Research Article ISSN 2639-944X

Journal of Medical - Clinical Research & Reviews

A Comprehensive Safety Profile of Oleoylethanolamide

Narendra S. Deshmukh[1], Sedratul Muntha[2], Silma Subah[2], Paul Clayton[3*]

[1]*Intox Private Ltd, 375, Urawade, Tal. Mulshi, Dist. Pune 412 115, Maharashtra, India.*

[2]*Gencor Pacific Limited, Hong Kong.*

[3]*Senior Scientific Consultant to Gencor Pacific, USA.*

***Correspondence:**
Paul Clayton, Senior Scientific Consultant to Gencor Pacific, USA.

Received: 01 Mar 2024; **Accepted:** 05 Apr 2024; **Published:** 15 Apr 2024

Citation: Deshmukh NS, Muntha S, Subah S, et al. A Comprehensive Safety Profile of Oleoylethanolamide. J Med - Clin Res & Rev. 2024; 8(4): 1-10.

Keywords
Oleoylethanolamide, Neuroinflammatory stress, Oleic acid.

Introduction

The endogenous fatty acid amide oleoylethanolamide (OEA) is a bioactive mono-unsaturated lipid mediator belonging to the acylglycerol and N-acylethanolamine family, with structural similarities to the endocannabinoids. OEA is synthesized from oleic acid and phosphatidylethanolamine, mainly in the small intestine, adipose tissues, neurons and astrocytes [1,2]. A substantial amount of research has elucidated OEA's main physiological roles as an anti-inflammatory mediator and a regulator of energy homeostasis [3,4]. The anorexigenic and lipolytic effects of OEA make it a promising candidate for weight management.

Despite structural similarities with the endocannabinoids, OEA does not act via the cannabinoid receptors CB1 and CB2. Whereas endocannabinoids such as anandamide activate CB1 receptors mainly in the mesolimbic system, leading to increased food intake, OEA is an anorexigen [5]. OEA has also been shown to improve memory and reduce stress and depression [6,7], via central and peripheral mechanisms.

OEA rapidly crosses the blood brain barrier (BBB) [8], and its ability to reduce neuro-inflammatory stress [8-10] makes it potentially useful in the management of various neurodegenerative and psychiatric disorders. The anti-neuroinflammatory mechanism is thought to involve central PPAR-α agonism, thereby suppressing synthesis of nuclear factor NF-κB and AP-1 [11,12].

This has been demonstrated in alcohol-intoxicated rats, in which OEA has anti-depressant and neuroprotective effects [6]. Alcohol abuse causes peripheral inflammation and neuroinflammation. Neuroinflammation is characterized by activation of the innate immunity toll-like receptor 4 (TLR-4), which has implications in many psychiatric disorders [13]. External stresses such as increased alcohol concentration in the serum activate TLR4 via NF-κB and AP-1 pathway and induce a pro-inflammatory response. Exogenous administration of OEA blocks the alcohol-induced TLR4-mediated proinflammatory cascade, thereby reducing proinflammatory cytokines and chemokines, oxidative and nitrosative stress, and ultimately preventing neural damage in the frontal cortex of rodents [10].

OEA also exerts potentially beneficial effects on inflammation and related disorders via peripheral actions. Damage to the gastrointestinal barrier caused by dysbiosis or by noxious stimuli such as alcohol permits translocation of enteric microorganisms and microbial products such as LPS into the portal vein and thence the systemic circulation, AKA endotoxemia. This affects many organs including the liver, blood vessels and the CNS, triggering chronic inflammation via receptors such as TLR4.

The breakdown of colonic epithelial barrier function is ultimately caused by elevated inflammatory, oxidative and nitrosative stress in the gut wall, which compromises epithelial junctional complexes such as tight junction (TJ) proteins (Claudin-3 and Occludin). OEA has been shown to modulate intestinal permeability in vitro via TRPV1 and PPAR-α mechanisms [14] as mentioned above, protecting the intestinal barrier and thereby reducing endotoxemia and neuroinflammatory stress. In OEA-pretreated animals, the integrity of TJ proteins was preserved upon alcohol induced damage [6]. OEA's ability to protect colonic epithelial barrier function and reduce endotoxemia may find applications in modulation of the gut-brain axis.

OEA's many biological functions in different domains make it a promising candidate for the clinical management of metabolic, inflammatory and neuroinflammatory conditions. A robust safety profile for this metabolite, however, does not yet exist. This paper aims to contribute to the debate, and perhaps facilitate the introduction of OEA into clinical use, by providing a series of detailed toxicity, safety and dose range studies for this novel autacoid.

Materials and Methods

Acute dose range of OEA

In the acute dose range study, groups of three male and three female Wistar rats were given oleoylethanolamide by oral gavage, for 7-days daily at doses of 2000, 3000 and 4000 mg/kg body weight, with a parallel vehicle control group of rats.

The animals were examined daily for signs of toxicity and mortality. Body weights were recorded at the start and end points. Blood and derived plasma samples of rats of group G1 and group G4 from the study were subjected to clinical hematology and chemistry evaluations respectively, at termination of the 7-day treatment. The animals were sacrificed at 7 days and subjected to a complete necropsy examination.

Chronic dose range of OEA

In the chronic dose range finding study, groups of ten Wistar IGS rats, five males and five females, were given oleoylethanolamide by oral gavage, at doses of 500, 1000, 2000 and 3000 mg/kg body weight for 28 consecutive days. Control group rats were administered with the vehicle only i.e. analytical grade water with Tween 80 (1% w/v) in a similar manner.

The animals were observed for signs of systemic toxicity. Their body weight and food intake were recorded. Blood and derived samples were collected on day 29 from the group and subjected to clinical hematology and chemistry evaluations respectively. On completion of 28 days of treatment, the rats were sacrificed and subjected to complete necropsy and histopathology evaluations.

