

## Pharmacokinetic and Safety Evaluation of MBZM-N-IBT, A Lead Against Chikungunya Virus

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Chikungunya virus (CHIKV) infection was previously found to be inhibited by MBZM-N-IBT both *in vitro* and *in vivo*. To further assess its suitability for *in vivo* application, toxicity and pharmacokinetics were investigated. It showed no acute toxicity orally with an estimated LD50 of more than 5000 mg/kg in rats. While it showed toxicity at 1000 mg/kg in the chronic toxicity study, it was better tolerated at 500 mg/kg by rats. At 50 mg/kg, it was found to be safe in a 9-month study. A pharmacokinetic study revealed T<sub>max</sub> less than the gastric emptying time. High plasma protein binding supported its higher elimination half-life. *In silico* analysis predicted 22 metabolites. The majority of these metabolites fall in OECD class 5 and support the low toxicity of MBZM-N-IBT.

**Keywords:** Antiviral; MBZM-N-IBT; Pharmacokinetic; Toxicity.

Infection by Chikungunya virus (CHIKV) is categorised by WHO as a “neglected tropical disease” (WHO, 2014)<sup>1</sup>. Although it has a tropical origin, it is no longer limited to tropical areas. Gradually, it has become a global pathogen in many countries with different climatic conditions<sup>2,3</sup>. Although no specific antiviral is approved for its management, there have been continued efforts to identify potential drug candidates for further development as antiviral for CHIKV<sup>4-9</sup>. Our group recently patented MBZM-N-IBT (Figure 1) for its *in vitro* effects against CHIKV infection (Indian Patent No. 347450). Chemically it is 1-[(2-methyl benzimidazole-1-yl) methyl]-2-oxo-indolin-3-ylidene] amino] thiourea<sup>10</sup>. Unlike the prior art in

its chemical class (methisazone), it was reported as a potent inhibitor of CHIKV<sup>10</sup>.

In addition to anti-CHIKV properties, it was shown as a potent inhibitor of the Herpes Simplex Virus (HSV1) *in vitro*<sup>11</sup>. The effects against both RNA (CHIKV) and DNA (HSV) virus suggest its broad spectrum of action and potential for further progress as an antiviral candidate. Pharmacokinetic study plays a vital role in drug development as it helps in the prediction of efficacy and toxicity-related events. Thus, to better understand the toxicity and the effectiveness of MBZM-N-IBT *in vivo*, it is necessary to assess its pharmacokinetics through a rapid, simple, and effective bio-analytical method<sup>12</sup>. MBZM-N-IBT

is a chemical substance and may cause acute toxicity within 24 hours following single-dose administration. When continuously administered, it could lead to chronic toxicity in the living system when used over a more extended period of time.<sup>13,14</sup> A key criterion to assess acute toxicity is the determination of the lethal dose (LD<sub>50</sub>), which is defined as the dose that kills 50% of the population of test animals.

Furthermore, it appears as a guiding dose for *in vivo* efficacy and toxicity studies<sup>15</sup>. Accordingly, the LD<sub>50</sub> was estimated in the present study before assessing the chronic toxicity. *In silico* models have been widely used during the toxicology analysis<sup>16-18</sup> to reduce safety-related attrition in drug development. So, the metabolites of MBZM-N-IBT were predicted through *in silico* models, and their potential toxicity was assessed to support the *findings of in vivo* toxicology. With these thoughts in mind, the goal of the current investigation was to evaluate both the toxicity and pharmacokinetics of MBZM-N-IBT to assess its potential for further progress as an antiviral candidate.

## MATERIAL AND METHODS

### Chemicals and Reagents

MBZM-N-IBT was synthesized and purified by column chromatography following our established protocol<sup>10</sup>. The purity (Å98%) was monitored by HPLC. Metformin, Metronidazole, Ranitidine, Paracetamol, Phenobarbital, Cefixime, Salicylic acid, Cetrizine, omeprazole, and Losartan were all obtained from industry with high purity (Å95%). The HPLC-grade solvents, such as acetonitrile, water, and methanol, have been purchased from Merck, India. Drugs/test compounds were added to 1% carboxy methyl cellulose (CMC) in distilled water at room temperature, and fresh crude suspensions were prepared.

### Animals and Ethical Statement

The Institutional Animal Ethics Committee (IAEC) of the School of Pharmaceutical Sciences of Siksha O Anusandhan (Deemed to be University), Bhubaneswar, India, (Reg. no. 1171/PO/Re/S/08/ CPCSEA) granted its approval to the experimental protocol. The Committee for the Purpose of Control and Supervision of Experiments

on Animals (CPCSEA) approved all experiments and supervised the way they were conducted. To evaluate acute and chronic toxicity, healthy Wistar albino rats of either sex, around 150-200g, were used. They were kept in polyacrylic cages with 12-hour light/dark cycles at optimal temperatures of 25±2°C and 60±5% relative humidity. The animals had complete access to commercially available food and water.

### Animal grouping and husbandry

Animals were grouped into subgroups according to the experiments after seven days of acclimation. For the acute toxicity study, animals were divided into 4 groups arbitrarily (n=3, female rats). For the chronic toxicity study, animals (both male and female rats) were randomly divided according to the dose and the time limit of the experiment. The *in vivo* pharmacokinetic study was performed by taking albino rats at each time point (n=6). All of the animals were housed in polypropylene cages with stainless steel covers that contained water bottles and feeders.

### Dosing

MBZM-N-IBT was mixed with 1% carboxy methyl cellulose to achieve the desired concentration. Dosing formulations were stored at 4°C. The formulations were brought to room temperature and thoroughly mixed before administration.

### Acute oral toxicity of MBZM-N-IBT

Following the OECD-423 guidelines for testing chemicals (OECD, 2000)<sup>19,20</sup>, this experiment was carried out to estimate the LD<sub>50</sub> of MBZM-N-IBT. Following an overnight fast of approximately 15 to 16 hours, all the animals were given a single oral dose of MBZM-N-IBT with unrestricted access to water. The dose volume was 10 mL/kg. The starting dose was 50 mg/kg body weight. The animals were next examined with 300 mg/kg and 2000 mg/kg single oral doses. The toxic impact of each and every experimental animal was observed for the entire day. For the first 4h of daily administration, animals were critically monitored for any signs of toxicity. The changes in behavior, lethargy, appearance, salivation, illness, body weight, and mortality were examined during this period. The animals were further observed for 14 days.

