

# Evaluation of Toxicity Profile of Zoledronic Acid in Wistar Rat: A Sub-chronic Toxicity Study

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## Abstract

The evolution of bisphosphonates has led to compounds with ever-increasing potency such as Zoledronic acid. It is a potent bisphosphonate that inhibits bone resorption. To evaluate the safety profile of this drug a subchronic toxicity study for twenty-eight days was conducted in rats (male and female) at three different doses such as 0.5 mg/kg, 1 mg/kg and 2 mg/kg. All parameters related to physiological, haematological and biochemical aspects were evaluated. There were no signs of toxicity observed at any dose level used in this study. No mortality was seen in any of the treatment groups. Haematological, biochemicals as well as physiological parameters were unaltered at all three dose levels of Zoledronic acid treated groups as compared to control. It was inferred from results of our study that the intravenous Zoledronic acid is non-toxic even at very high dose level as compared to human dose. The present study has proven the safety profile of Zoledronic acid hence is likely to become the treatment of choice for hypercalcemia of malignancy and osteoporosis with potentially minimal side effects.

## INTRODUCTION

Bisphosphonates, an important class of osteotropic compounds, are effective in treating benign as well as malignant skeletal diseases that are characterized by enhanced osteoclast-mediated bone resorption (i.e., osteoporosis, Paget's disease, and tumor-induced osteolysis) (Adami et al. 2002; Suzuki et al. 2008). These compounds are widely used and most effective agents for the treatment of established osteoporosis after the menopause and increase bone density thus reduces the risk of fractures (Woolf and Akesson 2003). At least 25% of patients with breast cancer develop skeletal metastases and various skeletal complications include hypercalcaemia, pathological fracture, bone pain requiring radiotherapy, and spinal cord or nerve root compression, causing the greatest morbidity. (Black et al. 2007; Woolf and Akesson 2003). Thus there is a real need for treatment to reduce skeletal complications and to improve the quality of life in these individuals.

Among various choices for these complications Zoledronic acid (ZA or zoledronate) is the most potent and established bisphosphonate that has been studied in clinical trials and found to be effective in osteoporosis and skeletal metastases (Suzuki et al. 2008). It is superior to other drugs of similar class in the treatment of cancer-related hypercalcemia as well as osteoporosis after the menopause (Maclaughlin et al. 2008). Because it has high potency (IC<sub>50</sub> values for

zoledronic acid were 48  $\mu$ M and 20  $\mu$ M for 24 hours and 72 hours, respectively), only small doses suffice over for the inhibition of bone resorption on intravenous administration, and long lasting intervals also is an additional pharmacokinetic property associated with the use of ZA (MacLean et al. 2008; Black et al. 2007). In both rats and dogs radiolabelled i.v. Zoledronic acid (0.15 mg/kg) was cleared rapidly in a

multiexponential manner from blood and plasma while early elimination half-life is 1.75 hours, and terminal elimination half-life is 167 hours. This pattern is similar to the PK data obtained from patients and mean C<sub>max</sub> in patients after infusion was reported as 409 ( $\pm$ 142) ng/mL to 462 ( $\pm$ 135) ng/mL (Andrew et al. 2008; Weiss et al. 2008, Amanat et al.).

Oral bisphosphonates were also widely used for treating osteoporosis and have been shown to increase bone mineral density and decrease the rate of fracture (Grbic et al. 2008; Lyles et al. 2007). However, long-term compliance, gastrointestinal intolerance, and poor and variable absorption from the gastrointestinal tract are the big hurdles in its wide uses (de Nijs and Westgeest 2007; Weiss et al. 2008). Because of low bioavailability, high oral doses may be required (Berenson 2005). To address these problems intermittent intravenous administration of ZA has been used.

It was proved to be effective in the treatment of skeletal complications in patients as compared to oral ZA (Major 2002; Reid et al. 2002) and intravenous ZA can increase bone mineral density in patients with osteoporosis (Conte and Guarneri 2004; Gouin et al. 2006; Kimmel 2007). A single infusion of intravenous ZA has been reported to decrease bone turnover and improve bone density (Conte and Guarneri 2004).