Acute oral toxicity of OEA

In this study, five nulliparous and non-pregnant female rats were administered oleoylethanolamide by oral gavage in sequence, at minimum of 48 hours intervals for 14 days. The rats were observed for mortality and signs of toxicity for a period of 14 days thereafter and sacrificed on day 15. Their body weights were recorded weekly. Necropsy was performed on all animals sacrificed at termination. The LD50 with 95% confidence intervals was estimated.

Chronic toxicity of OEA

In this study, groups of twenty male and twenty female Wistar rats were administered oleoylethanolamide by oral gavage, for 180 days daily at doses of 500 mg/kg, 1000 mg/kg and 2500 mg/kg. Additional concurrent groups of ten male and ten female rats at control and high dose levels were treated similarly but, after cessation of treatment were further observed for reversal of toxicity or delayed toxicity for a period of 28 days.

The rats were examined for signs of toxicity, mortality and potential neurotoxicity. Blood and plasma samples of all the surviving rats were subjected to clinical hematology and clinical chemistry evaluations. Endocrine functions, reproductive functions and immune toxicity were also assessed. Urinalysis was performed on rats from the control and high dose groups. All rats were sacrificed at the end of the treatment for a complete necropsy and histopathological evaluations.

In-Vitro Mammalian Chromosome Aberration Test

In this study, cultures of human peripheral blood lymphocytes were exposed to the test item at concentrations of 62.5, 31.25 and 15.625 pg/mL, in the absence of metabolic activation system, for the exposure of 3 hours; and 31.25, 15.625 and 7.8125 pg/mL in the presence of metabolic activation system for the exposure of 3 hours and the subsequent absence of metabolic activation system for 24 hours. Duplicate cultures were used at each concentration. Testing was conducted in three separate experiments, two in absence of supplementary metabolic activation system (S9) and one in presence of metabolic activation system.

In the first experiment, conducted in absence of S9, the cells were exposed to the test item for 3 hours and sampled at a time equivalent to about 1.5 normal cell cycle lengths. The second experiment in absence of S9 was performed with continuous treatment (24 hours) until sampling at a time equivalent to about 1.5 cell cycle length. In the third experiment, the cells were exposed to the test item for 3 hours in presence of S9 and sampled at a time equivalent to about 1.5 normal cell cycle length.

Vehicle and positive controls, both with and without metabolic activation were tested concurrently with the test item. DMSO was used as vehicle for test item formulation. The known mutagens methyl methane sulphonate and cyclophosphamide monohydrate, were employed as positive controls at the concentrations of 30 pg/mL and 60 pg/mL, for the experiment without and with metabolic activation system respectively.

All cell cultures were treated with colchicine (0.5 pg/mL) at 2-3 hours prior to harvesting. Each culture was harvested and chromosomal preparations were made and stained with Giemsa. Three hundred well-spread metaphases were evaluated microscopically for structural aberrations per test concentration. Cytotoxicity, the number of chromosome aberrations per cell and percentage of aberrated cells were measured.

Mammalian *In-Vivo* Erythrocyte Micronucleus Test

In this study, group of five male mice was administered with oleoylethanolamide formulated in analytical grade water with 1% tween 80 by oral gavage at the dose of 2000 mg/kg body weight. The animals were treated once a day for two consecutive days, at an interval of 24 hours.

A concurrent vehicle control group of five male mice received 10 ml/kg body weight of analytical grade water with 1% tween 80, while another concurrent positive control group of five male

mice received cyclophosphamide monohydrate at the dose of 15 mg/kg body weight. All the animals were observed following the treatment and were sacrificed at about 48 hours after the last treatment.

During the study period the animals were monitored for mortality, clinical signs and body weight. Blood from each mouse was collected, fixed and stained with Anti-Mouse CD71 and Anti-Rodent CD61 antibodies. About twenty thousand polychromatic erythrocytes (PCEs, i.e. immature erythrocytes) per animal were examined using flow cytometer to detect the incidence of micro-nucleated PCEs (MN-PCEs). In addition, the proportion of immature erythrocytes was assessed for each animal as a measure of potential toxicity.

Mutagenicity of OEA

In this Ames Test, oleoylethanolamide was evaluated to determine its ability to induce reverse mutation at selected histidine loci in five tester strains of Salmonella typhimurium viz. TA1535, TA97a, TA98, TA100 and TA102 in the presence and absence of metabolic activation system (S9). Dimethyl sulphoxide was used as a vehicle.

Liver S9 fraction, induced in rats with combination of sodium phenobarbitone and 8-naphthoflavone, was used as the metabolic activator. The exposed bacteria were plated onto minimal glucose agar medium supplemented with L-histidine D-biotin solution. The plates were incubated at 37 °C for about 49 hours after which the histidine revertant colonies were counted and their frequency was compared with that in vehicle control group. Concurrent positive control group was also included in all the experiments as specified by the guideline. Based on the results obtained in preliminary tests which were conducted to assess the solubility / precipitation and to assess the cytotoxicity to the tester strains

Results

Evaluation of acute dose range of OEA

Oleoylethanolamide at up to 4000 mg/kg body weight did not induce any abnormal clinical signs in the male and female rats and did not cause any deaths amongst the treated rats. Although the group mean body weights were statistically comparable (P>0.05) on days 4 and 7 of the study, oleoylethanolamide induced a remarkable lowering of body weight gain in male rats treated at the dose of 4000 mg/kg body weight. Similar reduction was not observed in female rats at the highest dose, while a mild reduction observed in females treated at 3000 mg/kg was considered as being possibly incidental.