### Chronic oral toxicity of MBZM-N-IBT

Chronic toxicity was conducted as per

the OECD-452 guidelines (OECD, 2008)<sup>21</sup>. As per the recommendations of this guideline, the highest dose that can be administered is 1000 mg/kg. The animals were divided into 4 groups arbitrarily. Each group contained male and female rats (n=20 for each sex). The Group-I (normal control) rats received 1% CMC. Group II, III, and IV rats received oral MBZM-N-IBT at 1000 mg/kg, 500 mg/kg, and 50 mg/kg doses. All doses were administered once per day with a fixed schedule. Animals were observed for gross behavioral, neurological, and autonomic effects. Also, changes in eyes, skin/fur, salivation, sleep, diarrhea, coma, and mortality were noted twice daily throughout the testing period. On a weekly basis, the rats' body weight was monitored. After the completion of the period, ketamine was injected intraperitoneally, and rats were euthanized. For histological analysis, vital organs like the liver, heart, lung, and kidney were carefully excised, weighed, and preserved in 10% buffered formalin.

#### **Bioanalytical method development and Pharmacokinetics of MBZM-N-IBT**

A binary gradient HPLC (Shimadzu, Japan) was employed, along with an LC-20AD pump and SPD-M20A prominence diode array detector. Samples were injected into a KROMASIL PROCHROME C18 analytical column (4.6 × 150mm, 5µm) using a Hamilton SYR 25iL syringe after being filtered through a syringe-driven nylon membrane filter (0.22µ, 13mm). With an isocratic flow rate of 1.0mL/min and water methanol (50: 50 v/v) as the mobile phase, the estimation of MBZM-N-IBT was carried out. The mobile phase was degassed in an ultrasonic bath after being filtered through a Millipore vacuum filter system with a 0.45µm membrane filter. Each injection ran for ten minutes. At 360nm, the analytes were observed. At ambient temperature, all experiments were carried out. Following ICH guidelines Q2 (R1), a reversed-phase HPLC analytical technique was developed and validated for the quantitative determination of MBZM-N-IBT from bulk<sup>22</sup>. The linearity, accuracy, precision, LOD, and LOQ were determined. Again, this method was verified and re-validated for assay from rat plasma matrix following USFDA guidelines for bio-analytical method validation<sup>23,24</sup>. The calibration curve was used to estimate MBZM-N-IBT in plasma. The *in vivo* Pharmacokinetic study was performed by

taking three albino rats at each time point (n=6). Animals were orally administered with MBZM-N-IBT 50 mg/kg body weight. At appropriate time points (0.5, 1, 1.5, 2, 4, 8, 12, 18, and 24 hr), the animals were euthanized under light ether anesthesia, and blood was obtained through the cardiac puncture, which was then collected into a pre-treated vial containing EDTA. Following blood collection, the samples were centrifuged at 15,000 rpm for 15 minutes at 4°C to separate the plasma. Then, clear supernatants were collected and subjected to deproteinization (Acetonitrile).

The collected samples were preserved at -80°C until they were analyzed. Different pharmacokinetic parameters have been determined, including AUC, AUMC, MRT,  $C_{max}$ ,  $T_{max}$ , and  $T_{1/2}$ .

#### **Protein binding study**

Plasma protein binding (PPB) was evaluated using a modified version of a previously reported procedure<sup>25</sup>. Drugs with reported PPB values ranging from 0% to 99% were selected for the research. Metformin, metronidazole, ranitidine, paracetamol, phenobarbital, cefixime, salicylic acid, cetirizine, omeprazole, and losartan were all obtained from industry with high purity (≥95%). An isocratic mobile phase, composed of a 0.9% sodium chloride solution at pH 7.0, was employed, and it was run at a flow rate of 0.5 mL/min. The study was performed using a chiral pack HSA column (150×4 mm, 5µm). A significant correlation was achieved by generating a graph that compared the derived capacity factors with the reported (known) PPB percentages.

#### **In silico analysis of metabolites and their LD50**

The OECD QSAR Toolbox, owned by the OECD and the European Chemical Agency, is a tool used for the assessment of chemicals and the mechanistic basis of their toxicology<sup>26</sup>. The classical interface of QSAR tool box4.6, 2023 was used for the prediction of metabolite and their toxicity. MBZM-N-IBT structure was used as input. Several metabolism simulators were used to predict the metabolites, including *in vivo* Rat metabolism, autoxidation, and hydrolysis (acidic, basic, and neutral). These metabolites were utilized as inputs for predicting acute toxicity using the OECD QSAR toolbox, which aids in categorizing chemicals into specific groups.

#### **Statistics**

All data are expressed as mean±SEM

(Standard Error Mean) and were analyzed by the XLSTAT software using one-way ANOVA followed by Bonferroni multiple comparison tests ( $p < 0.001$ ).

## RESULTS

### MBZM-N-IBT showed no acute oral toxicity

After administration of different single oral doses (50 mg/kg, 300 mg/kg, and 2000 mg/kg) of MBZM-N-IBT, the animals were observed for the first 4h and daily up to 14 days. There were no behavioral changes or mortality (Table 1) with different doses of MBZM-N-IBT. As a result, it was classified as “Category 5” or “unclassified” as per the Globally Harmonized System (GHS) for chemical classification. Accordingly, 5000 mg/kg was considered as the  $LD_{50}$ .

No significant chronic toxicity was found on chronic use of MBZM-N-IBT (50 mg/kg)

### MBZM-N-IBT (50 mg/kg) did not affect the food and water intake of animals

Physiological changes (Table 2) were observed following the administration of MBZM-N-IBT at a dose of 1000 mg/kg/day (the highest permissible dose) and 500 mg/kg/day. Rats treated daily with 500 mg/kg showed no abnormality for up to 28 days (Table 2). Beyond 42 days, their food/water intake capacity was observed to decrease (Figure 2). A simultaneous decrease in body weight was also observed, and it became significant after 60 days. Rats treated with 50 mg/kg/day showed no abnormality even after 120 days of administration (Table 2). The average food intake and water consumption (Figure 2) were observed to be average at this dose. Although after 270 days, most of the parameters were normal (Table 2), It exhibited mild sedation, along with

an 11% reduction in body weight. The colour and consistency of the stool were yellowish and loose. The dose at 1000mg/kg/day showed toxicity (Figure 3) in rats after 28 days of continuous administration. Since mortality was found with the highest dose, this was discontinued.