These Nitrogen-containing bisphosphonates (nBPs) are bone-specific agents that inhibit farnesyl diphosphate synthase which has detrimental effect on bone structure (Body et al. 1999). Zoledronic acid, nBP, has strong affinity for bone as compared to other tissues; which provides specific inhibition of bone resorption and bone remodelling activity, with limited potential for adverse effects in non-skeletal tissues (Lipton et al. 2002). Apart from this ZA reduces osteoporotic fracture risk by 50–60% in persons with low bone mass or prior osteoporotic fracture, and skeletal-related events by one-third in cancer patients. The efficacious dose of ZA for cancer patients ranges between seven to ten times that in osteoporosis patients (Reid et al. 2002). Pharmacokinetic studies indicated that approximately half of any nBP dose reaches the skeleton, with an early half-life of ten days, and a terminal half-life of about ten years. Finally it is not metabolized and is excreted unchanged via the kidney (Kimmel et al. 2007, Andrew et al. 2008).

Despite of being frequently prescribed drug very limited data is available on safety of ZA on intravenous use. The current study was designed to investigate associated toxicities of ZA treatment by sub chronic dosing in rat to establish safety profile of this potential agent.

## **MATERIAL AND METHOD**

### **ANIMALS**

Healthy Wistar rats (males and females; 150-175 g weight) were randomly divided into four groups (three treatment groups and one control group). Each group consisted of 6 male and 6 female animals. Animals were provided with a standard diet (pellets) supplied by Amrut feed India (manufacturer and city of production details needed) and water was given ad libitum. They were group housed in polyurethane cages (three in each) at controlled room temperature of  $29 \pm 2^\circ\text{C}$  and a relative humidity of 50.5%, and a constant light-dark schedule (12 hours light and 12 hour dark cycle). The study protocol for these experiments was approved by the Institutional Animal Ethics Committee

of the Institute for Toxicological Studies, Pune, India.

### **REAGENTS**

ZA was procured from Venus Remedies Limited. All other reagents were purchased from Sigma Aldrich (Bangalore, India).

### **EXPERIMENTAL DESIGN AND DRUG TREATMENT**

ZA was administered intravenously at three dose levels i.e. 0.5 mg/kg, 1 mg/kg and 2 mg/kg body weight correspond to low dose, intermediate dose and high dose respectively for twenty eight days. Current study was conducted to outline safety profile of ZA. This study was designed keeping OECD guidelines in consideration. According to regulatory guidelines the doses of toxicity study in rodents shall be maximum tolerable dose and other two doses shall be reduced in geometric progression. Hence doses were selected as 2 mg kg (maximum tolerable), 1mg/kg and 0.5 mg/kg. The doses were 7.5 times, 15 times and 30 times higher as compared to the dose intended to use in human (4 mg/day). Normal saline was administered to the animals of control group as sham treatment. Treatment was conducted once daily for 28 days.

Physical parameters (body weight, food and water intake), local injury (tissue damage or necrosis at site of injection, inflammation, any other abnormal signs) were studied throughout the treatment. Mortality if any, in all the groups, during the course of treatment was also recorded. An autopsy was conducted if the animal died during course of treatment. All vital organs of dead animals isolated and observed for any abnormal signs i.e. liver damage/necrosis, morphological changes in kidney, heart etc. At the end of treatment hematological, biochemical (liver function tests & renal function tests) and histological parameters were studied.

### **HEMATOLOGICAL PARAMETERS**

Blood was collected by cardiac puncture. Blood samples were analyzed for routine hematological parameters. Blood cell count was conducted with blood smears.

