Zoledronic acid induces micronuclei formation, mitochondrial-mediated apoptosis and cytostasis in kidney cells

Soma Shiva Nageswara Rao Singireesu\textsuperscript{a,b,}, Sujan Kumar Mondal\textsuperscript{b}, Suresh Yerramsetty\textsuperscript{c}, Sunil Misra\textsuperscript{a}

\textsuperscript{a} Genetic Toxicology Laboratory, Pharmacology and Toxicology Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500007, India
\textsuperscript{b} Biomaterials Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500007, India
\textsuperscript{c} Chemical Biology Division, CSIR-Indian Institute of Chemical Technology, Hyderabad 500007, India

\begin{abstract}
Aims: Zoledronic acid (ZA), a FDA approved drug has used widely in the treatment of bone metastasis complications, has been linked to renal toxicity with unclear mechanism. The present study is aimed at investigating the genotoxic and cytotoxic effects of ZA in renal epithelial cells.

Main methods: The genotoxic effect of ZA in Vero and MDCK cells determined by cytokinesis block micronucleus (CBMN) assay. The cytotoxic effect assessed by analysing cell cycle profile, cell death and mitochondrial membrane potential by flow cytometry using propidium iodide, AnnexinV-FITC/PI and JC1 dye staining, respectively, BAX and Bcl-2 expression by Western blotting and caspase activity by spectrofluorimetry.

Key findings: The cytotoxic effect of ZA based on MTT assay revealed variable sensitivities of Vero and MDCK cells, with IC\textsubscript{50} values of 7.41 and 109.58 \textmu M, respectively. The CBMN assay has shown prominent dose-dependent induction of micronuclei formation in both cells, indicating ZA's clastogenic and aneugenic potential. Further, the ZA treatment led the cells to apoptosis, evident from dose-dependent increase in the percentage of cells in subG1 phase and display of membranous phosphatidylserine translocation. Studies also confirmed apoptosis through mitochondria, evident from the prominent increase in BAX/Bcl-2 ratio, mitochondrial membrane depolarization and caspase-3/7 activity. In addition, ZA reduces cytokinetic activity of renal cells, evident from dose-wise lowered replicative indices.

Significance: The study depict ZA's potential genotoxic effect along with cytotoxic effect in renal epithelial cells, could be key factors for the development of renal complications associated with it, which prompts renal safety measures in lieu with ZA usage.

\end{abstract}

1. Introduction

Zoledronic acid (ZA) chemically designated as (1-Hydroxy-2-imidazol-1-yl-phosphonoethyl) phosphonic acid monohydrate, is a potent inhibitor of osteoclastic bone resorption [1], employed in the treatment of osteoporosis, Paget's disease of bone, tumor induced osteolysis due to bone metastasis and also as a combination drug for multiple myeloma [2]. ZA reduces bone breakdown, thus lowering circulating calcium levels in blood [3]. ZA is non-metabolizable, either taken up by bone or and recovered in urine, indicating its elimination via the kidneys in its native form [4]. FDA reported 72 renal failure cases, who underwent 18 months of ZA medication and here found to partial recovery upon drug-discontinuation [5]. Similarly, acute tubular necrosis (ATN) in ZA treated (3 to 9 months) Paget's disease and multiple myeloma patients was also reported [6]. Studies also conveyed renal impairment incidents in patients (multiple myeloma, breast, prostate and non-small-cell lung cancer), who received ZA infusion [7,8].

Generally, nitrogen-containing bisphophonates (N-BP) inhibit farnesyl diphosphate synthase, a key enzyme of mevalonate pathway that leads to the inhibition of prenylation of cellular proteins, which is essential for the functioning of GTPases and thus, cell survival [9,10]. Even though bisphophonates (BP) are widely used in various medical procedures, the knowledge about their probable induction of renal cytotoxicity at molecular level is very limited. In a recent study, Verhulst et al. [11] revealed higher levels of ZA accumulation in human renal tubular cells through fluid phase endocytosis. The ZA exposure in kidney cells also induced lowering of prenylated protein levels through inhibition of FPP synthase, which is similar to the mechanism of therapeutic effect of ZA as in osteoclasts [12]. It is highly essential to characterize ZA's possible toxic effects in renal cells at molecular level,
which would give ideas about the preventive measures to be taken to keep the renal system safe.

In the present study, we investigated potential genotoxic effect of ZA in renal epithelial cells i.e., Verda reno (Vero) and Madin-Darby canine kidney (MDCK) cells using cytokinesis block micronucleus assay and cytotoxic effect by assessing cell cycle profile, mode of cell death, mitochondrial stress. Results revealed prominent genotoxic and cytotoxic effect of ZA in renal cells under in vitro condition, raises the essentiality of in-depth investigation further, in order to avoid possible adverse effects of ZA.