Oleoylethanolamide at up to 4000 m/kg body weight did not induce any adverse changes with respect to the hematology parameters viz. hemoglobin (Hb), hematocrit (PCV), erythrocyte count, total erythrocyte count (total RBC), total leukocyte count (total WBC), differential leukocyte (WBC) count, erythrocyte indices viz. mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count and total platelet count of the treated rats. Few hematological parameters of high dose male and female rats treated at 4000mg/kg body weight, were statistically significant (P<0.05), and those occurring in the control group were considered to be incidental.

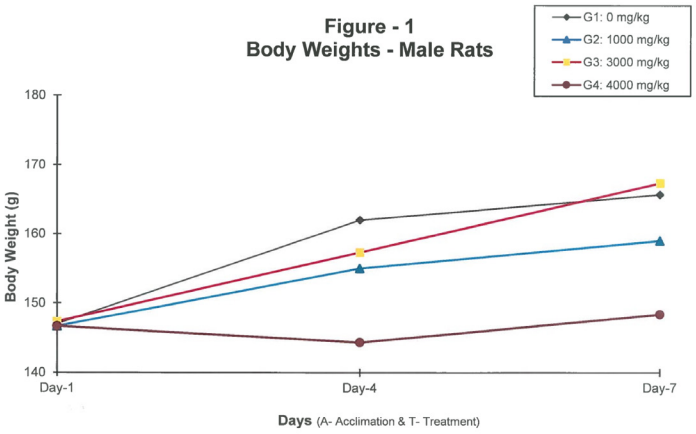


Figure 1: Body Weight Chart (Male Rats).

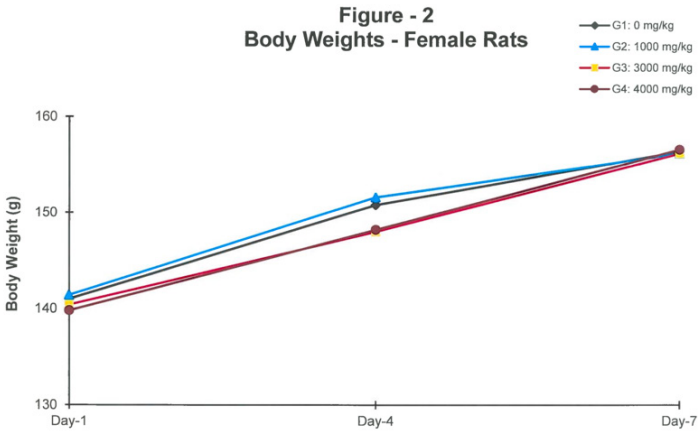


Figure 2: Body Weight Chart (Female Rats).

Treatment of rats with oleoylethanolamide up to the dose of 4000 m/kg body weight did not induce any adverse changes with respect to their plasma levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, protein (total), albumin, glucose creatinine, sodium, potassium and urea nitrogen.

The necropsy examinations did not reveal any gross pathological alterations in any tissues / organs examined at termination of the study period, or any changes suggestive of systemic toxicity.

Evaluation of Chronic Dose Range of OEA

The administration of oleoylethanolamide to Wistar IGS rat by oral gavage over a period of 28 days at doses of 500, 1000, 2000 and 3000 mg/kg body weight resulted in no remarkable clinical abnormalities in the male and female rats treated at and up to the dose of 3000 mg/kg. There were no treatment-related deaths.

Table 1 Summary of Haematology Data Male Rats

Group	Dose mg/kg		Hb (g/dl)	PCV (%)	Total RBC ($X10^6$/cmm)	RBC Indices			Total WBC ($X10^3$/cmm)	Differential WBC(%)					Platelets ($X10^3$/cmm)
						MCH (pg)	MCV (fl)	MCHC (g/dl)		N	L	M	E	B	
Vehicle Control															
G1	0	Mean	13.77	45.57	7.61	18.10	59.90	30.21	10.14	13.53	80.40	3.10	0.63	0.67	1240.33
		±S.D.	0.12	0.38	0.08	0.10	0.38	0.03	0.91	3.87	3.39	0.52	0.40	0.12	52.54
		n	3	3	3	3	3	3	3	3	3	3	3	3	3
Test Item: Oleoylethanolamide															
G4	4000	Mean	**14.83**$^{S+}$	**48.20**$^{S+}$	**8.29**$^{S+}$	17.93	58.28	30.78	9.87	16.37	73.60	4.53	2.93	**0.93**$^{S+}$	1186.67
		±S.D.	0.15	0.85	0.39	1.04	3.68	0.26	1.19	3.97	3.90	1.53	1.86	0.06	141.05
		n	3	3	3	3	3	3	3	3	3	3	3	3	3

S+: Values significantly higher($p<0.05$) than the control group.

Table 2 Summary of Haematology Data Female Rats

Group	Dose mg/kg		Hb (g/dl)	PCV (%)	Total RBC ($X10^6$/cmm)	RBC Indices			Total WBC ($X10^3$/cmm)	Differential WBC(%)					Platelets ($X10^3$/cmm)
						MCH (pg)	MCV (fl)	MCHC (g/dl)		N	L	M	E	B	
Vehicle Control															
G1	0	Mean	14.33	45.80	7.91	18.14	58.00	31.29	10.00	16.70	76.20	3.67	1.03	0.73	1262.0
		±S.D.	0.55	1.28	0.52	0.54	2.16	0.37	0.33	1.35	2.60	0.68	1.02	0.15	104.79
		n	3	3	3	3	3	3	3	3	3	3	3	3	3
Test Item: Oleoylethanolamide															
G4	4000	Mean	13.77	45.17	7.69	17.91	58.78	**30.48**$^{S-}$	11.35	13.90	76.53	4.30	0.67	0.93	1250.67
		±S.D.	0.67	1.90	0.54	0.47	1.65	0.32	3.95	6.02	3.72	1.35	0.45	0.49	87.08
		n	3	3	3	3	3	3	3	3	3	3	3	3	3

S-: Values significantly lower ($p<0.05$) than the control group.

