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The anti-parasite action of imidazole derivatives likely involves oxidative stress but not HIF-1α signaling

Oluyomi Stephen Adeyemi [a,b,*], Abiodun Omokehinde Eseola [c,d], Winfried Plass [d], Kentaro Kato [e], Chiagoziem A. Otuechere [c], Oluwakemi Josephine Awakan [a,b], Olubunmi Atolani [f], David Adeiza Otohinoyi [g], Tobiloba Christiana Elebiyo [a,b], Ikponmwosa Owen Evbuomwan [a,h]

[a] Landmark University SDG 3 (Good Health and Well-being Research Group), Nigeria
[b] Department of Biochemistry, Medicinal Biochemistry, Nanomedicine & Toxicology Laboratory, Landmark University, PMB 1001, Omu-Aran, 251101, Nigeria
[c] Department of Chemical Sciences, Redeemer's University, Ede, Nigeria
[d] Friedrich-Schiller-Universität Jena, Institute of Inorganic and Analytical Chemistry, Humboldtstraße 8, 07743, Jena, Germany
[e] Laboratory of Sustainable Animal Environment, Graduate School of Agricultural Science, Tohoku University, 232-3 Yomogida, Naruko-onsen, Osaki, Miyagi, 989-6711, Japan
[f] Department of Chemistry, University of Ilorin, Ilorin, Nigeria
[g] All Saints University, College of Medicine, Belair, Saint Vincent and the Grenadines
[h] Department of Microbiology, Cellular Parasitology Laboratory, Landmark University, PMB 1001, Omu-Aran, 251101, Nigeria

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ABSTRACT

Background: Therapeutic options for toxoplasmosis are limited. This fact underscores ongoing research efforts to identify and develop better therapy. Previously, we reported the anti-parasitic potential of a new series of derivatives of imidazole.

Objective: In the current investigation, we attempted the investigation of the possible action mechanism of few promising anti-parasite imidazole derivatives namely C1 (bis-imidazole), C2 (phenyl-substituted 1*H*-imidazole) and C3 (thiophene-imidazole)

Methods: We evaluated if oxidative stress, hypoxia as well as metabolic reprogramming of host L-tryptophan pathway form part of the parasite growth inhibition by imidazoles. Anti-parasite assay was performed for imidazoles at concentrations ranging from 0 to 10 μM, while pyrimethamine was used as reference drug to validate assay.

Results: Imidazole compounds restricted parasite growth dose-dependently. However, in the presence of an antioxidant (Trolox), L-tryptophan and/or $CoCl_2$ (chemical inducer of hypoxia), the growth inhibitory efficacy of imidazoles was appreciably abolished. Further, imidazole treatment led to elevated level of reactive oxygen species, while reducing parasite mitochondrial membrane potential compared with control. In contrast, imidazole had no effect on host HIF-1α level suggesting its exclusion in the anti-parasite action.

Conclusion: Taken together, imidazole-based compounds might restrict parasite growth by causing oxidative stress. The findings provide new insight on the likely biochemical mechanisms of imidazoles as prospective anti-parasite therapy. Data gives new perspective that not only underscores the anti-parasite prospects of imidazoles, but implicates the host L-tryptophan pathway as a feasible treatment option for *T. gondii* infections.

1. Introduction

Toxoplasmosis, caused by Toxoplasma gondii, is a common parasitic zoonosis globally. *T. gondii* is a holoparasite that requires human and veterinary hosts to complete its life-cycle, causing an array of maladies, including abortions and congenital disabilities in infected hosts [1]. The life cycle of *T. gondii* has been described in two excellent reviews [2,3]. Briefly, the parasite's sexual reproduction takes place in the intestine of cats producing oocysts. Upon sporulation, oocytes produce infectious sporozoites that are sources of infection in warm-blooded vertebrates. In

* Corresponding author. Oluyomi Stephen at Landmark University, Omu-Aran, Nigeria.
E-mail address: yomibowa@yahoo.com (O.S. Adeyemi).