2. Materials and methods

2.1. Chemicals

Zoledronic acid was purchased from Novartis Pharmaceuticals Corp. (New Jersey, USA). The chemicals were procured from various suppliers based on the experiments e.g. Flow cytometry (Life Technologies, CA, USA); Western blot and primary antibodies - Anti-BAX, and Anti-Bcl2 (Cell Signaling Technology, MA, USA); Secondary antibodies - goat anti-mouse and anti-rabbit IgG Alkaline Phosphatase (Calbiochem, SD, USA); Caspase-3/7 assay (AnaSpec, CA, USA); cell culture consumables and other fine chemicals (Sigma-Aldrich, Missouri, USA).

2.2. Cell lines and culture condition

Vero and MDCK cell lines obtained from National Centre for Cell Science (India) were cultured in DMEM media supplemented with 10% FBS, 2 mM l-glutamine, 1 mM Sodium pyruvate, 60 μg/ml penicillin, 100 μg/ml streptomycin and maintained in 5% CO2 incubator with 37 °C temperature. Cells were sub-cultured every 2–3 days.

2.3. MTT assay

The cytotoxic activity of ZA against renal cells was predicted using MTT assay [13]. Cells seeded in 96 well plates (104 cells/well) were allowed to attain proper morphology and attachment by incubating in a 5% CO2 humidified incubator. Different concentrations of ZA was added to Vero (1, 2, 4, 6, 8 and 10 μM) and MDCK cells (10, 20, 40, 60, 80, 100 and 120 μM) along with DMSO control, in triplicates and incubated for 24 h. Afterwards, media was replaced with serum-free media and incubated for another 3 h. Once the plates were dried completely, 100 μl DMSO was added to each well and OD readings were taken at 570 nm. From the concentration versus percentage cellular growth inhibition graph, regression equation was derived and the IC10, IC30 and IC50 values of ZA were determined for renal cell types.

The assessment of genotoxic effect and cell death induction of ZA was performed on renal cell lines (Vero and MDCK cells), which were treated with their respective IC10, IC30 and IC50 concentrations of ZA.

2.4. CBMN assay

The cytokinesis block micronucleus (CBMN) assay was performed on renal cells (2 X 105 cells/100 mm dish) that were treated with different concentrations of ZA (IC10, IC30 and IC50), 2.5 μM Mitomycin C (positive control) and DMSO (vehicle control) according to the procedure described by Bollu et al. [14] with slight modifications. After treatment completion (24 h), cells were additionally incubated with 5 μg/ml of Cytochalasin B for 18 h, to induce the cytokinesis block in cells. The cells were then trypsinized, centrifuged (1500 rpm/5 min) and suspended for 10 min in 0.9% Sodium citrate at 8 °C. Afterwards, cells were re-centrifuged (1200 rpm for 5 min) and suspended in low volume Sodium citrate. The cell suspensions were smeared gently on clean, grease-free glass slides and kept overnight at 37 °C in 85% relative humidity. Later, the slides were stained with 0.5% Giemsa and observed under microscope to determine the frequency of binucleated micronucleated (BNMN) cells. At least 1000 cells per set in triplicates for each treatment were screened along with the controls. Parallely, the cytokinetic activity of cells in the presence of different concentrations of ZA was also assessed by measuring their replicative indices (RI). RI refers to the proliferation rate of treated cells relative to the untreated cells and was calculated using the formula [15].

\[
\text{RI} = \frac{[(\text{Total binucleated cells}) + (2\times \text{Total multinucleated cells})]}{[(\text{Total binucleated cells}) + (2\times \text{Total multinucleated cells})]} \times 100
\]

The cytostatic effect was in turn calculated from the measured RI values.

Cytostasis = 100 – RI

2.5. Flow cytometry

2.5.1. PI FACS

The cell cycle profile of ZA treated renal cells was studied by propidium iodide (PI) staining and flow cytometry [16]. After treatment (24 h), the cells were trypsinized, washed with PBS and fixed overnight at −20 °C in 70% ethanol. Later, the cells were given two more PBS washes and then incubated in PI staining solution (0.2% Triton X-100, 50 μg/ml PI and 0.1 mg/ml RNase A in PBS) for 30 min in dark light at room temperature. After giving PBS wash, cells were immediately analysed with Mo Flo Legacy flow cytometer (Beckman Coulter) and Summit V.3 software (Beckman Coulter).