### **BIOCHEMICAL PARAMETERS**

Biochemical parameters were analysed by Merck semi auto analyzer (Selectra Junior) using kits for Serum glutamic oxaloacetic transaminase (SGOT), Ecoline ASAT (tris) (GOT) (11766400011730) kit, for Serum glutamic pyruvate transaminase (SGPT) Ecoline ALAT (tris) (GPT)

(11766100011730) kit, Blood Urea , Ecoline Urea kit (11761800011730), serum levels of alkaline phosphatase by Ecoline Alk Phosphatase kit(1176500011730), GOD-POD kit for Glucose from Accurex India. Other parameters were estimated such as total protein, blood urea nitrogen (BUN, URSL-0455, SEPPIM) and creatinine ( Merckotest Creatinine 10338500011730) as signs of nephrotoxicity.

**HISTOLOGICAL EXAMINATION**

At the end of treatment animals were sacrificed and various organs including the liver, kidney, brain, heart and gonads were collected. The organs were quickly blotted, weighed on digital balance and processed for histological assessment. All the organs were immediately fixed in 10% buffered formalin. The organ body weight ratio of each organ was calculated and tissues were processed for H& E staining.

**STATISTICAL ANALYSIS**

Results are expressed as Mean ± SD. Significance of difference between groups was evaluated by using ANOVA. If ANOVA shows significant differences, post hoc analysis was performed with Tukey test. P<0.05 was considered as statistically significant.

**RESULTS**

**PHYSICAL PARAMETERS**

No physical changes were observed throughout the dosing period. There was no significant change in the mean body weight of the animals in ZA treated groups as compared to vehicle treated control group at the end of treatment.

**HEMATOLOGY**

There were no significant changes observed in red blood cell (RBC), hemoglobin (Hb), total leukocyte counts (TLC) and platelet counts in all Zoledronic acid treated male groups as compared to respective control groups (Table 1). HCT dose-dependently decreased in treated groups but all changes were not significant.

**Figure 1**

Table 1: Effect of sub acute dose of Zoledronic acid on hematological parameters in male rat. RBC, Red Blood Cells, Rt, Reticulocytes; HCT, Hemocrit; MCV, Mean Corpusular Volume; MCH, Mean Corpusular Hemoglobin; MCHC, Mean Corpusular Hemoglobin Concentration; WBC, White Blood Cells; E, Eosiniophils; L, Leukocytes; M, Monocytes; N, Neutrophils.

Parameters	Control	Zoledronic acid 0.5 mg/kg	Zoledronic acid 1 mg/kg	Zoledronic acid 2 mg/kg	
Haemoglobin (g%)	15.01 ± 1.20	14.82 ± 1.40	14.14 ± 2.20	13.73 ± 1.90	
Total RBC (X10 <sup>6</sup> /mm <sup>3</sup> )	5.98 ± 0.80	5.78 ± 0.80	6.05 ± 0.90	5.57 ± 0.60	
Rt (%)	1.15 ± 0.30	1.22 ± 0.30	1.23 ± 0.40	1.30 ± 0.40	
HCT (%)	44.07 ± 4.10	43.8 ± 3.90	42.80 ± 6.80	41.07 ± 6.40	
MCV (µm <sup>3</sup> )	74.52 ± 10.5	77.60 ± 16.80	71.80 ± 13.50	74.53 ± 15.30	
MCH (pg)	25.38 ± 3.40	26.26 ± 5.90	23.74 ± 4.50	24.92 ± 4.80	
MCHC(%)	34.10 ± 0.90	33.77 ± 0.90	33.07 ± 1.50	33.50 ± 0.50	
Platelets (X10 <sup>5</sup> / mm <sup>3</sup> )	8.45 ± 0.90	8.60 ± 0.60	7.97 ± 1.10	7.92 ± 1.10	
Total WBC(X10 <sup>3</sup> / mm <sup>3</sup> )	6.37 ± 1.20	6.25 ± 1.40	6.08 ± 0.90	5.58 ± 0.70	
Differential %	N	22.00 ± 2.40	22.17 ± 2.30	22.00 ± 1.90	23.17 ± 2.90
	L	76.00 ± 3.00	76.67 ± 3.00	76.33 ± 2.30	76.50 ± 3.00
	E	1.67 ± 0.50	1.83 ± 0.30	2.00 ± 0.60	1.83 ± 0.50
	M	0.83 ± 0.05	0.5 ± 0.05	0.67 ± 0.10	0.67 ± 0.12

All values expressed as Mean ± SD (n=6).