Table 3: Summary of Overall Food Consumption.

Sex	Group	G1 (Vehicle Control)	G2 500 mg/kg	G3 1000 mg/kg	G4 2000 mg/kg	G5 3000 mg/kg
Male Rats	Average*	25.3	26.0	23.3	23.4	20.9
	As% of Control	100	103	92	93	83
Female Rats	Average*	18.9	19.0	17.7	16.5	16.5
	As% of Control	100	101	94	87	87

*Study average of four weeks.

Table 4: Statistically Significant Haematological Alternation.

Sr. No.	Parameter	Sex, Group and Change	Mean ± SD (control mean in parenthesis)	Remark	Conclusion
1	Total WBC count ($x10^3$ cell / µL	Males G5 ↑	14.75 ± 0.88 (8.71±1.32)	Not dose dependent; comparable to the historical control range of 5.27 to 14.33	Incidental/ Induced, but Non-adverse
2	Eosinophill (%)	Males G5 ↓	0.63 ± 0.05 (1.36±0.47)	Values comparable to HCR of 0.87 to 1.91	Incidental

↑or↓: Mean value is higher (↑) or lower(↓) than the control group mean at $P<0.05$.
*HCR – Historical control range in the test facility for Wistar SPF rats (ADVIA 2120I Haematology System, Siemens Health Diagnosis Ltd.)

Table 5: Statistically Significant (P<0.05) Clinical Chemistry Alternations (Female Rats).

Sr. No.	Parameter (unit)	Group and Change	Mean ± SD (control mean in parenthesis)	Remark	Conclusion
1	Total Protein (g/dL)	G5↓	6.64 ± 0.25 (7.56 ± 0.39)	Somewhat dose dependent; Well within historical control ranges of 5.5 to 8.2 (Total Protein), 4.9 to 6.7 (Globulin); 0.0 to 0.3 (A/G Ratio). With lowered total protein, and minimally elevated albumin levels, the globulin levels show minimal lowering.	Could be Induced, but non-adverse
2	Globulin (g/dL)	G3↓ G5↓	G3: 5.64 ± 0.55 G5: 5.40 ± 0.33 (6.56 ± 0.60)		
3	Albumi/Globulin (A/G) Ratio	G3↑ G5↑	G3: 0.22 ± 0.01 G5: 0.23 ± 0.04 (0.18 ± 0.04)		
4	ALT(IU/L)	G3↑ G4↑ G5↑	G3: 69.00 ± 14.92 G4: 72.80 ± 25.65 G5: 77.60 ± 39.43 (29.40 ± 4.51)	Dose dependent increase; Higher than historical control range of 24 to 43. Correlated to a 12-14.5% increase in relative liver weights in female rats in these groups. But no histopathological correlation noted in liver.	Induced, non adverse effect
5	Sodium (mmol/L)	G5↑	140.80 ± 1.30 (138.40 ± 0.55)	Well within historical control range of 132 to 155; No correlated changes.	Incidental
6	Phosphorus (mg/dL)	G4↑ G5↑	G4 : 6.60 ± 0.44 G5: 6.86 ± 0.25 (5.74 ± 0.29)	Somewhat dose dependent; Well within historical control range of 5.1 to 8.8	Could be induced but non-adverse

control group value is presented in parenthesis
↑and↓: Mean value is lower(↓)or higher(↑) than the control group mean at P<0.05.
HCR – Historical control range in the test facility for this strain of rat but from a different source(Taconic Inc).

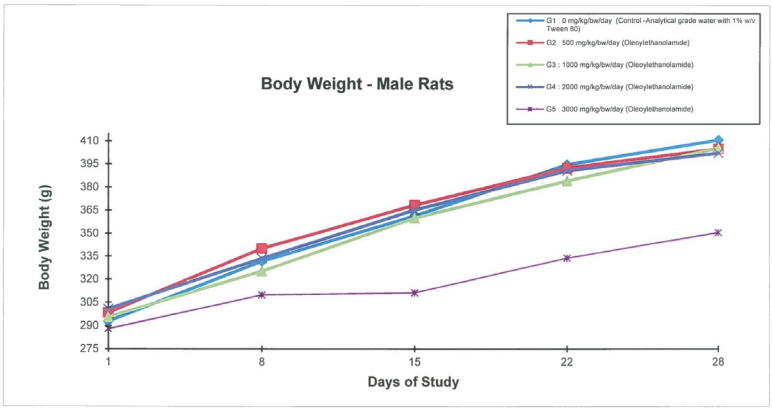


Figure 3: Body Weight (Male Rats).

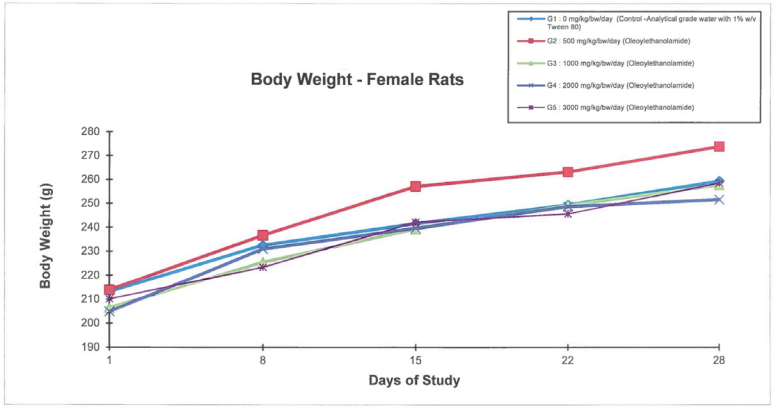


Figure 4: Body Weight (Female Rats).