2.5.2. AnnexinV-FITC/PI FACS

The apoptotic/necrotic death induction by ZA in renal cells was characterized by using a FITC tagged AnnexinV and PI double stain apoptosis detection kit (BioLegend). After 12 h of ZA (IC30 & IC50) treatment, cells were trypsinized, washed with PBS and suspended in 300 μl AnnexinV/PI staining solution for 15 min at room temperature in dark. Immediately, the cells were analysed with Mo Flo Legacy flow cytometer (Beckman Coulter).

2.5.3. JC1 dye FACS

The quantification of cells with lowered mitochondrial membrane potential was done by JC1 dye staining and flow cytometry [17]. In brief, the Vero and MDCK cells grown in 6 well plates (4 × 105 cells/well) were treated with IC50 and IC30 concentrations of ZA for 12 h, detached by trypsinization, incubated with 2.5 μg/ml JC1 dye for 15 min in dark and finally, analyzed in Mo Flo Legacy flow cytometer (Beckman Coulter).

2.6. Western blot analysis

The expression of BAX and Bcl-2 proteins in renal cells was analysed using Western blot technique [13]. After treating the cells with different concentrations of ZA (IC10, IC30 & IC50) for 24 h, the cellular protein lysates were prepared by using RIPA buffer extraction procedure. Cells were lysed thoroughly in a low volume RIPA buffer (Millipore) in ice for 15 min and centrifuged at 14000 rpm at 4 °C for 20 min. After estimating the collected supernatant's protein concentration by using BCA assay kit (Thermo), each sample's protein (40 μg) was subjected to a gradient of 12% SDS PAGE. Later, the protein bands were electro-transferred on to a PVDF membrane (Millipore) and then, the blot was incubated in a blocking solution (5% fat-free milk powder in TBST) for 1 h at 4 °C. After giving three times TBST wash, the blot was incubated with primary antibodies i.e., 1:1000 of Rabbit pAb anti-Bax (clone 2772, Cell Signalling), Rabbit pAb anti-Bcl-2 (clone 2876, Cell Signalling) and Rabbit pAb anti-β-Actin (clone AC-74, Sigma-Aldrich).
overnight at 4 °C. After giving three times TBST wash, the blot was subsequently incubated for an hour with alkaline phosphatase conjugated secondary antibody. Finally, BCIP/NBT chromogenic substrate (Millipore) was applied on to the blot to visualize the specific protein bands and their intensities were quantified by using ImageJ software (NIH).

2.7. Caspase-3/7 assay

Caspase-3/7 assay was performed by using a fluorescence based kit method (AnaSpec). Briefly, cells in 6 well plates (3 × 10^5 cells/well) were treated for 24 h, separately with 2.5 μM Mitomycin C (positive control), 20 μM Z-DEVD-FMK (caspase inhibitor; BD Biosciences) and different concentrations of ZA (IC_{30} & IC_{50}) along with untreated cells. The fluorescence intensity of a protein lysate, proportional to its caspase activity was measured with Bio Lumin 960 (Molecular Dynamics, USA) multimode reader, set with an excitation at 400 nm and emission at 505 nm wave length. The net caspase-3/7 activity of test sample was determined by comparing with that of the untreated cells.

2.8. Statistical analysis

All the experiments were carried in triplicates. The experimental significance between the treatments was measured by performing analysis of variance (ANOVA) followed by Dunnett’s posttest (GraphPad Prism 7.01, USA). The experiments for which, the ‘P’ value ≤ 0.05 in comparison to the control were considered as statistically significant.

3. Results

In order to assess the cytotoxic effect of Zoledronic acid (ZA) in renal epithelial cells, MTT assay was performed for Vero and MDCK cells after 24 h of treatment with different concentrations of ZA. The cellular growth limiting effect of a drug in comparison to the untreated condition was based on the conversion of MTT into insoluble formazan crystals by the metabolically active cells [18]. Both Vero and MDCK cells treated with differing concentrations of ZA have shown dose-dependent reduction in cell growth in comparison to the untreated cells (Fig. 1). Among Vero cells, the lowest concentration of ZA (1 μM) treated cells have shown slight reduction in cell growth (5.5%) in comparison to the untreated cells, which was found to be statistically insignificant (P = 0.15). Whereas, the Vero cells treated with ZA at 2, 4, 6, 8 and 10 μM concentrations have shown significantly (P < 0.001) reduced cell growth of 16.25, 30.5, 42.5, 52.25 and 59.75%, respectively, in comparison to the untreated cells. Similarly, the ZA treated MDCK cells at 10, 20, 40, 60, 80 and 120 μM concentrations have shown significantly (P = 0.04; P = 0.02; P < 0.001; P < 0.001; P < 0.001; P < 0.001) reduced cell growth of 8.65, 9.46, 13.31, 20.26, 29.9, 42.63 and 58.34%, respectively, in comparison to the untreated cells. From the concentration verses percentage cell growth reduction graph (Fig.1), regression equation was derived. By using the equation, the concentration values of ZA, where the cells underwent 10, 30 and 50% growth reduction (IC_{10}, IC_{30} and IC_{50}) were calculated (Table 1). On comparing the inhibitory concentration values (IC), it was found that the renal cell lines (Vero and MDCK cells) possess variable levels of sensitivity toward ZA and their IC_{50} values differed largely from each other. Vero cells appeared to be highly sensitive, as their respective IC_{50} value (7.41 μM) was much lower in comparison to MDCK cells (IC_{50} = 109.58 μM).