### Histopathology revealed safety of MBZM-N-IBT (50 mg/kg)

The rat liver tissues (Figures 3a, 4a, and 5a) from the control group showed normal hepatic morphology, but MBZM-N-IBT (1000 mg/kg for 28 days) treated rat showed activated kupffer cells, infiltration of lymphocytes at the portal/central vein, sinusoidal obstruction, fibrous connective tissue and necrotic plaques (Figure 3b). In the case of MBZM-N-IBT (500mg/kg for 60 days), hepatocytes were loosely arranged, and vacuolar degeneration of hepatocytes was observed (Figure 4b). Following MBZM-N-IBT (50 mg/kg for 270 days) treatment, the liver showed alteration of cytoarchitecture (the arrowhead points to hepatic lobules, where the

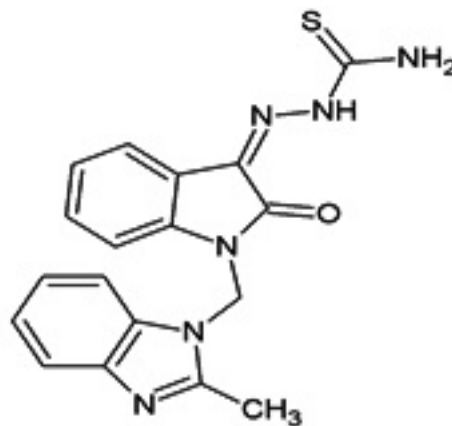


Fig. 1. Structure of MBZM-N-IBT

Table 1. Acute oral toxicity observation table

Step	Dose (mg/kg)	Number of Animals	Number of moribund/ deceased Animals	Subsequent dead animal
1	50	3	0	0
2	50	3	0	0
3	300	3	0	0
4	300	3	0	0
5	2000	3	0	0
6	2000	3	0	0

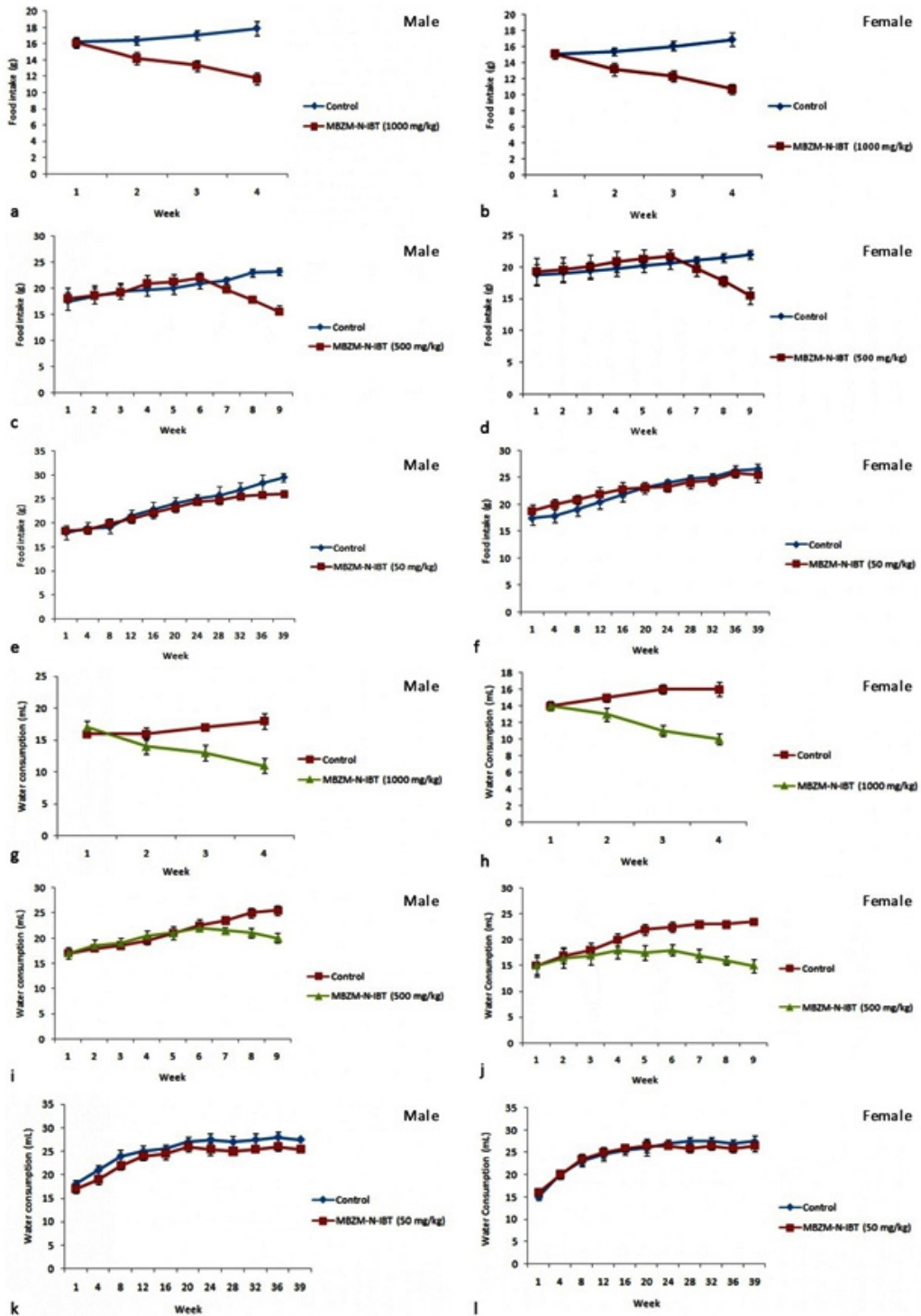
trabecular structure appears slightly blurred, while the remaining lobules exhibit a distinct blurring). Empty vacuolar spaces were seen in the cytoplasm of some cells. Kupffer cells were additionally detected along the sinusoidal walls (Figure 5b). The kidney tissues (Figures 3c, 4c, and 5c) from the normal control group showed the presence of normal renal corpuscle and kidney tubules. The MBZM-N-IBT (1000 mg/kg for 28 days) treated rat showed the presence of granular cast, focal proximal tubular epithelial necrosis, and unusual morphological pattern with the degeneration of the renal corpuscles (Figure 3d). Kidney tissues from MBZM-N-IBT (500 mg/kg for 60 days) treated rats showed cellular cast and Protein and reduction in the renal corpuscles (Figure 4d). The section of the kidney showed a normal appearance (the arrowhead highlights that the collecting tubules are lined with a simple cuboidal epithelium, and there is adjacent interstitial tissue) in case of treatment at 50 mg/kg/day for 270 days (Figure 5d). The stomach tissues (Figures 3e, 4e, and 5e) from the normal control group showed the presence of apparent normal mucosa and submucosa and thickened mucosal layer. Stomach tissues from

MBZM-N-IBT (1000 mg/kg for 28 days) treated rats showed minor epithelial ulceration covered by fibrin entangling neutrophils involving the reduction of gastric mucosal thickening (Figure 3f). Whereas 500 mg/kg for 60 days (Figure 4f) and 50 mg/kg for 270 days (Figure 5f) treatment showed no alteration in stomach cytoarchitecture of the rats. The lung tissues (Figures 3g, 4g, and 5g) from the normal control group showed polygonal alveoli and blood vessels. The presence of thin interalveolar septa and well-inflated alveoli indicate normal lung architecture. The MBZM-N-IBT (1000 mg/kg for 28 days) treated rat showed collapsed alveoli, suggesting severe alveolar damage (Figure 3h).

