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New imidazoles cause cellular toxicity by impairing redox balance, mitochondrial membrane potential, and modulation of HIF-1α expression

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ABSTRACT

Background: Our previous reports demonstrated the prospects of a new series of imidazoles as a source of alternative anti-parasite treatments, thus warranting further studies that include toxicity profiling.
Objective: In this study, we evaluated three imidazoles: bis-imidazole (compound 1), phenyl-substituted 1*H*-imidazole (compound 2), and thiopene-imidazole (compound 3) for cellular toxicity and possible mechanisms.
Methods: The three (3) compounds were assessed for *in vitro* cytotoxic action. Additionally, we probed likely mechanistic actions of these imidazoles. Findings showed dose-dependent cellular toxicity by these imidazoles.
Results: In the presence of antioxidant (Trolox), cytotoxicity was improved for compounds 2 and 3 but not for compound 1. Meantime, compound 7 promoted reactive oxygen species (ROS) production, which was abated in the presence of a standard antioxidant (Trolox). Additionally, the three (3) imidazoles impaired mitochondrial membrane potential (MMP). While MMP was not restored after treatment removal, the addition of antioxidant (Trolox) improved MMP for compounds 2 and 3 treatment. Additionally, compound 1 elevated expression of hypoxia-inducing factor 1-alpha (HIF-1α). This may not be unconnected with the capacity of compound 1 to cause oxidative stress.
Conclusion: We show evidence that supports the cytotoxic action of imidazoles involves likely impairment to redox balance and mitochondrial membrane potential. The findings help our understanding of the mechanistic action of these imidazoles in living cells, and altogether may boost their prospects as new and alternative anti-protozoans.

1. Introduction

Imidazole derivatives have been reported to have diverse pharmacological properties, some of which include; anti-fungal, anti-parasitic, anti-bacteria and anti-cancer properties [1]. For example, imidazoles such as a derivative of 2-substituted-N-[4-(1-methyl4,5-diphenyl-1*H*-imidazole-2-yl)phenyl] acetamide has been implicated for cytotoxicity toward cancer cells [2]. In addition, new series of N-(6-substituted-benzothiazol-2-yl)-2-[[4,5-dimethyl1-((*p*-tolyl/4-nitrophenyl)amino)-1*H*-imidazole-2-yl] thio]acetamide derivatives were also revealed to possess higher cytotoxic activity against tumor cells than that of Cisplatin which is a known -anticancer drug [3]. On the other hand, cytotoxic actions by imidazoles and derivatives have been linked with DNA damage and oxidative stress in HepG2 cells [4].

Furthermore, heterocyclic compounds, including imidazoles, serve as biologically potent scaffolds. The activities associated with imidazoles include anti-mycobacterial, antiparasitic, antiprotozoal,

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and antihelminthic [5]. In our laboratories, we had earlier identified some novel imidazole compounds with activities against the growth of *Trypanosoma* and *Toxoplasma gondii* [6,7]. In agreement with our findings on imidazoles, a global approval was recently granted for the use of Fexinidazole (a derivative of 5-nitroimidazole) as an oral treatment of trypanosomiasis and Chagas' disease [8]. From all intents and purposes, the application of medicinal chemistry and modification of lead imidazole-derived compounds could be the way forward in the development of alternative and minimally toxic antidotes to trypanosomiasis.

Several workers have reported the toxicity of imidazoles and their derivatives to various cells, such as MCF-7 [9], Hep G2 cells [10], and human lung carcinoma A549 cells and l BEAS-2B bronchia cells [11]. However, there is limited information about the toxicological and mechanistic profiles of imidazoles. In this current study, we investigated the cellular mechanisms of toxicity of three imidazole derivatives: bis-imidazole (C-1), phenyl-substituted 1*H*-imidazole (C-2), and thiophene-imidazole (C-3) using Human foreskin fibroblast (HFF) cells as an experimental model. This study will significantly contribute to knowledge in terms of the cellular mechanisms of toxicity of imidazoles, and possibly promote their utility as anti-protozoan and/or therapeutic agents.

2. Materials and methods

2.1. General

Chemicals and reagents were of analytical grade and used as supplied.