3.1. Genotoxic effect

The clastogenic and aneugenic potential of ZA in Vero and MDCK cells was assessed using CBMN assay. The treatment with ZA at different concentrations (IC_{10}, IC_{30} & IC_{50}) in both Vero (1.4, 3.94 & 7.41 μM ZA) and MDCK (23.86, 79.9 & 109.58 μM ZA) cells has shown prominent induction of micronuclei (MN) formation in binucleated (BN) cells in a dose-dependent manner (Table 2). In case of Vero cells, the lowest concentration of ZA (IC_{10}/1.4 μM ZA) treated cells have shown slightly higher MN formation in comparison to the untreated cells, which was found to be statistically insignificant (P = 0.294). Whereas, the cells treated with ZA at higher concentrations (IC_{30} & IC_{50}/3.94 & 7.41 μM ZA) and positive control (2.5 μM Mitomycin C) have shown significantly (P < 0.001; P < 0.001; P = 0.044) higher MN formation in comparison to the untreated cells. Similarly, in case of MDCK cells, the treatment with lowest concentration of ZA (IC_{10}/23.86 μM) has shown slightly higher MN formation in comparison to the untreated cells, which was found to be statistically insignificant (P > 0.99). Whereas, the MDCK cells treated with ZA at higher concentrations (IC_{30} & IC_{50}/79.9 & 109.58 μM ZA) and positive control (2.5 μM Mitomycin C) have shown significantly (P < 0.001; P < 0.001; P = 0.005) higher MN formation in comparison to the untreated cells. The higher induction of micronuclei formation indicate potential genotoxicity of ZA in Vero and MDCK cells. The microscopic observation also revealed varied sizes of MN (approximately ranging from one sixth to one third in comparison to the main nucleus) in BN MN (binucleated micronucleated) cells, suggesting the induction of both clastogenic and aneugenic effects by ZA in renal cells.

3.2. Cell death

To find whether the ZA treatment leading the Vero and MDCK cells to cell cycle arrest or/and cell death depending on dosage, cell cycle analysis was performed after 24 h treatment with varying concentrations of ZA (IC_{10}, IC_{30} & IC_{50}). The results have shown prominent cell death induction potential of ZA in both Vero and MDCK cells as there was a dose-dependent increase in the percentage of subG1 phase cell population (Fig. 2A; Table S1). In case of Vero cells, the IC_{10}, IC_{30} and IC_{50} concentrations of ZA (1.4, 3.94 & 7.41 μM ZA) treated cells have shown significantly (P = 0.025; P < 0.001; P < 0.001) higher percentage of subG1 population of 11.89, 32.99 and 48.58%, respectively, in comparison to the untreated cells (4.56%; Fig. 2B). Similarly, the MDCK cells treated with IC_{10}, IC_{30} and IC_{50} concentrations of ZA (23.86, 79.9 & 109.58 μM ZA) have shown significantly (P = 0.003; P < 0.001; P < 0.001) higher percentage of subG1 population of 11.37, 30.29 and 48.51%, respectively, in comparison to the untreated cells (0.99%; Fig. 2C).