In contrast, rats treated with a lesser dose (500mg/kg for 60 days) showed slight interalveolar septa thickening. Whereas this suggests a relatively low level of toxicity, isolated focal regions with inflammatory cell infiltration and the appearance of clogged, thickened blood vessels indicate adverse effects on the lungs with continued treatment (Figure 4h). Following MBZM-N-IBT (50mg/kg for 270 days) treatment, normal intrapulmonary bronchioles could be seen (Figure 5h). The cardiac tissues (Figures 3i, 4i, and 5i) from the normal

**Table 2.** Behavioural changes in the animal during chronic toxicity studies following treatment with MBZM-N-IBT

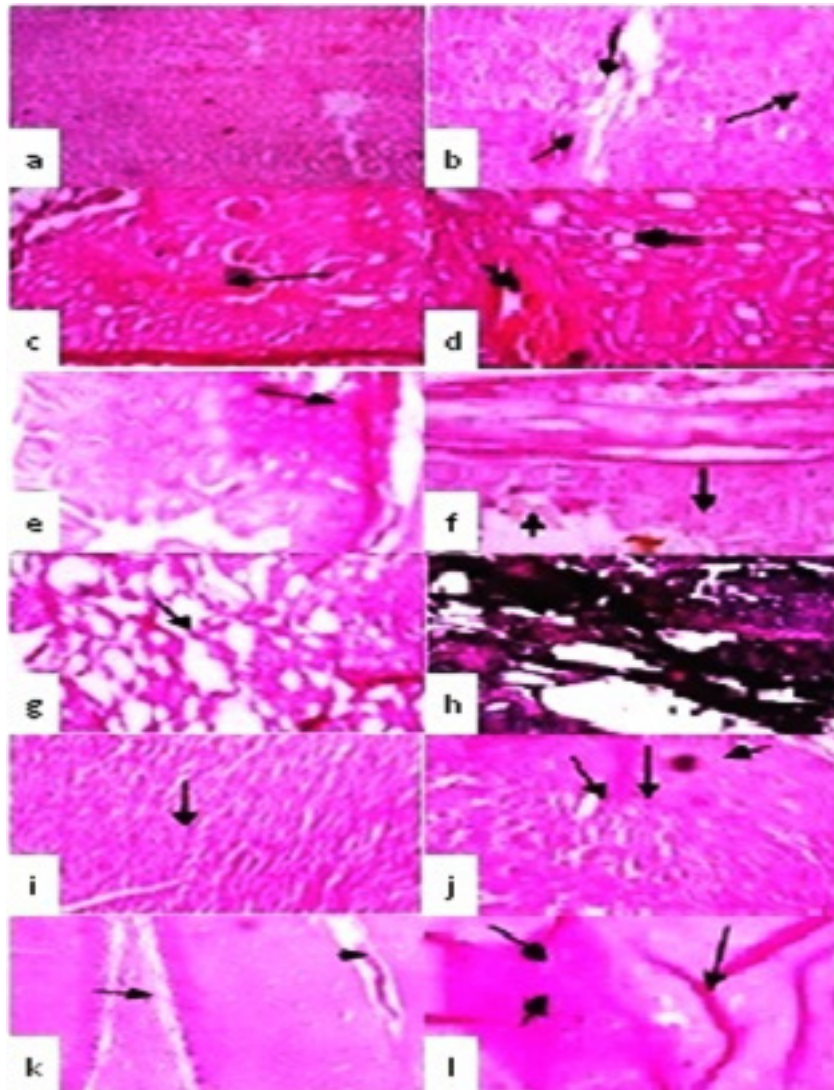
Parameters	1000 mg/kg	500 mg/kg	50 mg/kg	50 mg/kg
	28 days	60 days	120days	270 days
Condition of fur	Abnormal	Normal	Normal	Normal
Skin	Normal	Normal	Normal	Normal
Subcutaneous swelling	Nil	Nil	Nil	Nil
Eyes dullness	Present	Nil	Nil	Present
Breathing abnormalities	Present	Nil	Nil	Nil
Food intake	Decreased after 7days	Normal till 28 days	Normal	Normal
Colour/consistency of feces	Abnormal	Normal	Normal	Yellowish/loose
Alertness	Abnormal after one week	Normal	Normal	Normal
Convulsions	Not observed	Not observed	Not observed	Not observed
CNS	Sedation	Sedation	Sedation	Sedation
Body temperature	Normal	Normal	Normal	Normal
Bodyweight	Decreased after one week	Decreased after 42 days	Normal	Reduced by 11%
Grooming	Absent	Present	Present	Present
Death	3 animals were found dead during the testing period	No death after 60 days	No death	No death



**Fig. 2.** Mean food and water intake of animals. Food (a & b) and water (g & h) intake of animals treated with 1000 mg/kg up to 28 days. Food (c & d) and water (i & j) intake following oral use of 500 mg/kg of MBZM-N-IBT for 60 days. Food (e & f) and water (k & l) intake of animals administered at 50 mg/kg up to 270 days.

control group showed no necrosis throughout the musculature, no foci of cellular infiltration, normal nuclei, and normal cytoplasm. However, treated rats with MBZM-N-IBT (1000 mg/kg for 28 days) displayed distinct foci of cellular infiltration, having large nuclei and specific areas that showed general cellular ultrastructure disruption (Figure 3j). The rat treated with MBZM-N-IBT (500mg/kg for 60 days) displayed loss of nuclei, indicating dead cells and internalization of large nuclei indicative

of the myocyte regeneration process (Figure 4j). The histological section of the myocardium (Figure 5j) showed a normal appearance (arrowhead indicates muscle fiber and blood vessel) in the case of MBZM-N-IBT (50mg/kg for 270 days). The brain tissues (Figures 3k, 4k, and 5k) from the normal control group showed the cerebral cortex with regular ependyma of the ventricle, pyramidal cells, and perivascular space. The MBZM-N-IBT (1000mg/kg for 28 days) treated rat showed blood