Zoledronic acid treatment, at the doses administered, did not shown any deleterious effects on hematological parameters in the female rat group (Table 2). Blood cell count and hemoglobin in all treated groups were observed comparable to control female rat group (Table 2).

**Figure 2**

Table 2: Effect of sub acute dose of Zoledronic acid on hematological parameters in female rat. RBC, Red Blood Cells, Rt, Reticulocytes; HCT, Hemocrit; MCV, Mean Corpusular Volume; MCH, Mean Corpusular Hemoglobin; MCHC, Mean Corpusular Hemoglobin Concentration; WBC, White Blood Cells; E, Eosiniophils; L, Leukocytes; M, Monocytes; N, Neutrophils.

Parameters	Control	Zoledronic acid 0.5 mg/kg	Zoledronic acid 1 mg/kg	Zoledronic acid 2 mg/kg	
Haemoglobin (g%)	14.78 ± 0.90	15.43 ± 1.60	14.72 ± 1.20	14.33 ± 1.20	
Total RBC (X10 <sup>6</sup> / mm <sup>3</sup> )	5.80 ± 0.90	5.75 ± 0.90	5.63 ± 0.90	6.02 ± 1.10	
Rt (%)	1.12 ± 0.20	1.28 ± 0.30	1.18 ± 0.40	1.37 ± 0.40	
HCT (%)	43.28 ± 5.20	45.53 ± 4.80	43.40 ± 3.80	42.87 ± 5.70	
MCV (µm <sup>3</sup> )	75.48 ± 10.30	79.88 ± 7.60	79.30 ± 16.90	72.65 ± 13.70	
MCH (pg)	25.78 ± 3.50	27.07 ± 2.60	26.83 ± 5.60	24.36 ± 5.10	
MCHC(%)	34.18 ± 0.80	33.89 ± 0.20	33.80 ± 0.20	33.44 ± 0.90	
Platelets (X10 <sup>5</sup> / mm <sup>3</sup> )	8.33 ± 1.10	8.08 ± 1.10	7.85 ± 0.90	7.37 ± 1.20	
Total WBC(X10 <sup>3</sup> / mm <sup>3</sup> )	6.15 ± 0.90	5.83 ± 0.90	5.68 ± 0.80	5.90 ± 0.80	
Differential %	N	22.67 ± 2.50	22 ± 1.80	22.50 ± 2.50	24.17 ± 1.50
	L	75.5 ± 3.40	76.33 ± 2.70	74.60 ± 1.20	73.5 ± 2.10
	E	1.83 ± 0.70	2.33 ± 0.50	2.00 ± 0.60	2.50 ± 0.10
	M	0.67 ± 0.30	0.33 ± 0.20	0.67 ± 0.10	0.83 ± 0.05

All values expressed as Mean ± SD (n=6).

**BIOCHEMICAL PARAMETERS**

There were no significant changes observed in male and female rat total protein levels in all the dose groups when compared to control group. No significant increases were observed at even the high dose level in serum glucose, BUN,

GPT, GOT activities, alkaline phosphatase activities when compared to control group. All changes were not statistically significant (Table 3 and 4).

