118640_10_1	Compound 1_Low_Dose	18	48	0.3492
R118641_10_1	Compound 1_Low_Dose	19	48	0.6477
R118642_10_1	Compound 1_Low_Dose	20	48	0.4450
R118643_10_1	Compound 1_High_Dose	21	48	1.0283
R118644_10_1	Compound 1_High_Dose	22	48	0.8835
R118645_10_1	Compound 1_High_Dose	23	48	0.8293
R118646_10_1	Compound 1_High_Dose	24	48	0.9716
R118647_10_1	Compound 1_High_Dose	25	48	0.7369
R118648_10_1	Compound 1_High_Dose	26	48	0.8250
R118649_10_1	Compound 1_High_Dose	27	48	1.8515
R118650_10_1	Compound 1_High_Dose	28	48	0.8422
R118651_10_1	Compound 1_High_Dose	29	48	0.5643
R118652_10_1	Compound 1_High_Dose	30	48	0.5655
R118653_10_1	Compound 2_Low_Dose	31	48	0.6453
R118654_10_1	Compound 2_Low_Dose	32	48	0.3298
R118655_10_1	Compound 2_Low_Dose	33	48	0.3762
R118656_10_1	Compound 2_Low_Dose	34	48	0.7958
R118657_10_1	Compound 2_Low_Dose	35	48	0.1879
R118658_10_1	Compound 2_Low_Dose	36	48	0.5409
R118659_10_1	Compound 2_Low_Dose	37	48	0.2716
R118660_10_1	Compound 2_Low_Dose	38	48	0.3303
R118661_10_1	Compound 2_Low_Dose	39	48	0.3239
R118662_10_1	Compound 2_Low_Dose	40	48	0.3237
R118663_10_1	Compound 2_High_Dose	41	48	0.2358
R118664_10_1	Compound 2_High_Dose	42	48	0.2482
R118665_10_1	Compound 2_High_Dose	43	48	0.3576
R118666_10_1	Compound 2_High_Dose	44	48	0.3535
R118667_10_1	Compound 2_High_Dose	45	48	0.4627
R118668_10_1	Compound 2_High_Dose	46	48	0.4539
R118669_10_1	Compound 2_High_Dose	47	48	0.2073
R118670_10_1	Compound 2_High_Dose	48	48	0.2888
R118671_10_1	Compound 2_High_Dose	49	48	0.2743
R118672_10_1	Compound 2_High_Dose	50	48	0.3092

Table 3H: PAG levels in animals 3 days (72h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118674_10_1	Control	6	72	0.3258
R118675_10_1	Control	7	72	0.2822
R118676_10_1	Control	8	72	0.6112
R118677_10_1	Control	9	72	0.4703
R118678_10_1	Control	10	72	0.4345
R118679_10_1	Compound 1_Low_Dose	16	72	0.3674
R118680_10_1	Compound 1_Low_Dose	17	72	0.3499
R118681_10_1	Compound 1_Low_Dose	18	72	0.6478
R118682_10_1	Compound 1_Low_Dose	19	72	0.7468
R118683_10_1	Compound 1_Low_Dose	20	72	0.3577
R118684_10_1	Compound 1_High_Dose	26	72	1.9720
R118685_10_1	Compound 1_High_Dose	27	72	1.1100
R118686_10_1	Compound 1_High_Dose	28	72	0.7959
R118687_10_1	Compound 1_High_Dose	29	72	0.8368
R118688_10_1	Compound 1_High_Dose	30	72	1.5672
R118689_10_1	Compound 2_Low_Dose	36	72	0.3432
R118690_10_1	Compound 2_Low_Dose	37	72	0.2797
R118691_10_1	Compound 2_Low_Dose	38	72	0.3031
R118692_10_1	Compound 2_Low_Dose	39	72	0.3459
R118693_10_1	Compound 2_Low_Dose	40	72	0.3244
R118694_10_1	Compound 2_High_Dose	46	72	0.3128
R118695_10_1	Compound 2_High_Dose	47	72	0.3653
R118696_10_1	Compound 2_High_Dose	48	72	0.2427
R118697_10_1	Compound 2_High_Dose	49	72	0.2488
R118698_10_1	Compound 2_High_Dose	50	72	0.3522