2.2. Imidazole compounds

Imidazole compounds C-1, C-2, and C-3 were designed and synthesized by Dr. O.A. Eseola (Institute of Inorganic and Analytical Chemistry, Friedrich-Schiller-Universität Jena, Humboldtstraβe 8, 07743 Jena, Germany). The synthesis and characterization of compounds C-1, C-2, and C-3 followed established protocols as reported elsewhere [6,12].

2.3. The cytotoxicity of imidazoles

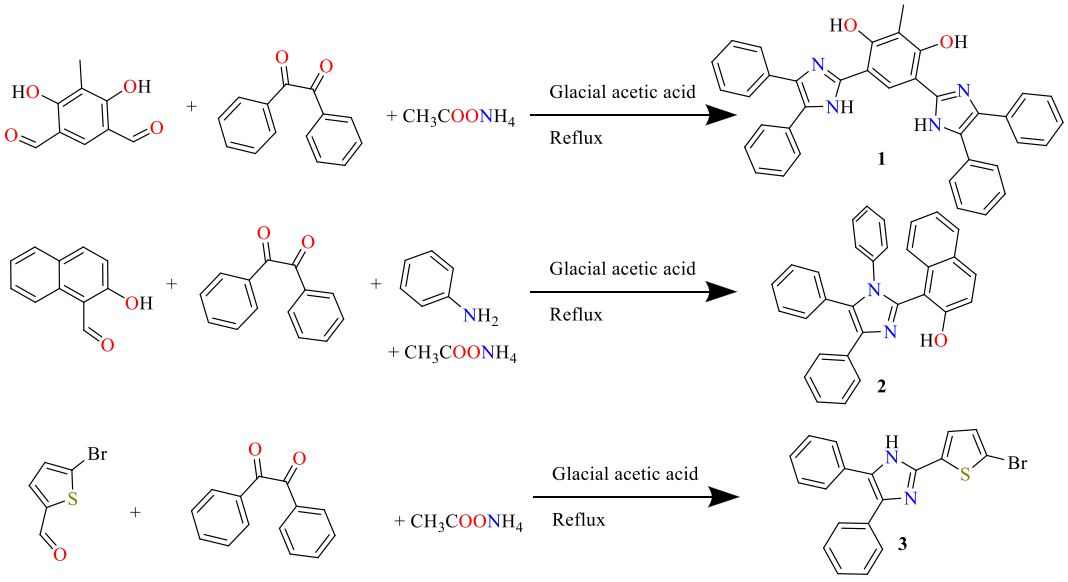
Human Foreskin Fibroblast (HFF) cells were grown in an atmosphere of 37 °C and 5% CO_2 in culture medium that consisted of Dulbecco's Modified Eagle's Medium (DMEM Nissui, Tokyo, Japan), 2 mM GlutaMAX™-I (Gibco, Invitrogen, UK), 10% (v/v) fetal calf serum (FCS; Gibco, Invitrogen, UK), and penicillin and streptomycin (100 U/mL; Biowhittaker, UK). Cells at 70–80% were sub-cultured into 96-well plates at a concentration of 1×10^5 cells per well. After incubating for 72 h at 37 °C and 5% CO_2, cells were treated with various concentrations of imidazoles (between 0.0 and 10 μM) in culture medium plus or minus Trolox (100 μM). The control well had only a culture medium. To validate the assay, staurosporine (1 μM final concentration) was used as a positive control drug. After a 72-h incubation, the colorimetric determination of cell viability using a CellTitre kit (Promega, Madison, USA), was performed at 490 nm on a microplate reader (Corona Electric, Hitachinaka, Japan). The biological evaluation was separately replicated three times and each assay done in triplicate.

2.4. Cellular reactive oxygen species (ROS) measurement

Assay for ROS in cells was performed as reported elsewhere [13]. The method is based on the oxidative formation of a fluorophore 2′,7′-dichlorofluorescein (DCF). In this assay, growing HFF cells were incubated with imidazoles (C-1, C-2, and C-3) at 1 x IC_{50} value plus or minus antioxidant (Trolox − 100 μM). After incubating for 24 h at 37 °C and 5% CO_2, the culture medium was removed, cells were rinsed and stained with 100 μM of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Sigma, St Louis, MO, USA) in PBS. After a 30–60 min incubation at 37 °C, fluorescence reading was at excitation of 485 nm and emission of 530 nm on a spectrofluorometer (Corona Electric, Japan). H_2O_2 (100 μM) was included as a positive control.