Although there was a mild (about 15%) lowering of body weight gain by male rats up to the dose of 3000 mg/kg, no effects were recorded on the body weight gain by female rats treated at and up to the dose of 3000 mg/kg. There was a mild lowering (11-14%) of food intake by male and female rats dosed at 3000 mg/kg body weight, and by female rats dosed at 2000 mg/kg body weight.

A statistically significant increase in the total white blood cells counts of male rats treated at the dose of 3000 mg/kg was recorded but assessed to be possibly incidental and non-adverse. There was also a statistically non-significant, mild (about 11% to 14.4%) increase in the absolute and relative liver weights of only the female rats, at the doses of 1000 mg/kg, 2000mg/kg and 3000 mg/kg body weight. This was considered to be a physiological response of liver, and non-adverse in nature.

A dose-related increase in alanine aminotransferase (ALT) levels (1.3 to 1.6 fold of control) was found only in female rats, at the doses of 1000 mg/kg, 2000 mg/kg and 3000 mg/kg body weight. There were no gross or microscopic pathological alterations in the liver, nor any sign of systemic toxicity.

Results of Acute Oral Toxicity of OEA

The test item did not cause any mortality and did not induce any abnormal clinical signs in the rats treated at 175 mg/kg, 550 mg/kg and 2000 mg/kg body weight.

Body weight gain in the rats was not affected during the 14days observation period post dosing. OEA did not induce any gross pathological alterations in any of the rats, as evident during their necropsy.

The LD 50 with 95% confidence intervals could not be determined due to absence of mortality amongst the treated rats. Therefore, an estimation has been presented: LD50 is greater than 2000 mg/kg body weight.

Table 6: AOT425stspgm (Version1.0) Test Results.

Test sequence	Animal ID	Dose(mg/kg)	Short-term Results	Long Term Results
1	RI2881	175	O	O
2	RI2882	500	O	O
3	RI2883	2000	O	O
4	RI2884	2000	O	O
5	RI2885	2000	O	O

(X=Died, O=Survived)

Results of Chronic toxicity of OEA

The administration of oleoylethanolamide resulted in no incidence of death and no remarkable clinical or ophthalmological abnormalities in male and female rats treated at and up to the dose of 2500 mg/kg. There were no significant / abnormal alterations in qualitative and quantitative neurological parameters and locomotor activity. There was no effect on body weights, weight gain and food consumption.

In hematological evaluations, no adverse changes were observed with respect to values of hemoglobin, hematocrit (PCV), total red blood cell (RBC) counts, erythrocyte indices (MCV, MCH, MCHC), total and differential white blood cell (WBC) counts, reticulocyte counts, platelet counts and coagulation parameters (prothrombin time and activated partial thromboplastin time) and in the morphology of their blood cells.

In clinical analysis and urinalysis, no adverse changes were observed with respect to plasma levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, glucose, total protein, albumin, globulin, albumin/globulin (A/G) ratio (calculated), urea nitrogen and urea (calculated), gamma glutamyl transpeptidase, creatinine, sodium, potassium, calcium, phosphorus, total bile acid, triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL). There was no alternation of urinalyses parameters.

In an endocrine evaluation, thyroid and reproduction hormone levels were monitored. The administration of oleoylethanolamide did not alter levels of thyroid hormones (T4, T3 and TSH) in the rats. There was no change in thyroid weights and no evidence of gross and microscopic pathology in thyroids. There were no abnormal clinical signs or body weight alterations suggestive of thyroid dysfunction. Oleoylethanolamide did not alter levels of the reproductive hormones FSH, testosterone, LH and estradiol.

No adverse effects were shown on absolute and relative (% of body weight) weights of liver, kidneys, adrenals, testes / ovaries, epididymis, prostate + seminal vesicles with coagulating gland, uterus with cervix, brain, spleen, thymus, heart, pituitary gland and thyroid gland (fixed). There were no gross pathological or histopathological alterations suggestive of systemic toxicity.

In the reproductive function panel, no significant changes were seen re. sperm concentration and sperm motility parameters including motile sperms, immotile sperms, total sperms, motility rate (%), straight line velocity (VSL-µm/sec), average path velocity (VAP- µm/sec), curvilinear velocity (VCL- µm/sec), linearity, straightness, amplitude of lateral head displacement (ALH- µm) and beat cross frequency (BCF). Organ weights, gross and microscopic pathology of reproductive organs along with assessment of stages of oestrus cycle of female rats, and levels of the reproductive hormones, did not show any sign of reproductive toxicity. In immune toxicity evaluation, administration of oleoylethanolamide did not adversely affect the immune system of male and female rats in this study, and did not present any potential immunotoxic hazard.

Results of *In-Vitro* Mammalian Chromosome Aberration Test

The test item induced 6.91, 17.59 and 21.51% cytotoxicity on the cultured human lymphocytes at the highest test concentrations of 62.5 pg/mL in experiment no. 1 and 31.25 pg/mL in experiment no. 2 and 3 respectively in the main study.