5

Table 3I: PAG levels in animals 4 days (96h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118700_10_1	Control	6	96	0.3697
R118701_10_1	Control	7	96	0.3249
R118702_10_1	Control	8	96	0.3650
R118703_10_1	Control	9	96	0.6536
R118704_10_1	Control	10	96	0.3976
R118705_10_1	Compound 1_Low_Dose	16	96	0.3038
R118706_10_1	Compound 1_Low_Dose	17	96	0.1334
R118707_10_1	Compound 1_Low_Dose	18	96	0.2845

R118708_10_1	Compound 1_Low_Dose	19	96	0.4082
R118709_10_1	Compound 1_Low_Dose	20	96	0.2536
R118710_10_1	Compound 1_High_Dose	26	96	0.4112
R118711_10_1	Compound 1_High_Dose	27	96	1.2414
R118712_10_1	Compound 1_High_Dose	28	96	0.7087
R118713_10_1	Compound 1_High_Dose	29	96	1.1422
R118714_10_1	Compound 1_High_Dose	30	96	0.5451
R118715_10_1	Compound 2_Low_Dose	36	96	0.4553
R118716_10_1	Compound 2_Low_Dose	37	96	0.3382
R118717_10_1	Compound 2_Low_Dose	38	96	0.3112
R118718_10_1	Compound 2_Low_Dose	39	96	0.3610
R118719_10_1	Compound 2_Low_Dose	40	96	0.3329
R118720_10_1	Compound 2_High_Dose	46	96	0.3558
R118721_10_1	Compound 2_High_Dose	47	96	0.3303
R118722_10_1	Compound 2_High_Dose	48	96	0.1808
R118723_10_1	Compound 2_High_Dose	49	96	0.2551
R118724_10_1	Compound 2_High_Dose	50	96	0.2381

Table 3J: PAG levels in animals 5 days (120h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118726_10_1	Control	6	120	0.3323
R118727_10_1	Control	7	120	0.2540
R118728_10_1	Control	8	120	0.2119
R118729_10_1	Control	9	120	0.4552
R118730_10_1	Control	10	120	0.4387
R118731_10_1	Compound 1_Low_Dose	16	120	0.2511
R118732_10_1	Compound 1_Low_Dose	17	120	0.1429
R118733_10_1	Compound 1_Low_Dose	18	120	0.1575
R118734_10_1	Compound 1_Low_Dose	19	120	0.2294
R118735_10_1	Compound 1_Low_Dose	20	120	0.3450
R118736_10_1	Compound 1_High_Dose	26	120	0.6995
R118737_10_1	Compound 1_High_Dose	27	120	1.2457
R118738_10_1	Compound 1_High_Dose	28	120	0.6107
R118739_10_1	Compound 1_High_Dose	29	120	2.4430
R118740_10_1	Compound 1_High_Dose	30	120	0.7116
R118741_10_1	Compound 2_Low_Dose	36	120	0.5135
R118742_10_1	Compound 2_Low_Dose	37	120	0.2422
R118743_10_1	Compound 2_Low_Dose	38	120	0.5300
R118744_10_1	Compound 2_Low_Dose	39	120	0.4505
R118745_10_1	Compound 2_Low_Dose	40	120	0.3250
R118746_10_1	Compound 2_High_Dose	46	120	0.4512
R118747_10_1	Compound 2_High_Dose	47	120	0.3983

R118748_10_1	Compound 2_High_Dose	48	120	0.1947
R118749_10_1	Compound 2_High_Dose	49	120	0.3433
R118750_10_1	Compound 2_High_Dose	50	120	0.3880