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the asexual phase of the life cycle, the sporozoites form the proliferative tachyzoites that later differentiate into the latent and encysted bradyzoites. While the bradyzoites form tissue cysts in the intermediate host, the tachyzoites invade many host tissues including the brain and muscles.

Consequently, *T. gondii* infections in immunocompromised individuals could cause encephalitis [4,5]. The menace of *T. gondii* infection is a public health concern because the symptoms are mostly latent in the immunocompetent hosts but could persist for periods spanning the host's lifetime. Unfortunately, latent toxoplasmosis had been implicated in mental disorders [6,7], testosterone imbalance [8], and autoimmune diseases [9].

The *Toxoplasma* parasite, endowed with intrinsic adaptive potentials, regulates its replication and infection in host cells via several survival pathways. Specifically, IFN-γ and STAT1, through their enhancement of the host immune response, are key signaling pathways in the control of parasite infection. Hence, it logically follows that impairing these pathways demonstrates an evasion mechanism for *T. gondii.* Precisely, STAT1, as a signal transducer of IFN-γ, controls the inflammatory proteins that are required for parasite control. Parasite inhibition of STAT1 results in the blockage of vital proteins such as interferon regulatory factor 1 (Irf1), iNOS, indoleamine 2, 3-dioxygenase 1(IDO). *T. gondii*, being a tryptophan auxotroph, is further favored by the diminution of IDO, an enzyme that catalyzes the rate-limiting step in tryptophan catabolism [10,11].

Interestingly, the effector functions of IDO could also be abolished under hypoxic conditions leading to a consolidation of the host tryptophan pool necessary for parasite growth [12]. Several studies establishing the role of oxidative stress in the pathophysiology of *T. gondii* infections have been reported [13,14]. To curtail oxidative burst during infection, host immune cells release superoxide anions as part of their protective mechanisms. Subsequently, an oxidative imbalance that impedes the replication of parasites is generated in the microenvironment. However, *T. gondii* deploys its intricate antioxidant network comprising of superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, thioredoxin peroxidase, thioredoxin reductase, and glutathione to resist oxidative damage [14]. In addition, increased expression of hypoxia inducible factor 1 (HIF-1) has been previously linked with *T. gondii* infection [15,16]. HIF-1 is a heterodimer composed of two subunits namely, α and β. Available evidence showed that HIF-1 is critical to survival and replicative growth of *T. gondii* in the host by regulating pro-parasite genes including glycolytic metabolic genes, transferrin receptor, and vascular endothelial growth factors [17–19]. Moreover, increased expression of HIF-1 protein and its stabilization are not restricted to hypoxic stress as many pathogens including *T. gondii* could activate HIF-1 [20], and loss of the HIF-1α subunit has been implicated to result in a significant decline in the growth of parasite at physiological oxygen levels [16].

Despite the advances made in elucidating the mechanisms of action of *T. gondii*, treatment options for toxoplasmosis remain limited. Current treatment regimens are predominantly combination therapies, including pyrimethamine plus sulfadiazine, trimethoprim plus sulfamethoxazole, and atovaquone plus sulfadiazine. Unfortunately, these drugs show an effect only in the active phase of the parasite, in addition to elicit adverse events in patients. Antibiotic medicines such as azithromycin, clarithromycin, and spiramycin used as alternative treatment options are poorly tolerated and also have no effect on the bradyzoite form of the parasite [21–23].

Consequently, studies focusing on safer and alternative antiparasitic therapy with novel mechanisms of action warrant further attention. Earlier, we had reported some novel imidazole compounds showed promising growth-inhibiting potential against *Trypanosoma* sp and *Toxoplasma gondii* [24,25]. Consistent with our findings, a 5 - nitroimidazole derivative, fexinidazole, is currently approved as therapeutics for human African trypanosomiasis [26]. In the present study, we attempted to investigate the anti-parasite action mechanism of a new series of imidazole derivatives in human foreskin fibroblast (HFF) cells infested with *T. gondii viz-a-viz* the examination of the role played by oxidative stress and host cellular factors such as the modulation of the tryptophan pathway, and HIF-1α expression level.