2.5. Mitochondrial membrane potential determination

The assay for mitochondrial membrane potential was as previously reported [13]. In this assay, growing HFF monolayers were treated with imidazoles (C-1, C-2, and C-3) at 1 x IC_{50} value plus or



Scheme 1. Representative scheme for the synthesis of imidazole derivatives.

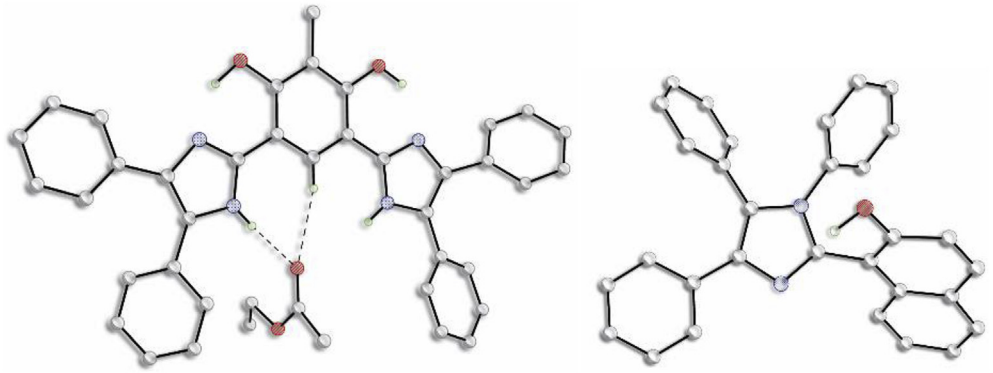


Fig. 1. Perspective views of the previously reported structures of **1** (left) and **2** (right). Some hydrogen atoms and co-crystallized solvent molecules have been omitted for clarity [6].

minus antioxidant (Trolox − 100 μM). After incubating for 24 h at 37 °C and 5% CO_2, the culture medium was replaced with a staining solution containing 200 nM MitoRed (Dojindo Molecular Technologies Inc. Japan) as per the manufacturer's guidelines. Fluorescence reading was at excitation of 560 nm and emission of 580 nm on a spectrofluorometer (Corona Electric, Japan).

2.6. Hypoxia-inducing factor 1-alpha (HIF-1α) detection and measurement

HIF-1α level was determined by using a cell-based human ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) as reported elsewhere [7]. In this assay, growing HFF cells were incubated with imidazoles (C-1, C-2, and C-3) at 1 x IC_{50} value. After incubating for in an atmosphere of 37 °C and 5% CO_2 for 24 h, cells were treated as per the manufacturer's instructions. Detection of HIF-1α was accomplished by staining with solutions of primary (anti−HIF−1α antibody) and secondary antibodies (horseradish peroxidase-conjugated). This was followed by luminescent measurement using a multi-mode reader (Promega, Madison, WI, USA). The inclusion of a known inhibitor of HIF-1α expression [14,15], 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), and $CoCl_2$, a chemical inducer of hypoxia [16] validated the assay.

2.7. Statistical analysis

A one-way ANOVA (GraphPad, San Diego, CA, USA) was used to analyse the results. The data are presented as the mean of three replicates ± standard error of mean (SEM). The Dunnet post-hoc test was used to compare the groups, and mean values were taken significantly at $p < .05$. The concentration that caused a 50% reduction in cell viability (IC_{50}) was estimated from a dose-response curve of the compound concentrations versus the cell viability. The curve was fitted using a non-linear regression analysis.

3. Results

3.1. Imidazole derivatives

The imidazole compounds are as shown in Scheme 1. The design of the azole derivatives was such that it allows for modifications based on electronic and steric considerations. For example, an electronic differential in the bis-imidazole (C-1), phenyl-substituted 1*H*-imidazole (C-2), or thiophene-imidazole (C-3) allows for electron-releasing or electron-withdrawing exchanges. Therefore, the compounds are likely to differ greatly in terms of the intermolecular interaction potential. The identities of these compounds have been previously established [6].