Table 3K: PAG levels in animals 6 days (144h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118752_10_1	Control	6	144	0.3102
R118753_10_1	Control	7	144	0.3237
R118754_10_1	Control	8	144	0.2400
R118755_10_1	Control	9	144	0.4053
R118756_10_1	Control	10	144	0.5319
R118757_10_1	Compound 1_Low_Dose	16	144	0.2149
R118758_10_1	Compound 1_Low_Dose	17	144	0.1279
R118759_10_1	Compound 1_Low_Dose	18	144	0.2475
R118760_10_1	Compound 1_Low_Dose	19	144	0.2412
R118761_10_1	Compound 1_Low_Dose	20	144	0.2688
R118762_10_1	Compound 1_High_Dose	26	144	outlier
R118763_10_1	Compound 1_High_Dose	27	144	0.9646
R118764_10_1	Compound 1_High_Dose	28	144	0.3602
R118765_10_1	Compound 1_High_Dose	29	144	1.7596
R118766_10_1	Compound 1_High_Dose	30	144	0.8435
R118767_10_1	Compound 2_Low_Dose	36	144	0.2186
R118768_10_1	Compound 2_Low_Dose	37	144	0.2282
R118769_10_1	Compound 2_Low_Dose	38	144	0.3879
R118770_10_1	Compound 2_Low_Dose	39	144	0.4435
R118771_10_1	Compound 2_Low_Dose	40	144	0.3302
R118772_10_1	Compound 2_High_Dose	46	144	0.4526
R118773_10_1	Compound 2_High_Dose	47	144	0.3196
R118774_10_1	Compound 2_High_Dose	48	144	0.2000
R118775_10_1	Compound 2_High_Dose	49	144	0.2590
R118776_10_1	Compound 2_High_Dose	50	144	0.3791

Table 3L: PAG levels in animals 7 days (168h) after treatment with vehicle, compound 1 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-methyl-N-[6-(4-methyl-piperazin-1-yl)-4-o-tolyl-pyridin-3-yl]-isobutyramide) or compound 2 (2-(3,5-Bis-trifluoromethyl-phenyl)-N-[4-(2-chloro-phenyl)-6-morpholin-4-yl-pyridin-3-yl]-N-methyl-isobutyramide).

Spectrum_ID	Group	Animal	Time	PAG
R118778_10_1	Control	6	168	0.2738
R118779_10_1	Control	7	168	0.1810

R118780_10_1	Control	8	168	0.1795
R118781_10_1	Control	9	168	0.3639
R118782_10_1	Control	10	168	0.4539
R118783_10_1	Compound 1_Low_Dose	16	168	0.1903
R118784_10_1	Compound 1_Low_Dose	17	168	0.1053
R118785_10_1	Compound 1_Low_Dose	18	168	0.1619
R118786_10_1	Compound 1_Low_Dose	19	168	0.1736
R118787_10_1	Compound 1_Low_Dose	20	168	0.2033
R118789_10_1	Compound 1_High_Dose	27	168	0.2991
R118790_10_1	Compound 1_High_Dose	28	168	0.1745
R118791_10_1	Compound 1_High_Dose	29	168	1.9217
R118792_10_1	Compound 1_High_Dose	30	168	0.6983
R118793_10_1	Compound 2_Low_Dose	36	168	0.2454
R118794_10_1	Compound 2_Low_Dose	37	168	0.2715
R118795_10_1	Compound 2_Low_Dose	38	168	0.3498
R118796_10_1	Compound 2_Low_Dose	39	168	0.3157
R118797_10_1	Compound 2_Low_Dose	40	168	0.3238
R118798_10_1	Compound 2_High_Dose	46	168	0.3469
R118799_10_1	Compound 2_High_Dose	47	168	0.2856
R118800_10_1	Compound 2_High_Dose	48	168	0.1695
R118801_10_1	Compound 2_High_Dose	49	168	0.2008
R118802_10_1	Compound 2_High_Dose	50	168	0.2243

Example 3: Electron microscopic examination (Determination of Phospholipidosis)

Blood samples (as much as possible) were withdrawn from all animals at necropsy at about 48 hours after dosing (subgroup A) and about 168 hours after dosing (subgroup B)

The samples were collected from the abdominal aorta or by cardiac puncture under terminal anaesthesia (CO_2). A total of at least 2ml blood was required to gain buffy coat (buffy coat = leukocyte band) samples.