When the treated groups were compared to the vehicle control group, no significant ($p > 0.05$) increase was observed in the incidence of structural chromosome aberrations in the human peripheral blood lymphocytes, exposed at the concentrations

COMPUTATION OF MITOTIC INDICES

Experiment No.1
Treatment period: 3 hours Without S9

Group / Concentration (µg/mL)	Sr. No.	Slide ID.	No. of MPs	Total No. of Cells	Mitotic Index		
					per Slide	Average	% Lowering Over Vehicle Control
Vehicle Control (DMSO)	A	VC-1	96	1000	9.60		
	B	VC-4	92	1000	9.20		
	Total		**188**	**2000**		**9.40**	-
Treatment 62.5	A	62.5-4	87	1000	8.70		
	B	62.5-2	88	1000	8.80		
	Total		**175**	**2000**		**8.75**	6.91
Treatment 31.25	A	31.25-2	82	1000	8.20		
	B	31.25-2	84	1000	8.40		
	Total		**166**	**2000**		**8.30**	11.70
Treatment 15.625	A	15.625-1	82	1000	8.20		
	B	15.625-1	90	1000	9.00		
	Total		**172**	**2000**		**8.60**	8.51
Positive Control (MMS) 30	A	PC-4	94	1000	9.40		
	B	PC-1	90	1000	9.00		
	Total		**184**	**2000**		**9.20**	2.13

MPs = Metaphases
MMS = Methyl Methane Sulphonate

Figure 6: Cytotoxicity Results Experiment No. 1.

COMPUTATION OF MITOTIC INDICES

Experiment No.2
Treatment period: 24 hours Without S9

Group / Concentration (µg/mL)	Sr. No.	Slide ID.	No. of MPs	Total No. of Cells	Mitotic Index		
					per Slide	Average	% Lowering Over Vehicle Control
Vehicle Control (DMSO)	A	VC-2	98	1000	9.80		
	B	VC-2	101	1000	10.10		
	Total		**199**	**2000**		**9.95**	-
Treatment 31.25	A	31-2	83	1000	8.30		
	B	31-2	81	1000	8.10		
	Total		**164**	**2000**		**8.20**	17.59
Treatment 15.625	A	15.625-2	92	1000	9.20		
	B	15.625-2	95	1000	9.50		
	Total		**187**	**2000**		**9.35**	6.03
Treatment 7.8125	A	7.8125-1	94	1000	9.40		
	B	7.8125-1	97	1000	9.70		
	Total		**191**	**2000**		**9.55**	4.02
Positive Control (MMS) 30	A	PC-1	95	1000	9.50		
	B	PC-1	90	1000	9.00		
	Total		**185**	**2000**		**9.25**	7.04

MPs = Metaphases
MMS = Methyl Methane Sulphonate

Figure 7: Cytotoxicity Results Experiment No. 2.

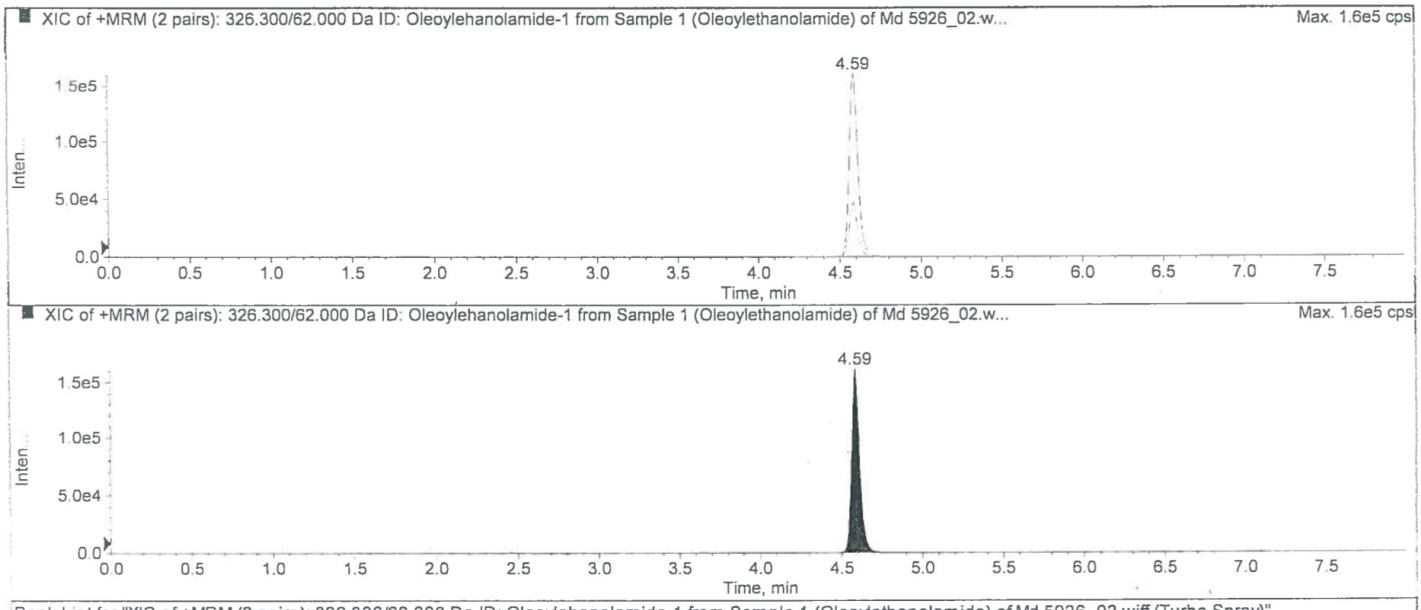
COMPUTATION OF MITOTIC INDICES

Experiment No.3
Treatment period: 3 hours With S9

Group / Concentration (µg/mL)	Sr. No.	Slide ID.	No. of MPs	Total No. of Cells	Mitotic Index		
					per Slide	Average	% Lowering Over Vehicle Control
Vehicle Control (DMSO)	A	VC-1	92	1000	9.20		
	B	VC-1	94	1000	9.40		
	Total		**186**	**2000**		**9.30**	-
Treatment 31.25	A	31-1	70	1000	7.00		
	B	31-1	76	1000	7.60		
	Total		**146**	**2000**		**7.30**	21.51
Treatment 15.625	A	15.625-3	78	1000	7.80		
	B	15.625-3	80	1000	8.00		
	Total		**158**	**2000**		**7.90**	15.05
Treatment 7.8125	A	7.8125-1	75	1000	7.50		
	B	7.8125-1	83	1000	8.30		
	Total		**158**	**2000**		**7.90**	15.05
Positive Control (CPM) 60	A	PC-2	84	1000	8.40		
	B	PC-2	81	1000	8.10		
	Total		**165**	**2000**		**8.25**	11.29

MPs = Metaphases
CPM-Cyclophosphamide monohydrate

Figure 8: Cytotoxicity Results Experiment No. 3.