In order to find the mode of cell death (apoptotic or necrotic/both) induced by ZA in Vero and MDCK cells, AnnexinV-FITC/PI staining and flow cytometry was performed (Fig. 2D). In case of an apoptotic cell, the membranous phosphatidylserine (PS) moieties translocate toward outer side. The cell impermeable FITC tagged AnnexinV specifically bind to the externalized PS moieties and emits fluorescence, which helps in quantifying the number of apoptotic cells. The results have
shown prominent apoptosis induction of ZA in both Vero and MDCK cells, as there was a dose-dependent increase in the percentage of cells displaying externalized membranous PS moieties. The Vero cells treated with ZA at IC_{30} and IC_{50} concentrations (3.94 & 7.41 μM ZA) for 12 h have shown significantly (P < 0.001) higher percentage of apoptotic cells of 17.57 and 25.06%, respectively in comparison to the untreated cells (2.47%; Fig. 2E). At the same time, they have shown a significantly (P < 0.001) higher percentage of apoptotic cells of 28.46 and 37.96%, respectively in comparison to the untreated cells (3.92%; Fig. 2F). At the same time, they have shown prominent apoptosis induction of ZA in both Vero and MDCK cells treated with different concentrations of ZA (24 h) growth inhibition assessed by MTT.

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Polynomial regression equation</th>
<th>(R^2)</th>
<th>Concentration (in μM)</th>
<th>IC_{50} (mean ± SD)</th>
<th>IC_{90} (mean ± SD)</th>
<th>IC_{95} (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>y = −0.349x^2 + 9.737x − 2.9954</td>
<td>&gt; 0.99</td>
<td>1.40 ± 0.2</td>
<td>3.94 ± 0.47</td>
<td>7.41 ± 0.86</td>
<td></td>
</tr>
<tr>
<td>MDCK</td>
<td>y = 0.0037x^2−0.0271x−8.5395</td>
<td>&gt; 0.99</td>
<td>23.86 ± 6.44</td>
<td>79.9 ± 6.37</td>
<td>109.58 ± 7.44</td>
<td></td>
</tr>
</tbody>
</table>

a: Cytotoxic activity: Exponentially growing cells treated with different concentrations of ZA (24 h) growth inhibition assessed by MTT.

b: Polynomial regression equation: y = ax^2 + bx + c; where, x: independent variable; y: dependent variable; a: quadratic co-efficient; b: linear co-efficient; c: constant

c: R^2: co-efficient of determination. Estimates the fit of regression method

d: IC_{10, 30, 50} is defined as the concentration, where 10, 30 and 50% decrease in cell growth compared to the untreated cells.

### Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sample</th>
<th>Total cells screened</th>
<th>Total BNMN cells</th>
<th>Total MN</th>
<th>MN/1000 cells (mean ± SD)</th>
<th>%MN increase in relation to vehicle control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>DMSO</td>
<td>3596</td>
<td>43</td>
<td>54</td>
<td>15.3 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>ZA</td>
<td>IC_{50}</td>
<td>3185</td>
<td>61</td>
<td>64</td>
<td>20.67 ± 3.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IC_{30}</td>
<td>3110</td>
<td>91</td>
<td>102</td>
<td>34.0 ± 6.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IC_{60}</td>
<td>3110</td>
<td>94</td>
<td>112</td>
<td>36.3 ± 3.06</td>
<td></td>
</tr>
<tr>
<td>MDCK</td>
<td>DMSO</td>
<td>3027</td>
<td>8</td>
<td>8</td>
<td>2.67 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>ZA</td>
<td>IC_{50}</td>
<td>3107</td>
<td>9</td>
<td>9</td>
<td>3.00 ± 1.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IC_{30}</td>
<td>3040</td>
<td>67</td>
<td>73</td>
<td>24.3 ± 2.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IC_{60}</td>
<td>3077</td>
<td>75</td>
<td>77</td>
<td>25.7 ± 1.53</td>
<td></td>
</tr>
<tr>
<td>Mit C</td>
<td>3354</td>
<td>78</td>
<td>84</td>
<td>24.33 ± 3.21</td>
<td>59.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3010</td>
<td>23</td>
<td>34</td>
<td>9.00 ± 2.00</td>
<td>237.08</td>
<td></td>
</tr>
</tbody>
</table>

The significant difference of treatments in comparison to the negative control (DMSO) * P < 0.05 (Dunnett's post test)

a: Total BNMN cells: Total number of binucleated micronucleated cells

b: MN/1000 cells: number of micronuclei per 1000 cells

c: RI: replicative index, refers to the proportion of cell division cycles completed in a treated sample relative to the untreated sample till the chemical exposure period.

d: Mit C: 2.5 μM Mitomycin C (positive control).

e: IC_{10, 30, 50} is defined as the concentration, where 10, 30 and 50% decrease in cell growth compared to the untreated cells.