After fixation in 2.5% glutaraldehyde, the buffy coat samples from all animals were embedded in Epon. Semithin and thin sections were prepared from samples of all vehicle, all compound 1 treated and compound 2 high dose treated animals (animals treated with the lower dose of compound 2 were not examined as the high dose animals did not show any lymphocytes with lamellar bodies). All thin sections were examined ultrastructurally. If possible, 200 lymphocytes per sample were examined for lamellar bodies. The results per sample include the total number of lymphocytes examined, the total number of positive lymphocytes (i.e. with lamellar bodies), the percentage of positive lymphocytes and a grading of severity of phospholipidosis (criteria see table 4)

Results

Lymphocytes containing cytoplasmic lamellar bodies were seen in animals treated with compound 1 only. Animals treated with compound 2 were not affected.

Lamellar bodies occurred dose dependently in animals treated with compound 1. The incidence of affected lymphocytes per animal was quite variable within the treatment groups.

In animals given 300 mg/kg/day of compound 1 and necropsied 48 hours after application the incidence of affected lymphocytes ranged from 5-12 % (data from 3 animals). Phospholipidosis was considered to be minimal to slight. At the same dose and necropsy 168 hours after application 0-1 % of the lymphocytes were affected only indicating partial or complete recovery. Phospholipidosis was considered to be minimal or not present at this time point.

In animals given 1500 mg/kg/day compound 1 and necropsied 48 hours after application the incidence of affected lymphocytes ranged from 15-31 % (data from 4 animals) and phospholipidosis was considered to be moderate to marked. In animals sacrificed 168 hours after application the incidence of affected lymphocytes ranged from 21-31 % (data from 4 animals) and phospholipidosis was again considered to be moderate to marked. There was a minimally increased mean incidence in affected lymphocytes in animals sacrificed 168 hours after application when compared to animals sacrificed 48 hours after application. However, this increase was considered to be due to individual variations within the small sample size (4 animals only) rather than a real effect.

Discussion and Conclusion

Lymphocytes containing cytoplasmic lamellar bodies indicating a compound-induced phospholipidosis were seen in animals treated with compound 1 only. Lamellar bodies occurred dose dependently and were already seen 48 hours after application. At 300 mg/kg/day of compound 1 the incidence of affected lymphocytes was about 5-12 % and partial or complete recovery was seen 168 hours after application. At 1500 mg/kg/day of compound 1 15-31% of the lymphocytes were affected. There was no obvious difference in the incidence of affected lymphocytes between the two different time points, i.e. there were no indications of recovery within 168 hours after application. Animals treated with compound 2 did not show any lymphocytes containing cytoplasmic lamellar bodies.

Table 4: Individual Animal Data for phospholipidosis.

n.d. = not determined; [1] = insufficient number of lymphocytes; [2] = very limited number of lymphocytes (data not included in final assessment); [3] = no findings in higher dose group; [4] = no buffy coat available (decedent)

Buffy coats, grade PL (severity of phospholipidosis): ≤ 5%: minimal , >5-15%: slight, >15-25%: moderate, >25: marked.

Dose Group	Compound	Dose [mg/kg/day]	No. of animal: Necropsy after application		lymphocytes			Result, PL	
			48 hours	168 hours	counted	positive	%, pos.	-	+
1	Vehicle	0	1		103	0	0	-	
			2		90	0	0	-	
			3		182	0	0	-	
			4		167	0	0	-	
			5		109	0	0	-	
				6	178	0	0	-	
				7	200	0	0	-	
				8	133	0	0	-	
				9	200	0	0	-	
				10	190	0	0	-	
2	Compound 1	300	11		n.d.[1]	n.d.[1]	n.d.[1]		n.d.
			12		200	10	5		1
			13		n.d.[1]	n.d.[1]	n.d.[1]		n.d.
			14		141	8	5.7		2
			15		189	23	12.2		2
				16	82	0	0		-
				17	174	2	1.1		1
				18	200	2	1		1
				19	200	0	0		-
				20	118	1	0.8		1