**Figure 3**

Table 3: Effect of sub acute dose of Zoledronic acid on biochemical parameters in female rat. BUN, Blood urea nitrogen; SGOT, Serum glutamic oxaloacetic transaminase; SGPT, Serum, Glutamic pyruvic transaminase activities; SAP, Serum Alkaline Phosphat

Parameters	Control	Zoledronic acid 0.5 mg/kg	Zoledronic acid 1 mg/kg	Zoledronic acid 2 mg/kg
Total Serum protein (g%)	6.27 ± 0.60	6.45 ± 1.20	6.27 ± 0.90	6.28 ± 0.80
BUN (mg%)	21.80 ± 2.30	23.50 ± 2.88	26.50 ± 4.80	27.17 ± 4.10
SGPT (IU/L)	51.20 ± 10.90	55.83 ± 9.90	63.50 ± 13.50	65.50 ± 13.60
SGOT (IU/L)	101.00 ± 15.30	109.00 ± 23.90	106.50 ± 20.90	110.50 ± 23.90
SAP (IU/L)	2750 ± 53.70	347.17 ± 65.00	370.83 ± 91.60	368.00 ± 97.30
Blood Sugar (mg%)	94.20 ± 7.30	98.33 ± 5.20	95.33 ± 6.20	99.67 ± 3.30

All values expressed as Mean ± SD (n=6)

**Figure 4**

Table 4: Effect of sub acute dose of Zoledronic acid on biochemical parameters in male rat. BUN, Blood urea nitrogen; SGOT, Serum glutamic oxaloacetic transaminase; SGPT, Serum Glutamic pyruvic transaminase activities; SAP, Serum Alkaline Phosphatase

Parameters	Control	Zoledronic acid 0.5 mg/kg	Zoledronic acid 1 mg/kg	Zoledronic acid 2 mg/kg
Total Serum protein (g%)	6.77 ± 1.10	5.92 ± 1.10	6.11 ± 0.70	6.20 ± 0.90
BUN (mg%)	21.20 ± 2.20	23.50 ± 2.90	24.33 ± 4.30	25.67 ± 5.70
SGPT (IU/L)	50.50 ± 12.10	56.33 ± 1.30	61.25 ± 12.10	61.83 ± 14.90
SGOT (IU/L)	96.00 ± 10.40	107.17 ± 19.10	110.00 ± 22.00	105.67 ± 20.50
SAP (IU/L)	251.00 ± 44.50	284.00 ± 65.60	370.00 ± 93.80	354.50 ± 85.00
Blood Sugar (mg%)	95.00 ± 6.90	97.67 ± 4.80	96.67 ± 5.70	98.33 ± 5.90

All values expressed as Mean ± SD (n=6)

**HISTOLOGICAL EXAMINATION**

There were no significant treatment related histopathological changes observed in organs of any of the treated groups (tabel 5& 6)

**Figure 5**

Table 5: Effect of sub acute doses of Zoledronic acid on organ weight in male mice

Parameters	Control	Zoledronic acid 0.5 mg/kg	Zoledronic acid 1 mg/kg	Zoledronic acid 2 mg/kg
Body weight (g)	256.00±5.57	269.75±6.97	259.00±8.25	258.68±4.76
Liver (g)	4.31±0.46	4.57±0.47	4.88±0.4	4.71±0.34
Kidney (g)	0.71±0.05	0.76±0.05	0.78±0.03	0.77±0.04
Heart (g)	0.43±0.04	0.46±0.04	0.48±0.04	0.47±0.04
Brain (g)	1.56±0.05	1.60±0.06	1.67±0.04	1.59±0.03
Testes (g)	1.32±0.04	1.33±0.03	1.40±0.05	1.23±0.04

(values expressed as Mean±SD, n=6).

**Figure 6**

Table 6: Effect of sub acute doses of Zoledronic acid on organ weight in female mice

Parameters	Control	Zoledronic acid 0.5 mg/kg	Zoledronic acid 1 mg/kg	Zoledronic acid 2 mg/kg
Body weight (g)	110±6.63	110.08±3.54	109.35±6.1	109.93±7.12
Liver (g)	4.37±0.48	4.44±0.54	4.85±0.37	4.85±0.35
Kidney (g)	0.73±0.05	0.74±0.05	0.79±0.03	0.77±0.02
Heart (g)	0.45±0.05	0.45±0.03	0.48±0.04	0.49±0.03
Brain (g)	1.68±0.03	1.56±0.05	1.60±0.04	1.55±0.06
Ovary(g)	0.17±0.04	0.18±0.03	0.16±0.01	0.20±0.02

(values expressed as Mean±SD, n=6)

**DISCUSSION**

Zoledronic acid, a third-generation nitrogen-containing bisphosphonate, is the most potent member of the bisphosphonate family, to date, ever investigated in clinical trials (Body et al. 1999). It is a highly effective inhibitor of osteoclast-mediated bone resorption. Because of its high potency, small doses and long intervals are sufficient enough to inhibit bone resorption and can easily be added to complex therapeutic regimens (Body et al. 1999; Lipton et al. 2002).