Peak List for "XIC of +MRM (2 pairs): 326.300/62.000 Da ID: Oleoylehanolamide-1 from Sample 1 (Oleoylethanolamide) of Md 5926_02.wiff (Turbo Spray)"

	Time (min)	Area (counts)	% Area	Height (cps)	% Height	Width (min)	Baseline Type
1	4.5857	5.8674e5	100.0000	1.6200e5	100.0000	0.2665	Base to Base

Figure 9: Chromatogram - Plasma Analysis.

of 62.5, 31.25 and 15.625 pg/mL in the absence of metabolic activation system for the exposure of 3 hours and 31.25, 15.625 and 7.8125 pg/mL presence of metabolic activation system for the exposure of 3 hours and in the absence of metabolic activation system for 24 hours.

Positive Controls: Sensitivity of the test system and activity of S9 mix were demonstrated in the positive control group. The positive controls, directly acting mutagen Methyl methane sulphonate and the promutagen cyclophosphamide monohydrate, induced a significant increase ($p < 0.05$) in chromosome aberration frequencies over the concurrent controls.

Results of Mammalian *In-Vivo* Eryhtrocyte Micronucleus Test

There were no overt abnormal clinical signs in any of the groups. All treated animals survived till termination.

The average percentage incidence of PCEs (mean ± SD) was 1.39 ± 0.26 in mice treated with oleoylethanolamide at the dose level of 2000 mg/kg body weight, while the corresponding values in vehicle and positive control groups were 1.37 ± 0.23 and 0.84 ± 0.19 respectively.

The average percentage incidence of micronucleated PCEs (mean ± SD) among the total PCEs was 0.19 ± 0.03 in mice treated with oleoylethanolamide at the dose level of 2000 mg/kg body weight, while the corresponding values in vehicle and positive control groups were 0.20 ± 0.02 and 0.83 ± 0.19 respectively. Results of the present study indicate that there was no increase in the percentage of micronucleated (immature) erythrocytes in the peripheral blood of mice treated with oleoylethanolamide at the dose of 2000 mg/kg body weight ($p > 0.05$). The positive control (cyclophosphamide monohydrate) significantly increased ($p < 0.05$) micronuclei in immature erythrocytes (MN-PCE). The vehicle and the positive control results were as expected, confirming the sensitivity of the test system and the validity of the assay.

Table 7: Proportion of Immature Erythrocytes and Micronucleated Erythrocytes.

Group/Treatment		Dose mg/kg	Parameters	Incidence	
				%PCE	%MN-PCE
G2	Analytical grade water with 1% Tween 80 (Vehicle Control)	10	Mean	1.37	0.20
			SD	0.23	0.02
			No. of Animals	5	
G3	Oleoylethanolamide (Test Item)	2000	Mean	1.39	0.19
			SD	0.26	0.03
			No. of Animals	5	
G4	Cyclophosphamide Monohydrate (Positive Control)	15	Mean	0.84*	0.83*
			SD	0.19	0.19
			No. of Animals	5	

Sampling time: ~ 48 hours after the last dose
*: $p<0.05$
PCE: Polychromatic erythrocyte
Dose Volume: 10 mL/kg body weight
SD – Standard deviation
MN-PCE: Micronucleated PCE

Analysis of plasma samples confirmed that animals were systemically exposed to oleoylethanolamide. In the plasma samples collected at 1 hour, 3.5 hours and 5 hours after second dosing, the average concentration of oleoylethanolamide was 0.0004 mg/mL, 0.0001 mg/mL and 0.0001 mg/mL respectively.

Results of Mutagenicity of OEA

Histidine revertant colonies in the treatment groups in tester strain TA97a, TA98, TA100, TA102 and TA1535 at all concentrations were found to be within the expected frequency ranges, comparable to those in the vehicle control group and with the laboratory historical control data at INTOX, both in presence and absence of metabolic activation system.

Observations during both the experiments did not reveal any dose dependent or two-fold (3-fold for TA1535) increase when compared with the vehicle control group according to the criteria of evaluation findings of this AMES test during plate incorporation method.

Plate counts for the spontaneous histidine revertant colonies in the vehicle control groups were comparable with the range reported in the literature. Concurrent positive controls demonstrated sensitivity of the assay with and without metabolic activation.

Discussion

OEA is a simple derivative of oleic acid with promising potential as an anti-obesity and anti-inflammatory compound, with immuno-modulatory applications [13]. The activation of PPAR-a receptors, TRPV1 and G protein coupled receptor GPR119 are considered to be responsible for its anti-inflammatory and anorexigenic effects [14]. OEA's ability to reduce liposaccharide-induced oxidative/nitrosative stress and to prevent endothelial cell damage[13] introduces other potential applications. These cannot be developed, however, without adequate safety data.

In a very limited number of previous clinical trials, doses of 250 mg/d of OEA oral intake exhibited satisfactory safety when compared to placebo for 3 months [15,16]. However, a detailed safety profile for this compound has not been previously available. In this paper we aimed to bridge the gap between the pre-existing science and the wider clinical use of OEA by providing a robust safety profile including acute, chronic, pre-natal and mutagenic toxicity studies.