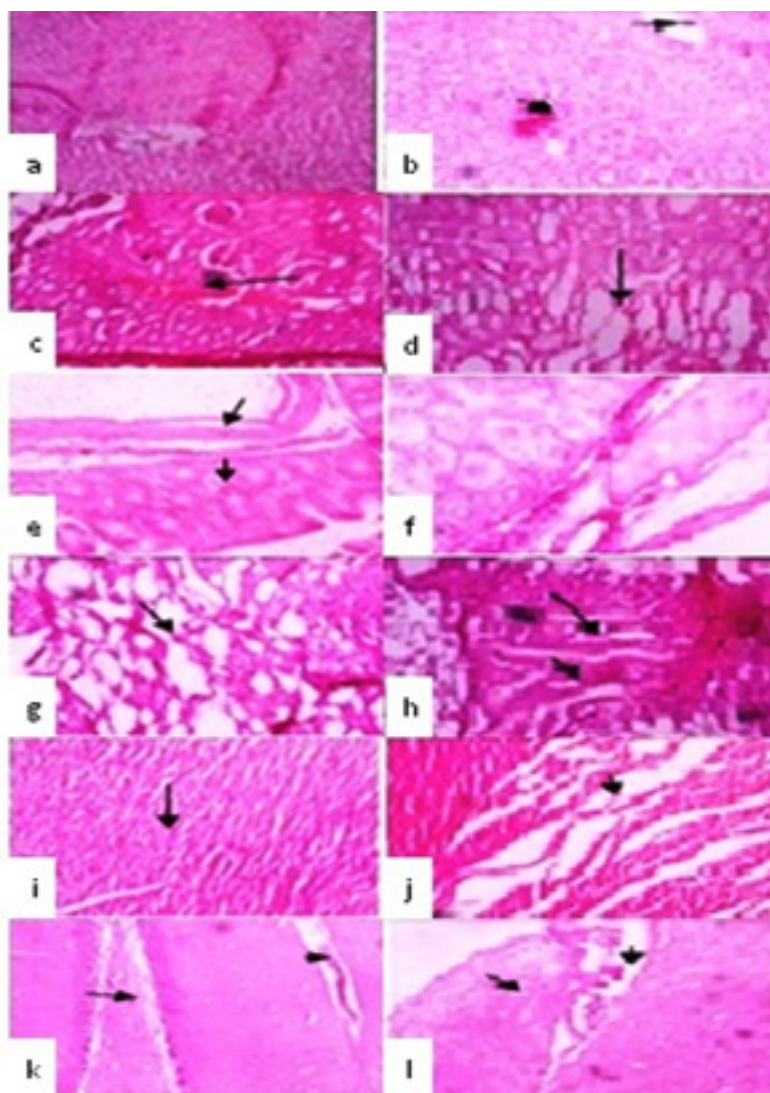
**Fig. 3.** Histopathology of rat tissues after the administration of MBZM-N-IBT (1000 mg/kg) for 28 days. The liver tissues (a), kidney tissues (c), stomach tissues (e), lung tissues (g), cardiac tissues (i), and brain tissues (k) of normal control group is compared with the liver tissues (b), kidney tissues (d), stomach tissues (f), lung tissues (h), cardiac tissues (j) and brain tissues (l) of MBZM-N-IBT (1000 mg/kg) treated group.

vessels dilated with increased perivascular spaces and intervening edema in the form of vacuoles in the brain substance and ischaemic necrosis (Figure 3l). Brain tissues from MBZM-N-IBT (500 mg/kg for 60 days) treated rats showed pyknotic nuclei and increased perivascular space with hemorrhage and vacuoles in the brain (Figure 4l). Meanwhile, the brain tissue (Figure 5l) showed a normal appearance (arrowhead indicates different sizes and shapes of nerve cells and nerve fibers) in the case

of MBZM-N-IBT (50 mg/kg for 270 days). This suggests that the dose of 50 mg/kg/day is relatively safe for oral administration.

#### **Bioanalytical Method Development and Pharmacokinetics of MBZM-N-IBT**

A novel RP-HPLC (Reverse Phase High-Performance Liquid Chromatography) method was established and validated in compliance with the ICH (International Council for Harmonization) guidelines, specifically ICH Q2 (R1). All the

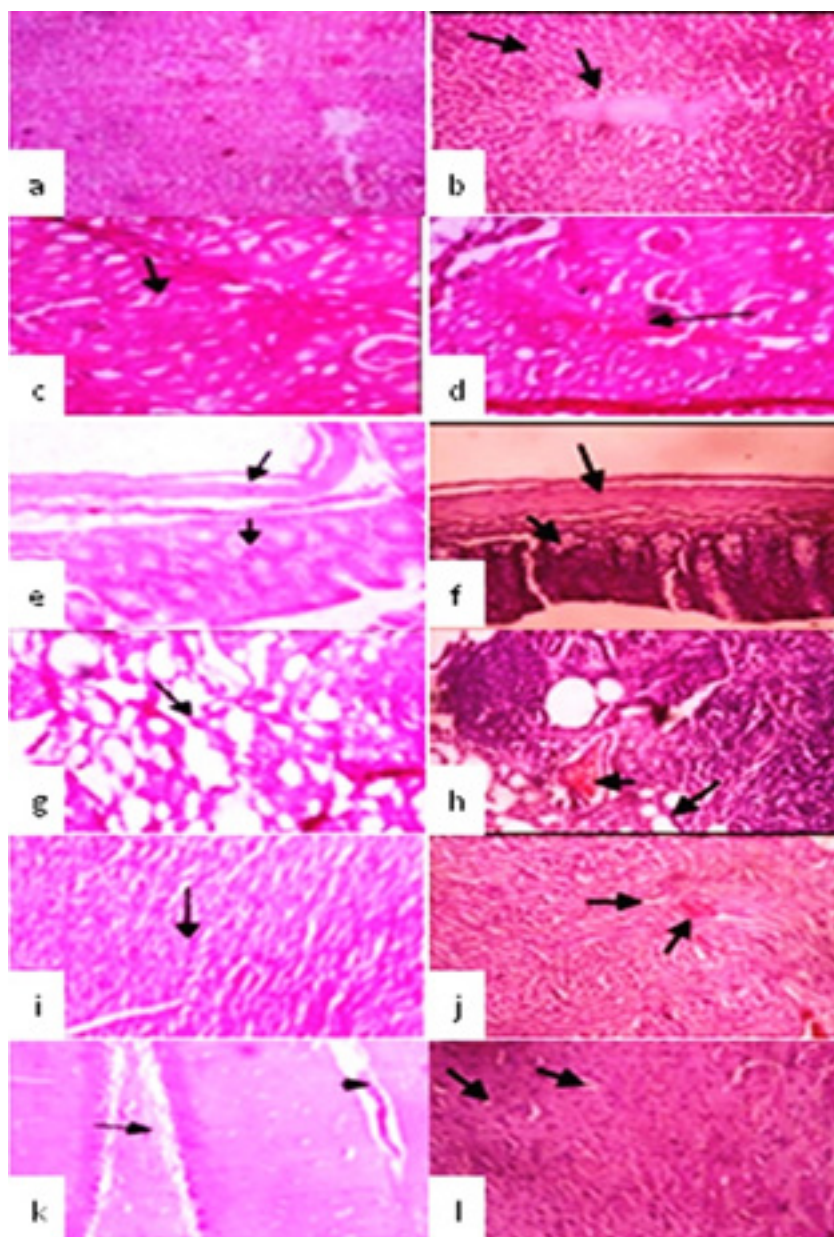


**Fig. 4.** Histopathology of rat tissues after the administration of MBZM-N-IBT (500 mg/kg) for 60 days. The liver tissues (a), kidney tissues (c), stomach tissues (e), lung tissues (g), cardiac tissues (i), and brain tissues (k) of normal control group is compared with the liver tissues (b), kidney tissues (d), stomach tissues (f), lung tissues (h), cardiac tissues (j) and brain tissues (l) of MBZM-N-IBT (500 mg/kg) treated group.