### 3.3 Mitochondrial mediated apoptosis

The apoptosis induction may involve alteration in the expression of pro- and anti-apoptotic protein members of Bcl-2 family, which influence the mitochondrial membrane integrity [19]. The Western blot analysis was performed to check the alteration in expression levels of pro-apoptotic ‘BAX’ and anti-apoptotic ‘Bcl-2’ proteins in Vero and MDCK cells treated with ZA (Fig. 3A). Vero cells at IC_{10}, IC_{30} and IC_{50} concentrations of ZA (1.4, 3.94 & 7.41 μM ZA) have shown higher BAX/Bcl-2 ratios of 0.76 (P = 0.07), 2.19 (P < 0.001) and 6.05 (P < 0.001), respectively, when compared to the untreated cells (BAX/Bcl-2 = 0.24). Similarly, MDCK cells at IC_{10}, IC_{30} and IC_{50} concentrations of ZA (23.86, 79.9 & 109.58 μM ZA) have shown significantly (P = 0.03; P < 0.001; P < 0.001) higher BAX/Bcl-2 ratios of 0.42, 0.68 and 2.1, respectively, in comparison to the control (0.07). The results indicate dose-dependent increase in BAX/Bcl-2 ratio in ZA treated cells as a consequence of proportional increase and decrease in the expression of BAX and Bcl-2 proteins, respectively, with the increase in concentration. The increase in BAX/Bcl-2 ratio causes elevation in the mitochondrial membrane permeability of cells.

The alteration in mitochondrial membrane permeability in renal cells upon exposure (12 h) to ZA was confirmed by JC1 dye staining and flow cytometry. In healthy cells, the JC1 dye accumulate as polymeric (aggregate) form in mitochondria due to higher membrane potential and emits red fluorescence (FL2). In case of cells under stress condition, the JC1 dye remain dispersed as monomeric form in mitochondria due to lower membrane potential and emits green fluorescence (FL1). The results have shown dose-dependent increase in the population of cells exhibiting mitochondrial membrane depolarization in both ZA treated Vero and MDCK cells (Fig. 3B). The Vero cells treated with IC_{50} and IC_{90} concentrations of ZA (3.94 & 7.41 μM ZA) have shown significantly (P < 0.001) higher percentage of FL1′ population of 33.45 and 45.98%, respectively, in comparison to the untreated cells (2.58%; Fig. 3C). Similarly, the MDCK cells treated with IC_{50} and IC_{90} concentrations of ZA (79.9 & 109.58 μM ZA) have shown significantly (P < 0.001) higher percentage of FL1′ population of 27.39 and 42.87%, respectively, in comparison to the untreated cells (2.43%; Fig. 3D). Hence, the ZA treatment in both Vero and MDCK cells causes prominent increase in mitochondrial membrane permeability.

The increase in the mitochondrial membrane permeability results in the activation of caspases [20]. In order to determine the induction of caspases in ZA treated Vero and MDCK cells, a fluorescence based caspase-3/7 assay was performed. The substrate 7-amino-4-trifluoromethyl coumarin conjugated with Asp-Glu-Val-Asp (Ac-DEVD-
AFC) in the reaction mixture, gets cleaved in the presence of caspase-3/7 active form and emits a fluorescence signal. The caspase activity of Vero and MDCK cells was measured in the presence of Mitomycin C (positive control), which is a well-known inducer of caspase-3 [21] and caspase-3 inhibitor, Z-DEVD-FMK, in order to determine the lowest limit of caspase activity in renal cells [22]. The results have shown a dose-dependent increase in caspase activity in ZA treated renal cells (Fig. 3E, F and Table S2). The Vero cells treated with IC30 and IC50 concentrations of ZA (3.94 & 7.41 μM ZA) and positive control (2.5 μM Mitomycin C) have shown significantly (P < 0.001) higher levels of caspase activity of 4.83, 7.69 and 7.74, respectively in comparison to the untreated cells (1.65; Fig. 3E). Similarly, the MDCK cells treated with IC30 and IC50 concentrations of ZA (79.9 & 109.58 μM ZA) and positive control (2.5 μM Mitomycin C) have shown significantly (P < 0.001) higher levels of caspase activity of 7.19, 10.51 and 7.01, respectively in comparison to the untreated cells (3.63; Fig. 3F). At the same time, the Vero and MDCK cells treated (24 h) with caspase inhibitor have shown lower levels of caspase activity of 1.09 and 2.57, respectively, in comparison to the respective untreated cells (1.65 and 3.63, respectively) and were found to be statistically insignificant (P = 0.765; P = 0.362). Thus, the ZA treatment in renal epithelial cells induces mitochondrial-mediated apoptosis.