3.2. Cellular toxicity and biochemical mechanisms

Dose-dependent cellular toxicity was observed for C-1, C-2, and C-3 with IC_{50} values of 3.42, 19.81, and 52.76 μM, respectively (Fig. 1). However, while the addition of antioxidant (Trolox) was able to decrease cytotoxicity by C-2 and C-3, it made no appreciable

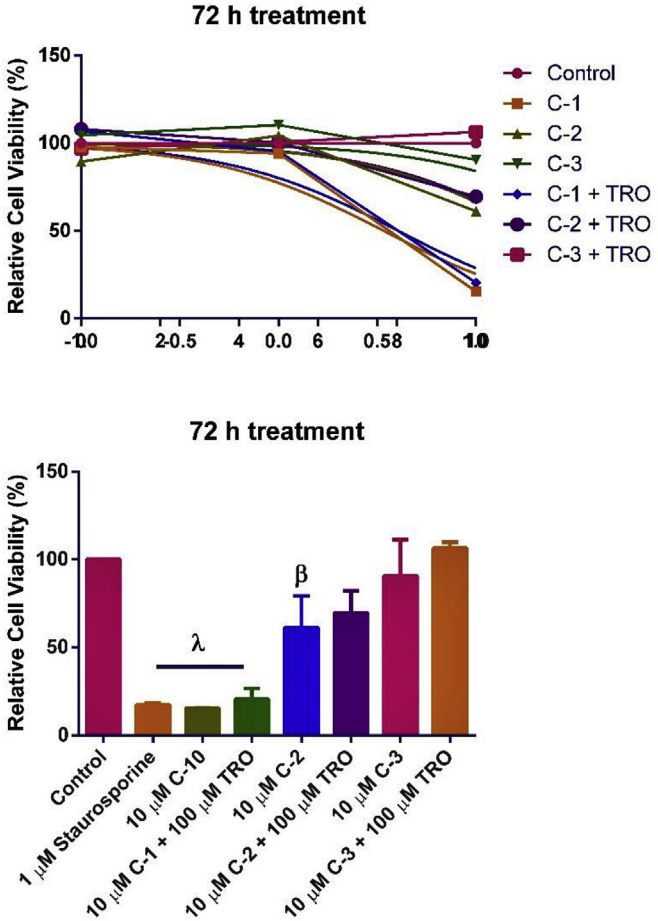


Fig. 2. Cytotoxicity of imidazoles [a] Dose-response curve after a 72-h treatment with imidazoles and [b] Single dose treatment in the absence and presence of Trolox (100 μM). Data are expressed as the mean ± standard error of mean (SEM). Experiment was repeated three times independently. β is significant at $p < .001$ while λ is significant at $p < .0001$ relative to the control.