parameters were validated, and the results are shown in Table 3. After this, the method was verified according to USFDA guidelines for bio-analytical method validation. Specificity was confirmed by the absence of peaks at the retention time of MBZM-N-IBT from the plasma. The calibration

curve was generated by adding a known quantity of MBZM-N-IBT to 500  $\mu$ L of rat plasma, resulting in concentrations of 2, 4, 6, 8, and 10  $\mu$ g/mL. MBZM-N-IBT in rat plasma was quantified by measuring the analyte's response against the calibration curve. The concentration of MBZM-N-IBT in six



**Fig. 5.** Histopathology of rat tissues after the administration of MBZM-N-IBT (50 mg/kg) for 270 days. The liver tissues (a), kidney tissues (c), stomach tissues (e), lung tissues (g), cardiac tissues (i), and brain tissues (k) of the normal control group is compared with the liver tissues (b), kidney tissues (d), stomach tissues (f), lung tissues (h), cardiac tissues (j) and brain tissues (l) of MBZM-N-IBT (50 mg/kg) treated group

replicates of plasma spiked with 4, 6, and 8 µg/mL of MBZM-N-IBT was evaluated using HPLC in a single day and repeated on three separate days to evaluate accuracy and precision. In accordance with FDA guidelines, the acceptance criteria for accuracy were set at 85-115%, and the coefficient of variation values (CV) was required to be  $\hat{A}$

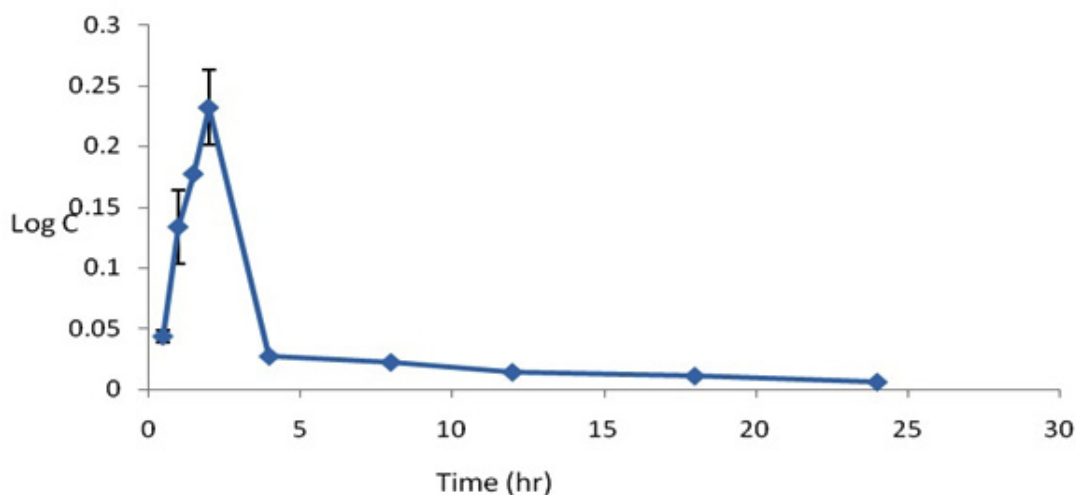
15%, except at the Lower Limit of Quantification (LLOQ), where accuracy was acceptable within the range of 80-120%, and the CV  $\hat{d}$  20% (Table 4). The pharmacokinetic plasma profiles of MBZM-N-IBT after 50 mg/kg oral administration are depicted in Figure 6, and pharmacokinetic parameters are summarized in Table 5. The estimated maximum

**Table 3.** Validation parameter for analysis of MBZM-N-IBT from bulk

Linearity	$r^2=0.999$	0.5-100 µg/mL		
Accuracy (%±SD)		100.16 ± 0.557		
Precision(RSD %)	Intra-day	Inter-day		
		Day 1	Day 2	Day 3
	0.722	0.55	0.57	1.31
LOQ	0.150 µg/mL			
LOD	0.050 µg/mL			

**Table 4.** Validation of analytical method for analysis of MBZM-N-IBT from plasma

Linearity	$r^2=0.999$	2-10 µg/ml				
Accuracy (%±SD)	LQC	96.7±0.094				
	MQC	97.5±0.113				
	HQC	93.9±0.244				
Precision(RSD %)	Intra-day	Inter-day				
		Day 1	Day 2	Day 3		
		LQC	2.55	3.36	2.85	3.31
		MQC	1.93	3.11	3.80	2.27
		HQC	3.24	1.92	3.29	2.89
LLOQ	2 µg/ml					
LOD	0.4 µg/ml					



**Fig. 6.** AUC of MBZM-N-IBT in rat plasma

**Table 5.** Pharmacokinetic parameters of MBZM-N-IBT

Parameter	Result
AUC (µg/mL h)	25.604±0.123
AUMC(µg/mL h <sup>2</sup> )	298.346±0.412
MRT (h)	11.652±0.040
C <sub>max</sub> (µg/mL)	1.709±0.069
T <sub>max</sub> (h)	2
K <sub>el</sub> (h <sup>-2</sup> )	0.085±0.031
t <sub>1/2</sub>	8.120±0.069

\*mean±SD (n=6)

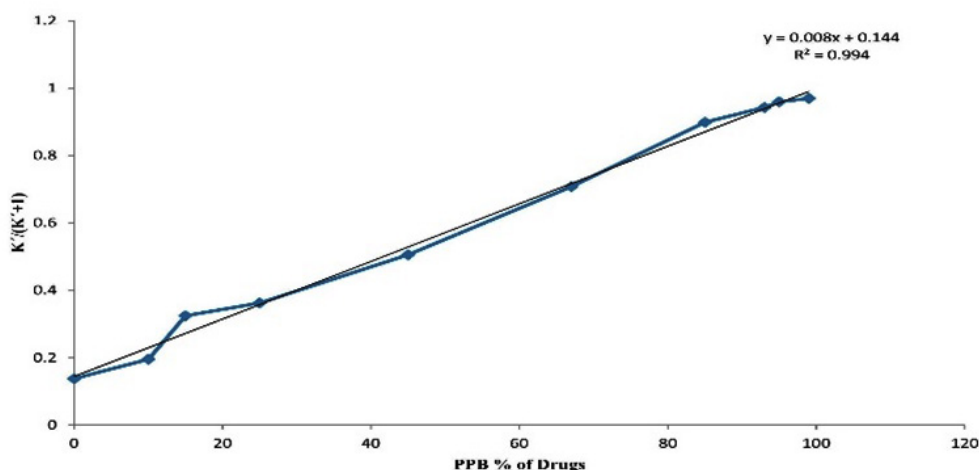
plasma concentration (C<sub>max</sub>) was 1.079±0.069 µg/mL, and the duration to achieve the maximum plasma concentration (T<sub>max</sub>) was 2h. This shows that absorption of MBZM-N-IBT is not slow and is within the normal gastric emptying time of 2-4 hours. The apparent elimination half-life (t<sub>1/2</sub>) was calculated as 8.120±0.069h, which indicates that more than 40h (five half-lives) may be required for complete clearance of MBZM-N-IBT from systemic circulation.