2. Materials and methods

2.1. Materials

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and pyrimethamine were sourced from Wako Pure Chemicals (Japan) while, L-tryptophan, cobalt II chloride ($CoCl_2$) and 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) were products of Sigma-Aldrich (St. Louis, MO, USA). In addition, all reagents were of analytical grade and used as supplied unless stated otherwise.

2.1.1. Imidazole compounds

Imidazole compounds (Fig. 1) C1 (4,6-bis(4,5-diphenyl-1*H*-imidazole-2-yl)-2-methyl-benzene-1,3-diol), C2 (1-(1,4,5-triphenyl-1*H*-imidazole-2-yl)naphthalen-2-ol), and C3 (2-(5-bromo-2-thienyl)-4,5-diphenyl-1*H*-imidazole) were synthesized as previously described [24,27].

2.2. Methods

2.2.1. Anti-parasite assay

2.2.1.1. Parasite strain. Determination of parasite growth inhibition assay was performed as reported previously [15]. A strain of *T. gondii* RH-2F expressing luciferase activity was used. The parasite was sustained through repeated passages in cultures of human foreskin fibroblast cells (HFF; ATCC, Manassas, VA, USA). The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Nissui,

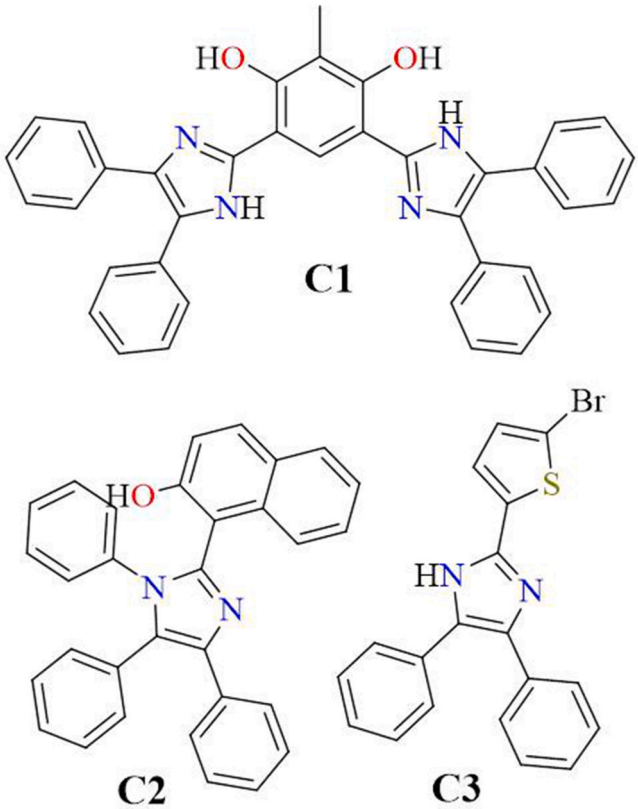

Fig. 1. Structures of the compounds investigated.

Tokyo, Japan), 2 mM GlutaMAX (Gibco, Invitrogen, Waltham, MA, USA), 10% (v/v) fetal calf serum (FCS; Gibco, Invitrogen, Waltham, MA, USA), and penicillin and streptomycin (10,000 U/mL; Leicestershire, UK). Activity of β-galactosidase (β-gal) was used to determine viability of *T. gondii* tachyzoite. Purification of parasite suspension was by lysing infected host cells, filtering and washing three times. Parasite count was done using a hemocytometer.

Our earlier report [24] informed the dose selection of imidazoles as well as guided the assays for parasite growth restriction studies. In this assay, a fresh parasite suspension free of contamination was inoculated into growing HFF cells monolayers in a 96-well solid white plate (Nunc; Fisher Scientific, Pittsburgh, PA, USA). Simultaneously, imidazoles at various concentrations (0–10 μM) were added. After incubating for 48-h in an atmosphere of 5% CO_2 and 37 °C, the viability of parasite was assessed by using a Beta-Glo luminescent kit on a Promega plate reader (Madison, WI, USA). Pyrimethamine was used as reference drug to validate assay while, the infected cells without imidazole treatment was taken as control. The biological experiment was in triplicate and repeated independently three times.