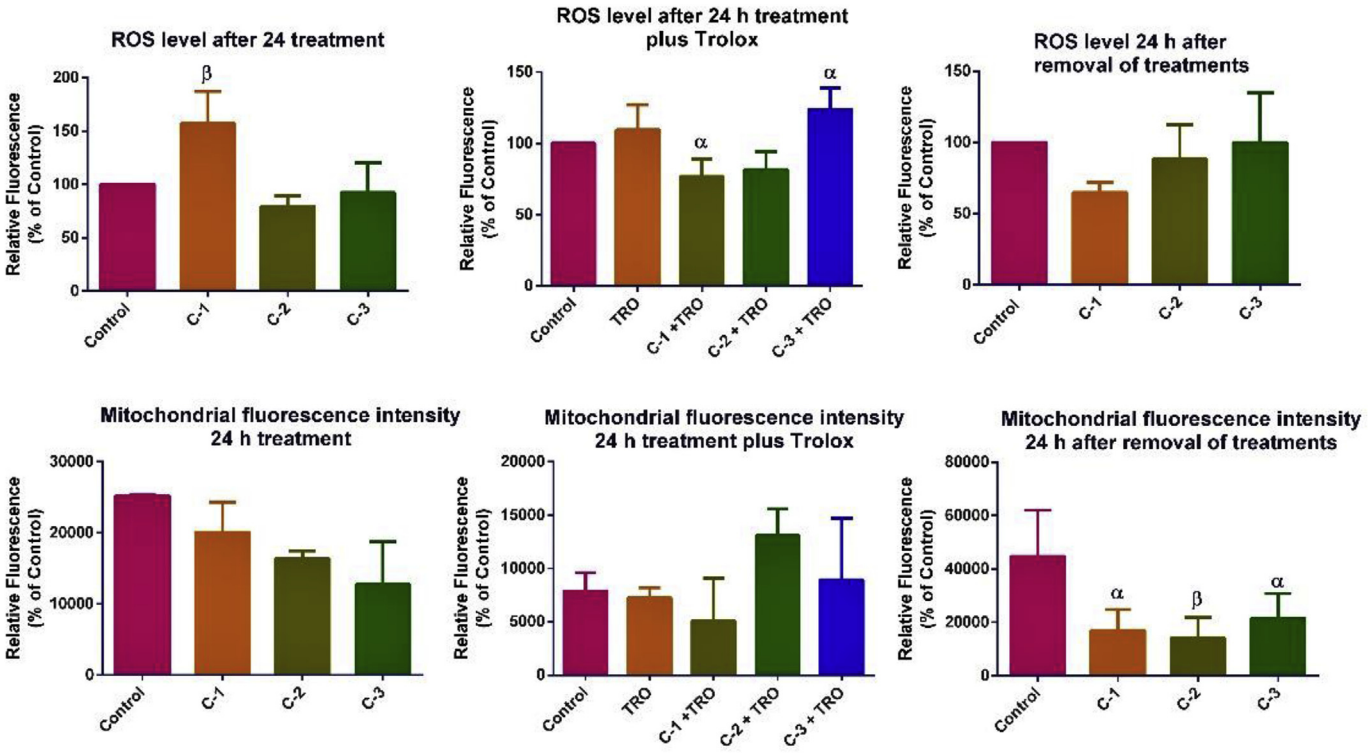


Fig. 3. ROS level and mitochondria fluorescence intensity after imidazole treatments minus/plus Trolox (100 μM). Data are expressed as the mean ± standard error of mean (SEM). Experiment was repeated three times independently. α is significant at $p < .05$ while β is significant at $p < .001$ relative to the control.

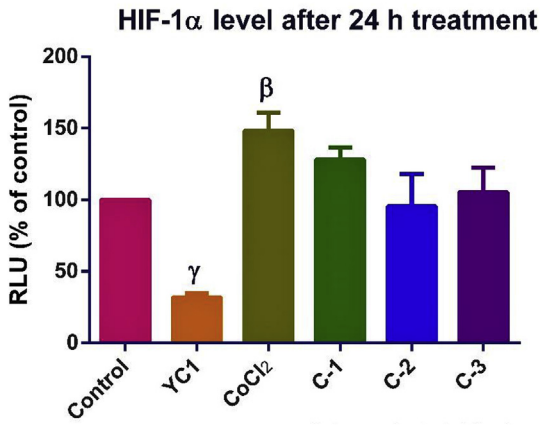


Fig. 4. Level of hypoxia inducing factor-1α (HIF-1α) after imidazole treatments. Data are expressed as the mean ± standard error of mean (SEM). Experiment was repeated three times independently. β is significant at $p < .001$ while γ is significant at $p < .0001$ relative to the control.

change to C-1 cellular toxicity. Nonetheless, IC_{50} values were elevated by an average of >5% for all the imidazole treatments in the presence of antioxidant (Trolox). Our cytotoxicity assay was validated by using a cytotoxic agent (staurosporine), and as expected, the drug reduced cellular viability drastically to <20% compared with control. At the highest dose (10 μM), the imidazoles reduced cell viability by ≥ 85%, ≥39%, and ≥10% respectively for C-1, C-2, and C-3.

To probe the cytotoxic mechanisms of these imidazoles, we determined the ROS level and mitochondrial membrane potential (MMP) in the presence and absence of Trolox. Findings showed that C-1 facilitated the production of ROS, which was reversible within 24 h after the removal of treatment (Fig. 2a–c). In the presence of antioxidant (Trolox), C-1 cytotoxic action was abated. Meantime, the presence of antioxidant (Trolox) appeared to potentiate the cytotoxic action of C-3. Besides, the imidazoles reduced the fluorescence intensity of the cellular mitochondria, suggesting an effect on cellular MMP (Fig. 2d–f). Meanwhile, the removal of imidazole treatments failed to restore the cellular MMP. In contrast, however, the presence of antioxidant (Trolox) restored cellular MMP for C-2 and C-3, but not for C-1 treatment. Further, we sought to evaluate if imidazoles affect HIF expression, considering its link with ROS and/or oxidative stress. To this end, we assayed for the HIF-1α level in cells after a 24 h treatment. Results showed that only C-1 treatment elevated HIF-1α level, though not significantly compared with control (Fig. 3). To validate HIF-1α assay, YC-1 (inhibitor of HIF-1α expression) and $CoCl_2$ (chemical inducer of hypoxia) were included. As expected, YC-1 and $CoCl_2$ respectively caused either >50% reduction or elevation in HIF-1α level compared with control (Fig. 4).