Intravenous ZA has proven to be very effective in the treatment of menopausal and glucocorticoid-induced osteoporosis, malignant hypercalcemia, and Paget’s disease (Adami et al. 2002; Aparicio et al. 1998; Boutsen et al. 2001). Clinical benefits in patients with cancer and multiple myeloma include bone pain improvement (Lipton et al. 2002; Reid et al. 2002), reduction, and delay in skeletal complications (Coukell and Markham 1998; Lipton et al. 2002). As earlier mentioned pharmacokinetics pattern is same in rat and patients, toxicity profile in animal can be extrapolated to human. However, very scant information is available so far on the toxicity and safety profile of this marketed drug (Lacerna and Hohneker 2003). To bridge this gap, a subchronic toxicity study was designed at three different dose levels. The highest dose selected was thirty times of the human dose.

The current study demonstrates the safety profile of this promising therapeutic choice ZA in rat. As this drug has very long duration of action hence safety should be established to ensure risks involve with the therapy.

To investigate possible toxic outcomes of ZA all physical and biochemical parameters were estimated. ZA inhibits osteoclast mediated bone resorption have been reported to occur in approximately 50% of patients with metastatic cancer, but certain cancers including multiple myeloma, breast, prostate, lung, kidney and thyroid carcinoma are more frequently associated with clinically symptomatic bone

lesions thus in these critical situations strongly requires monitoring of vital organs including liver and kidney. Another important aspect to consider was its wide use for the treatment of established osteoporosis after the menopause, increases bone density and reduction in the risk of fractures (Body et al. 1999; Black et al. 2007). The effects of ZA were studied and observed no physical changes were observed during the study period of twenty eight days in all three treatment groups. Increase in body weights and growth of treated animals of either sex were of similar pattern as in control groups.

ZA had no toxic effect on liver, as measured using the biochemical parameters described in this study suggested no significant changes in serum alkaline phosphatase, SGOT and SGPT activities in ZA treated groups of either sex as compared to the respective control group (Table 3 and 4). This confirms the safety profile of ZA injection in hepatic related aspects, in the rat. Blood was evaluated for potential hematological toxicity of this agent. Hemogram was estimated and results had shown no deleterious effect on blood cell count, haemoglobin and other related parameters in treated animals (Table 1 and 2).

ZA is eliminated unchanged through renal excretion, thus it is mandatory to estimate effects on kidney function (Kimmel 2007). Biochemical parameters related to kidney function were evaluated and no significant differences were observed in BUN, glucose and proteins with respect to control (Table 3 and 4). Previous reports also suggested ZA has demonstrated a favourable renal safety profile when compared with control (Gouin et al. 2006).

There were no signs of toxicity observed in any of the organs examined in the histopathological analysis conducted for the doses that were administered in male and female rats. Thus histopathological studies also support the safety data of other physiological, biochemical and hematological parameters after ZA injection. The limitations of this study were limited organs have been studied and effect on blood coagulation has not been explored. It reserved scope of detailed study keeping these points in consideration. This study does not give indication of NOAEL and only proved no toxic effects of ZA treatment in treated rats.

In summary, our data suggest that ZA is safe even at a very high dose level which is approximately 30 times higher than the dose intended to be used in humans. In this study it indicates no significant alterations of any of physiological and biochemical parameters.

It can be concluded that in preclinical settings (subchronic dosing), a parenteral therapy consisting of potent compound ZA offers no obvious toxicity at any dose level.

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