Based on the 7-day acute dose range study, 28-days chronic dose range study, acute oral toxicity study and 180-days toxicity study, it is concluded that OEA did not induce any adverse alterations at and up to the highest tested dose of 2500 mg/kg b.wt./day body weight. The No Observed Adverse Effect Level (NOAEL) of oleoylethanolamide is found to be 2500 mg/kg b.wt./day body weight under tested conditions.

In the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL), OEA induced no

significant increase in the incidence of chromosome aberrations using HBPL over the tested range. In the in vivo murine Micronucleus Test, OEA did not increase the percentage of micronucleated (immature) erythrocytes in the peripheral blood of mice at the dose of 2000 mg/kg body weight. In the mutagenicity test, the tester strains TA1535, TA97a, TA98, TA100 and TA102 did not reveal any dose dependent or 2-fold (3-fold for 1535) increase at any of the test concentrations in the study. OEA is therefore determined to be non-mutagenic.

Based on the above results, OEA is considered to be safe up to dosage at 2500 mg/kg b.wt./day body weight and can be further developed for future applications.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship and/or publication of this article: the study was conducted at INTOX Private LTD., 357, Urawade Tal. Mulshi, Maharastra India. SS and S are associated with Gencor Pacific Limited. Paul Clayton provides occasional consulting services to Gencor Pacific Limited.

Funding

The author(s) disclosed receipt of the following financial support for research, authorship, and/or publication of this article: The study was conducted under a grant from Gencor Pacific Limited, North Plaza, Discovery Bay, Lantau Island, Hong Kong.

References

1. Herrera MI, Kölliker-Frers R, Barreto G, et al. Glial Modulation by N-acylethanolamides in Brain Injury and Neurodegeneration. Front Aging Neurosci. 2016; 8: 81.
2. Hu SS, Mackie K. Distribution of the Endocannabinoid System in the Central Nervous System. Handb Exp Pharmacol. 2015; 231: 59-93.
3. Schwartz GJ, Fu J, Astarita G, et al. The lipid messenger OEA links dietary fat intake to satiety. Cell Metab. 2008; 8: 281-288.
4. Thabuis C, Tissot-Favre D, Bezelgues JB, et al. Biological functions and metabolism of oleoylethanolamide. Lipids. 2008; 43: 887-894.
5. Reyes-Cabello C, Alen F, Gómez R, et al. Effects of the anandamide uptake blocker AM404 on food intake depend on feeding status and route of administration. Pharmacol Biochem Behav. 2012; 101: 1-7.
6. Antón M, Alén F, Gómez de Heras R, et al. Oleoylethanolamide prevents neuroimmune HMGB1/TLR4/NF-kB danger signaling in rat frontal cortex and depressive-like behavior induced by ethanol binge administration. Addict Biol. 2017; 22: 724-741.
7. van Kooten MJ, Veldhuizen MG, de Araujo IE, et al. Fatty acid amide supplementation decreases impulsivity in young adult heavy drinkers. Physiol Behav. 2016; 155: 131-140.
8. Herrera MI, Kölliker-Frers R, Barreto G, et al. Glial Modulation by N-acylethanolamides in Brain Injury and Neurodegeneration. Front Aging Neurosci. 2016; 8: 81.
9. Pérez-Martín E, Pérez-Revuelta L, Barahona-López C, et al. Oleoylethanolamide Treatment Modulates Both Neuroinflammation and Microgliosis and Prevents Massive Leukocyte Infiltration to the Cerebellum in a Mouse Model of Neuronal Degeneration. Int J Mol Sci. 2023; 24: 9691.
10. Orio L, Alen F, Pavón FJ, et al. Oleoylethanolamide Neuroinflammation and Alcohol Abuse. Front Mol Neuro sci. 2019; 11: 490.
11. Plaza-Zabala A, Berrendero F, Suarez J, et al. Effects of the endogenous PPAR-alpha agonist oleoylethanolamide on MDMA-induced cognitive deficits in mice. Synapse. 2010; 64: 379-389.
12. Stahel PF, Smith WR, Bruchis J, et al. Peroxisome proliferator-activated receptors key regulators of neuroinflammation after traumatic brain injury. PPAR Res. 2008; 2008: 538141.
13. García Bueno B, Caso JR, Madrigal JL, et al. Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases. Neurosci Biobehav Rev. 2016; 64: 134-147.
14. Karwad MA, Macpherson T, Wang B, et al. Oleoylethanolamine and palmitoylethanolamine modulate intestinal permeability in vitro via TRPV1 and PPARα. FASEB J. 2017; 31: 469-481.
15. Ghaffari S, Roshanravan N, Tutunchi H, et al. Oleoylethanolamide A Bioactive Lipid Amide as A Promising Treatment Strategy for Coronavirus/COVID-19. Arch Med Res. 2020; 51: 464-467.
16. Romano A, Coccurello R, Giacovazzo G, et al. Oleoylethanolamide a novel potential pharmacological alternative to cannabinoid antagonists for the control of appetite. Biomed Res Int. 2014; 2014: 203425.
17. Tutunchi H, Ostadrahimi A, Saghafi-Asl M, et al. Oleoylethanolamide supplementation in obese patients newly diagnosed with non-alcoholic fatty liver disease Effects on metabolic parameters anthropometric indices and expression of PPAR-α UCP1 and UCP2 genes. Pharmacol Res. 2020; 156: 104770.
18. Payahoo L, Khajebishak Y, Asghari Jafarabadi M, et al. Oleoylethanolamide Supplementation Reduces Inflammation and Oxidative Stress in Obese People A Clinical Trial. Adv Pharm Bull. 2018; 8: 479-487.