Dose Group	Compound	Dose [mg/kg/day]	No. of animal: Necropsy after application		lymphocytes			Result, PL	
			48 hours	168 hours	counted	positive	%, pos.	-	+
3	Compound 1	1500	21		191	36	18.8		3
			22		126	19	15.1		3
			23		200	61	31		4
			24		(52) [2]	(3)	(5.8)		(2)
			25		113	29	25.7		4
				26	n.d. [4]	n.d. [4]	n.d. [4]		n.d.
				27	200	62	31		4
				28	200	42	21		3
				29	103	29	28.2		4
				30	104	25	24		3
4	Compound 2	300	31		n.d. [3]	n.d. [3]	n.d. [3]		n.d.
			32		n.d. [3]	n.d. [3]	n.d. [3]		n.d.
			33		n.d. [3]	n.d. [3]	n.d. [3]		n.d.
			34		n.d. [3]	n.d. [3]	n.d. [3]		n.d.
			35		.n.d. [3]	.n.d. [3]	.n.d. [3]		n.d.
				36	n.d. [3]	n.d. [3]	n.d. [3]		n.d.
				37	n.d. [3]	n.d. [3]	n.d. [3]		n.d.
				38	n.d. [3]	n.d. [3]	n.d. [3]		n.d.
				39	n.d. [3]	n.d. [3]	n.d. [3]		n.d.
				40	n.d. [3]	n.d. [3]	n.d. [3]		n.d.
5	Compound 2	1000	41		181	0	0	-	
			42		162	0	0	-	
			43		200	0	0	-	
			44		200	0	0	-	
			45		200	0	0	-	
				46	200	0	0	-	
				47	190	0	0	-	
				48	106	0	0	-	
				49	173	0	0	-	
				50	200	0	0	-	

Claims

1. A method for determining a risk of a phospholipidotic compound for inducing mitochondrial toxicity associated with a metabolic disorder, comprising
a) measuring the level of PAG in a body fluid sample,
b) comparing the level of determined PAG with the level of PAG of a control
wherein an increased level of PAG in comparison with said control is indicative that said compound induces mitochondrial toxicity which is associated with drug-induced phospholipidosis.

2. The method according to claim 1 wherein the metabolic disorder is drug-induced phospholipidosis, metabolic disorders caused by inborn errors or inherited metabolic disorders

3. The method according to claim 1 or 2 wherein the body fluid is blood or urine.

4. The method according to any one of claims 1 to 3 wherein PAG in the biological fluid is determined by methods comprising NMR spectrometry.

5. Use of PAG as marker for mitochondrial toxicity.

6. Use according to claim 5 wherein the mitochondrial toxicity is associated with metabolic disorders.

7. A kit comprising a means or an agent for measuring of PAG.

8. The kit according to claim 8 wherein the kit further comprises a user's manual for interpreting the results of any measurement with respect to determining the risk of a phospholipidotic compound inducing mitochondrial toxicity associated with a metabolic disorder.

9. The kit according to claim 8 or 9 wherein the metabolic disorder is drug-induced phospholipidosis, metabolic disorders caused by inborn errors or an inherited metabolic disorder.

10. Use of a kit according to any one of claims 7 to 9 for determining the risk of a phospholipidotic compound for inducing mitochondrial toxicity which is associated with a metabolic disorder.

11. Use according to claim 10 in a method according to any of claims 1 to 4.

12. Use according to any one of the claims 6, 10 or 11 wherein the metabolic disorders is drug-induced phospholipidosis, metabolic disorders caused by inborn errors or inherited metabolic disorders

13. Methods, uses and kits substantially as herein before described especially with reference to the foregoing examples

***