**Plasma protein binding of MBZM-N-IBT**

The HPLC method estimated Plasma protein binding using a human-HSA chiral column. The capacity factor measures the retention of

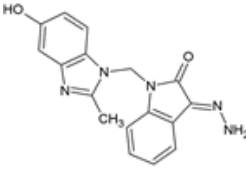
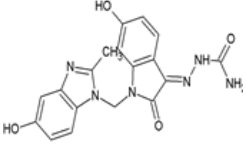
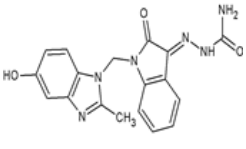
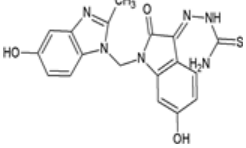
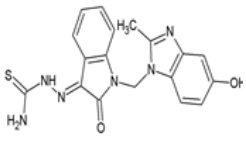
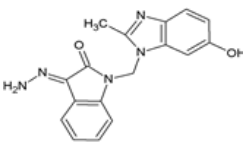
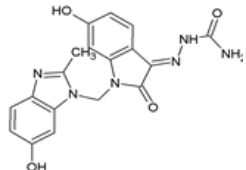
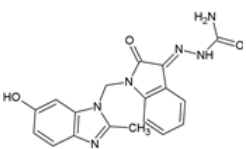
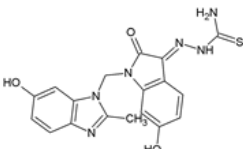
**Table 6.** Plasma protein binding % of different drugs

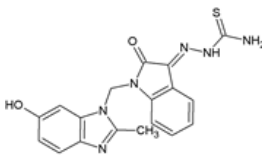
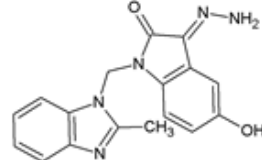
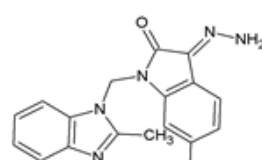
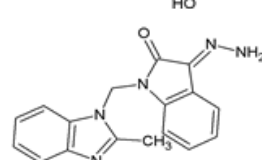
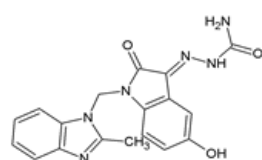
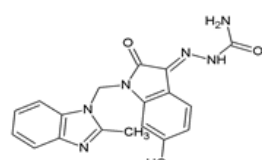
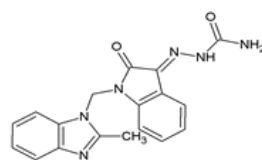
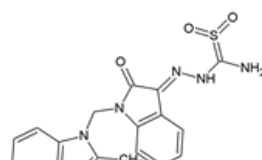
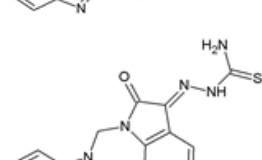
Drug	Reported PPB %	Rt	K'	K'/(K'+1)	Measured PPB %
Metformin <sup>27</sup>	0	3.161	0.161	0.138	0.00
Metronidazole <sup>28</sup>	10	3.546	0.244	0.196	12.5
Ranitidine <sup>29</sup>	15	4.032	0.482	0.325	22.625
Paracetamol <sup>30</sup>	25	4.494	0.571	0.363	27.375
Phenobarbital <sup>31</sup>	45	6.44	1.027	0.506	45.25
Cefixime <sup>32</sup>	67	2.798	2.436	0.708	70.05
Salicylic acid <sup>33</sup>	85	15.610	8.974	0.899	94.375
Cetirizine <sup>34</sup>	93	28.662	16.834	0.943	99.875
Omeprazole <sup>35</sup>	95	40.638	24.414	0.96	102
Losartan <sup>36</sup>	99	52.971	32.985	0.970	103.25
MBZM-N-IBT	-	51.962	17.248	0.94	99.5

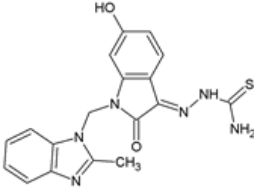
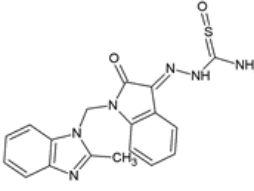
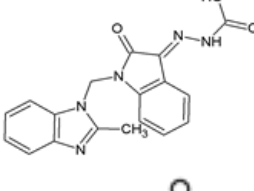
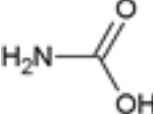


**Fig. 7.** Calibration curve for Plasma protein binding

**Table 7.** Predicted Metabolites of MBZM-N-IBT

S.No.	Metabolites	Structure	LD50	OECD class
1	M1		3000mg/kg	5
2	M2		3000mg/kg	5
3	M3		3000mg/kg	5
4	M4		3000mg/kg	5
5	M5		3000mg/kg	5
6	M6		3000mg/kg	5
7	M7		3000mg/kg	5
8	M8		3000mg/kg	5
9	M9		3000mg/kg	5

10	M10		3000mg/kg	5
11	M11		3000mg/kg	5
12	M12		3000mg/kg	5
13	M13		3000mg/kg	5
14	M14		3000mg/kg	5
15	M15		3000mg/kg	5
16	M16		3000mg/kg	5
17	M17		3000mg/kg	5
18	M18		3000mg/kg	5

19	M19		3000mg/kg	5
20	M20		3000mg/kg	5
21	M21		2100mg/kg	5
22	M22		681mg/kg	4

drugs in the column. Accordingly, the capacity factor of drugs while using a human-HSA chiral column represents its affinity towards human serum albumin. Since this is the major plasma protein, its binding correlates well with drugs' *in vivo* plasma protein binding. To corroborate this, various drugs with reported plasma protein binding in the range of 1-99% were considered. Their capacity factors were determined (Table 6). A significant correlation ( $r^2=0.994$ ) was found between the capacity factor and the known PPB% of these drugs. Accordingly, this showed a linear regression ( $Y=0.008X+0.144$ , Figure 7). The modified capacity factor ( $K'/(K'+1)$ ) of MBZM-N-IBT was determined to be 0.94. Accordingly, its PPB% was found to be 99.5 (Table 6). This suggests that MBZM-N-IBT has a high plasma protein binding capacity.