4. Discussion

In our recent investigations, we reported the prospects of imidazoles as a source of alternative anti-parasite therapies. In this light, it's plausible to profile these imidazoles for cellular toxicity as part of the needed critical pre-clinical data. Therefore, in the present study, we tested three (3) imidazoles (C-1, C-2, and C-3) for *in vitro* cellular toxicity and likely mechanisms.

Our findings showed that the imidazoles have dose-dependent cellular toxicity tendencies. While toxicity by C-2 and C-3 was improved in the presence of antioxidant (Trolox), C-1 cytotoxic action remained unabated. That cytotoxicity caused by these imidazoles was restored in the presence of antioxidant may indicate the involvement of ROS and/or oxidative stress. However, our study

did show that only C-1 treatment led to increased ROS level compared with control, and this was reversible within 24 h upon removal of treatment. In addition, ROS production by C-1 was ameliorated in the presence of antioxidant (Trolox). This contrasts what was observed for cytotoxic action by C-1, wherein antioxidant (Trolox) failed to improve cellular toxicity.

Moreover, our findings showed that all imidazoles (C-1, C-2, and C-3) caused a reduction in cellular MMP compared with control. This reduction in cellular MMP may not only lead to limited energy production in terms of ATP generation but cause mitochondrial damage committing to cell death. Meanwhile, investigations have shown that ROS and/or oxidative stress may lead to mitochondrial damage [13,17]. In addition, earlier findings have shown that intracellular ROS could negatively impact mitochondria leading to cell death [18,19]. More so, when imidazole treatments were removed, the cellular MMP was not restored, suggesting that mitochondrial damage might be beyond repair. In contrast, the presence of antioxidant (Trolox) restored cellular MMP for C-2 and C-3, but not for C-1 treatment. Taken together, findings implicate altered redox status and cellular MMP in the cytotoxic action by these imidazoles. In particular, cytotoxic action by C-1 might not preclude ROS and alteration of cellular MMP.

Furthermore, C-1 treatment raised the level of HIF-1α compared with control. This may not be unexpected if we consider that, in this study, C-1 caused oxidative stress by promoting ROS production. Meanwhile, oxidative stress and/or ROS have been linked to elevated HIF-1α level [7,20]. Considered together, oxidative stress and altered cellular MMP might be contributing to cytotoxic action by C-1, while the elevated HIF-1α level might be an adaptive mechanism for cellular survival following imidazole treatments.

Furthermore, we may explore the structure-activity relationship to explain the cytotoxic action of these imidazoles. Perhaps, in this regard, the electron-donating or -withdrawing effects and steric bulkiness due to the presence of aromatic rings might cause differential cellular toxicity. In addition, hydrophobicity of the phenyl compounds could also contribute to differential cytotoxic action by the imidazoles. The high molecular weight of compound C-1, probably due to the presence of large cyclic hydrocarbons, might contribute to its appreciable cellular toxicity. In the same manner, the electron-withdrawing capacity of the bis-imidazole (C-1) may be due to the presence of a phenanthrene group, and this might have resulted in the reactivity of the compound with the cellular macromolecules. This is consistent with our previous study, which showed that aromatic rings not only increased bulkiness but potentiated cytotoxicity [21]. More so, the ratio of carbon to hydrogen in C-1 was high, and this, in part, might be responsible for the observed cytotoxicity. In contrast, C-2, which had reduced bulkiness and possessed an electron-donating capacity, might explain low cytotoxicity compared with C-1. Furthermore, C-3, which had a brominated thiopene moiety, showed lesser cytotoxicity compared with C-1 and C-2. Together, the differential cytotoxicity by these imidazoles shows consistent with our previous investigations [6,21].

In conclusion, we have presented evidence that supports a dose-dependent cytotoxic action by imidazoles. Also, our findings revealed that the cytotoxic mechanisms of these imidazoles might not preclude oxidative stress and/or altered cellular MMP. Knowledge of the cellular mechanistic action of the imidazoles may boost therapeutic prospects.

Declaration of competing interest

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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