3.4. Cytostasis

The calculated replicative indices (RI) based on CBMN assay for Vero, and MDCK cells treated (24 h) with different concentrations of ZA (IC10, IC30 & IC50 i.e. 1.4, 3.94 & 7.41 μM ZA-Vero cells; 23.86, 79.9 & 109.58 μM ZA-MDCK cells) have shown prominent cytostatic effects in the range of 8.49–36.95% and 2.67–30.42% (Table 2), respectively, indicating a substantial percentage of cells remained in mono-nucleated or undivided state. The positive controls (2.5 μM Mitomycin C) of Vero and MDCK cells exhibited 6.88 and 4.53% cytostatic effect, respectively. This suggests ZA’s probable role in cytokinesis inhibition in...
addition to apoptosis induction.

4. Discussion

Zoledronic acid (ZA) used widely in the treatment of bone-related ailments has been known to induce renal toxicity associated with acute tubular necrosis and renal fibrosis [23], with the underlying mechanism/s remaining unclear. ZA 4 mg for infusion is given as single dose for patients with tumour-induced hypercalcaemia and once every 3–4 weeks for patients with advanced bone metastases. Approximately 40% of the administered dose gets excreted through urine within 24 h of dosage. The renal GFR (glomerular filtration rate) of ZA is only 50% (approximately) in cancer patients with bone metastases, when compared to normal condition [24]. The variations of kidney retention and plasma protein binding play a role in the renal safety issues of ZA in clinical use [25,26]. It is very much needed to establish a relation between administered doses, kidney retention levels of ZA and extent of deterioration of renal function, especially in patient groups having high risk of renal impairment.

Studies have shown inhibition of FPP synthase by ZA in renal cells also, similar to the mechanism of therapeutic effect as in osteoclasts. The effective cellular growth and FPP synthase enzyme inhibitory concentration values (EC50 and IC50, respectively) of ZA were largely varied [12], raises a suspicion of additional means of toxicity. In the present study, we hypothesized the implication of genetic toxic effect and its relevant cellular death induction by ZA in renal cells could play a role in its associated renal toxicity. Studies were oriented toward the assessment of genotoxic and cytotoxic potential of ZA in Vero and MDCK cells. ZA at a concentration range of 0, 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100 and 120 μM (corresponding to 0, 270, 540, 810, 1620, 2160, 2700, 2700 × 2, 2700 × 4, 2700 × 6, 2700 × 8, 2700 × 10 and 2700 × 12 ng/ml) has been used. The lowest concentration used in our experimental set-up precisely correspond to the serum levels in ZA treated patients, as the 15 min intravenous infusion of 4 mg ZA builds

![Fig. 2. (continued)](image-url)
Fig. 3. Mitochondrial mediated apoptosis in ZA treated Vero and MDCK cells. A Alteration in BAX and Bcl-2 protein levels determined using Western blot technique. The graphs quantitatively represent the change in BAX/Bcl-2 protein ratio based on concentration.

B JC1 dye FACS of cells treated (12h) with different concentrations of ZA.

C & D Quantitative presentation of ‘FL2’ and ‘FL1’ population of cells after treatment with ZA.

E & F Caspase-3/7 induction in ZA treated Vero and MDCK cells. By using a fluorescence based method, caspase-3/7 activity for different concentrations of ZA, 2.5 μM Mitomycin C (positive control) and caspase inhibitor (20 μM Z-DEVD-FMK) treated (24 h) cells were determined. The significant difference of treatments in comparison to the untreated cells, * = P < 0.05 (Dunnett's multiple comparison test).
serum ZA Cmax levels around 300 ng/ml [24]. We are able to demonstrate prominently ZA's possible induction of genotoxic as well as cytotoxic effect in renal cells by using higher concentrations of ZA (≤120 μM), which is supported with the observation of excess levels of ZA accumulation in renal tubular cells through fluid phase endocytosis [11]. The Vero cells are derived from rhesus monkey kidney, have been used as cell line model for characterizing renal toxicity of lead nitrate, snake venoms, mycotoxins and calcium oxalate [27–31]. Similarly, the MDCK cells are derived from cocker spaniel kidney, form “domes” and tight junctions in confluent monolayers resembling distal tubule cells [32,33] and are well-applicable in characterizing drug induced distal tubule toxicity [34,35].