No metabolite was found for metabolism in the autoxidation and dissociation simulator, indicating the molecule's stability. A simulator for hydrolysis under neutral conditions predicted 5 metabolites. Under acidic and basic conditions, the hydrolysis simulator predicted 12 and 13 metabolites, respectively. In the *in vivo* rat simulator, 13 metabolites were predicted. After

combining the results of all these simulators, 22 unique metabolites were predicted for MBZM-N-IBT (Table 7). While 21 metabolites were expected to be OECD class five chemicals with very high LD50, one metabolite was categorized as OECD class 4 with a potential LD50 of 681mg/kg. This indicates that the metabolites of MBZM-N-IBT are not likely to be toxic.

## DISCUSSION

MBZM-N-IBT is an important antiviral lead. It has a broad spectrum of antiviral action and the potential to interfere in multiple ways against CHIKV and HSV replication<sup>6,11</sup>. However, it has been demonstrated to be effective *in vivo* at a dosage of 15 mg/kg against CHIKV infection in mice; further preclinical validation is necessary to justify its suitability as an antiviral candidate<sup>37</sup>. Hence, assessing its pharmacokinetics and toxicology is essential to encourage further *in vivo* validation.

An oral acute toxicity study, as per OECD-423 guidelines, revealed that rats had no acute toxicity during the monitoring period (14

days) (Table 1). Accordingly, the oral LD<sub>50</sub> of the MBZM-N-IBT was calculated to be greater than 5000 mg/kg as per the OECD-423 guidelines. This suggested the acute oral safety of MBZM-N-IBT. In the chronic toxicity studies, MBZM-N-IBT administration at a high dose for 28 days (1000 mg/kg/day) led to a remarkable reduction in food intake and water consumption. Although this was normal in the first three weeks in animals treated with a moderate dose (500 mg/kg/day), in the later stages, there was a reduction in food intake and water consumption (Figure 2). At a lower dose (50 mg/kg/day), rats showed no significant change in food intake and water consumption for 270 days. Also, there were no signs of toxicity and behavioural changes (Table 2). However, on prolonged use, some reversible sedation was noted. Besides, there were some changes in stool and body weight (Table 2). In congruence with these, significant alterations were observed in the pathological study of the excised liver, lungs, kidney, stomach, heart, and brain (Figures 3 & 4). At the end of the 270-day study, some necrosis of excised cardiomyocytes at the sub-endocardium region in cardiac tissue was observed. In excised brain tissue, vascular congestion, and cellular infiltration were also marked (Figure 5).

Nevertheless, the pathological study of the excised liver, kidney, stomach, and lung tissue showed a normal appearance (Figure 5). The sedation on prolonged use suggests the ability to reach the brain. Nevertheless, till 120 days and beyond, it showed no effect on motor coordination, locomotion, and anxiety at a dose of 50 mg/kg. Since the antiviral application usually requires short-term drug administration, this study hints at the safety of this dose for further investigation.

The analytical and bio-analytical method validation parameters are shown in Tables 3 and 4. Following an oral dose of 50 mg/kg to rats, the AUC of MBZM-N-IBT was determined (Figure 6). It revealed that 2h is taken to achieve the highest systemic concentration. This shows that it is absorbed within 2h of oral administration. Considering the fact that the normal gastric emptying time is between 2-4h, the absorption rate of MBZM-N-IBT may be adequate.

Further, its elimination half-life was found to be 8 h (Table 5). Drugs with a half-life of 12-48 h are generally considered suitable for

once-daily oral dosing<sup>38</sup>. Although the half-life of MBZM-N-IBT is less than this, it can remain in circulation for 5 half-lives or 40h. Hence, its suitability for once-daily oral dosing cannot be ruled out without further validation. Moreover, it has been demonstrated to be effective against CHIKV infection in mice with a once-daily oral dosing of 15 mg/kg<sup>37</sup>.

The protein binding was estimated using the HPLC method by measuring its affinity for human serum albumin attached to the stationary phase. To develop this method, the modified capacity of different drugs that represent their affinity for human serum albumin was correlated to their known PPB%. A high correlation ( $r^2 = 0.994$ ) suggested the suitability of the regression equation that was used for the calculation of PPB% (Figure 7). As shown in Table 6, the calculated PPB% was very close to that of the reported values for most of the drugs. Hence, 99.5% PPB was estimated for MBZM-N-IBT. This high PPB is in agreement with the relatively high elimination half-life (8h) and may support the longer duration of action.

Further, the metabolism of MBZM-N-IBT was predicted through *in silico* analysis using the OECD *QSAR Toolbox*. The reliability of the OECD QSAR toolbox has been demonstrated for the prediction of the acute toxicity of organic chemicals<sup>39</sup>. It is supported by the Organisation for Economic Co-operation and Development (OECD) as well as the European Chemicals Agency (ECHA). Using these tools, 22 unique metabolites (Table 7) were predicted. While 21 of these metabolites were non-toxic (OECD-class 5), one was predicted to have an LD<sub>50</sub> of 681mg/kg (Table 7). Thus, like MBZM-N-IBT, the metabolites are also not likely to be toxic. Since these metabolites were mainly predicted in the rat simulator model, this may also support the safety in rats demonstrated in the chronic toxicity studies. However, further *in vivo* metabolite analysis is necessary to validate this.

## CONCLUSION

The no-observed-adverse-effect level (NOAEL) of MBZM-N-IBT can be taken into consideration to be 50 mg/kg under the specified circumstances based on the single-dose oral acute toxicity study and the lack of substantial toxicity or

mortality in the sub-chronic toxicity study. While the *in vivo* pharmacokinetics suggests adequate rates of absorption, elimination half-life (8h), and high PPB suggest a potentially longer duration of action *in vivo*. The predicted metabolites are not expected to be toxic and support the *in vivo* safety of MBZM-N-IBT observed in toxicity studies. However, it is necessary to closely evaluate its effect on renal and hepatic function for long-term therapy. Although at this dose, it does not affect motor coordination, locomotion, and anxiety, some sedation is observed. Hence, it is necessary to closely monitor its effect on the brain. Further investigation into mutagenicity and teratogenicity is also required. Thus, the present investigation can encourage additional studies to increase the scope for translational application of MBZM-N-IBT.

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#### Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Ethical Approval

The experimental procedure was approved by the Institutional Animal Ethics Committee (IAEC) of School of Pharmaceutical Sciences, Siksha O Anusandhan (Deemed to be University), Bhubaneswar, India (Reg. no. 1171/PO/Re/S/08/CPCSEA).

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