The MTT assay has predicted dose-dependent potential cytotoxic effect of ZA against Vero and MDCK cells with the IC50 values of 7.41 and 109.48 μM concentration, respectively (Fig. 1 & Table 1). The differences of cellular as well as species origin might attribute to their variation in sensitivity toward ZA. In order to explore ZA safely in cancer therapy, it is important to establish its relative toxicity toward the cells of both cancer and normal origin. Based on the earlier cytotoxicity studies on various cancer cell lines viz., renal cancer (CAKI-2, HEK-293, RBM1-IT4), reticulum sarcoma (J774), non-small cell lung cancer (A549), oral carcinoma (HSC3) and multiple myeloma (NCL-H929) cells [36–41]; the furnished IC50 values of ZA were found to be higher in comparison to that of the present studied Vero and MDCK cells (IC50 = 7.41 and 109.48 μM, respectively), indicating possible higher sensitivity of normal kidney cells toward ZA.

The growth limitation effect of ZA in renal cells might be due to the induction of genetic damages [42]. The micronuclei represent genetic damages that have been transmitted to daughter cells and are the extranuclear bodies containing whole chromosome/s due to their failure of getting incorporated into the nucleus during cell division and/or part of chromosome/s due to chromosomal breaks [43,44]. The CBMN assay revealed prominent dose-dependent increase in the number of binucleated micronucleated (BNMN) cells (Table 2) containing variable sizes of micronuclei in ZA treated Vero and MDCK cells, indicate the induction of both clastogenic and aneugenic effects. The morphological discrimination based on size, where the large and small sized micronuclei correspond to whole chromosome/s and part of chromosome/s, respectively, was found to be a reliable method [45]. The micronucleated cells constitute strong apoptotic signal and hence, gets eliminated by apoptosis [46–48]. The cell cycle analysis and AnnexinV-FITC/PI staining followed by flow cytometry of ZA treated Vero and MDCK cells revealed dose-dependent increase in the population of cells at subG1 phase and display of membranous phosphatidylserine translocation, respectively (Fig. 2), confirm apoptosis induction of kidney cells by ZA.

The genetic damages could promote mitochondrial-mediated apoptosis of cells [49–52]. It is well established fact that the alteration in expression levels of BAX and Bcl-2 proteins modulate mitochondrial membrane integrity [53–55]. The Western blot analysis for different concentrations of ZA treated Vero and MDCK cells revealed dose-dependent increase in BAX/Bcl-2 ratio (Fig. 3A) supports alteration in mitochondrial membrane permeability. This was further confirmed by JC1 dye staining followed by flow cytometry of ZA exposed Vero and MDCK cells revealed dose-dependent increase in the percentage of green fluorescence ‘FL1’/mitochondrial membrane depolarized cell population (Fig. 3C, D). The increased mitochondrial membrane permeability in turn causes the release of different apoptosis promoting factors from inter-mitochondrial membrane space into cytoplasm, thereby activating the caspases to initiate entire apoptosis process [56–58]. The caspase-3/7 assay of ZA treated Vero and MDCK cells revealed dose-dependent increase in caspase activity (Fig. 3E, F), further establishes the fact that apoptosis is indeed mediated through mitochondria. The apoptosis induction through mitochondria by ZA

![Fig. 3. (continued)](image-url)
was in turn reported in different types of cancer cells [59–63], indicating undistinguished mode of action of ZA against cells irrespective their origin (normal/cancer).

In addition to apoptosis induction, the dose-dependent reduction in replicative indices (RI) based on CBMN assay in both Vero and MDCK cells (Table 2) indicate lowering of cellular dividing potential of renal cells upon exposure to ZA. The replicative index (RI) determines the proliferation rate of treated cultures in correlation with the untreated culture [15]. The assessment of renal toxicity of chemicals based on endpoints like, cell growth, apoptosis and mitochondrial membrane potential considered to be reliable [64].

From the above observations, it is clear that ZA has the potential to induce both genotoxic effect and cytotoxic effect (mitochondrial-mediated apoptosis and cytostosis) in kidney cells.

5. Conclusion

Zoledronic acid exhibit potential genotoxic and cytotoxic activity against renal cells (Vero and MDCK cells). The prominent induction of micronuclei formation depicts higher genotoxic potential of ZA toward against renal cells (Vero and MDCK cells). The prominent induction of apoptosis and cytostasis in kidney cells.

Conflict of interest

All authors declare that they have no conflict of interest.

Acknowledgements

The author S.S.S.N. Rao (CSIR-UGC fellow), sincerely thank the University Grants Commission, New Delhi, for providing the fellowship. The authors thank Dr. Archnagiri, Associate professor, Centre for Biotechnology, JNTU for their valuable guidance in documentation and Dr. Bhuvaneswari Chodisetti (UGC-DSK PDF) for their guidance in the current study enlighten the essentiality of in-depth investigation of nephrotoxicity of ZA at various levels before exploring for treatment procedures.

References