2.2.2. Assay to determine production of reactive oxygen species (ROS) in parasite

Reactive oxygen species (ROS) was assessed as described elsewhere [28]. The assay involves the oxidative conversion of 2′, 7′-dichlorodihydro- fluorescein diacetate (H_2DCF-DA, Sigma, St Louis, MO, USA) to a fluorophore 2′, 7′-dichlorofluorescein (DCF). In this assay, purified tachyzoite suspension was inoculated into cultures of HFF cells and after a 24-h incubation in an atmosphere of 37 °C and 5% CO_2, imidazoles were added to the cells. After a further incubation for 24 h at 37 °C, a purified parasite suspension was stained with a solution of 100 μM H_2DCF-DA in PBS. After incubation in 37 °C atmosphere for 30–60 min fluorescence measurement was recorded on a spectrofluorometer (Corona Electric, Japan). Excitation was at 485 nm and emission at 530 nm H_2O_2 (100 μM) was used as a reference.

2.2.3. Assay to determine parasite mitochondrial membrane potential

The mitochondrial membrane potential of parasite assessed as described elsewhere [29]. In this assay, purified tachyzoite suspension was inoculated into cultures of HFF cells and after 24-h incubation in an atmosphere of 37 °C and 5% CO_2, imidazoles were added to the cells. After a further incubation for 24 h at 37 °C, the parasites were harvested, purified, and re-suspended in PBS containing 200 nM MitoRed (Dojindo Molecular Technologies Inc. Japan). Fluorescence reading was taken on a spectrofluorometer. Excitation was at 560 nm and emission at 580 nm. Ionophore, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 2 μM final concentration) was included to validate assay.

2.2.4. Assay to measure the expression of hypoxia-inducing factor 1-alpha (HIF-1α)

The HIF-1α expression in treated versus untreated was used to determine if hypoxia induction was successful. To determine expression level of HIF-1α, we used ELISA Kit (Cell Biolabs, Inc., USA) and performed assay strictly adhering to instructions provided by the manufacturer as previously described [15]. In this assay, growing HFF cells (1 x 10^6 cells/mL) in a 96-well plate were treated with imidazoles plus or minus RH-2F infection. HIF-1α expression level was measured by luminescence (Promega, USA) after staining with a solution of anti–HIF–1α and horseradish peroxidase (HRP)-conjugated antibody. YC-1, a HIF-1α inhibitor was included to validate assay.

2.3. Data presentation and analysis

A one-way ANOVA (GraphPad Software Inc., San Diego, CA) was used to analyze data and the results are provided as an average of three independent repetitions plus/minus the corresponding error of mean (SEM). To compare among groups, we used Tukey's post-hoc test, while statistical significance was taken at p-value <0.05. Dose-response curve was used to estimate the concentration of imidazoles exhibiting a 50% reduction in viability of parasite (IC_{50}) while, the curve fitting was by a non-linear regression method.

3. Results

3.1. Imidazoles caused oxidative stress and impacted parasite mitochondria fluorescence capacity

Following demonstration of growth inhibiting potential of the imidazole derivatives, we attempted to assess the likely mechanistic action. Initially, we included an antioxidant (Trolox) in the anti-parasite screening assay to determine if ROS contributes to parasite growth inhibition. Anti-parasite action of the imidazole derivatives decreased in the presence of Trolox, while the IC_{50} values appreciably increased (Fig. 2a–d and Table 1). Reduction of anti-parasite efficacy of the imidazole derivatives in the presence of Trolox, implicated oxidative stress. To this end, we went further to determine if ROS was generated after exposure of *T. gondii* to the imidazole derivatives. ROS level was determined by fluorescence measurement. Only bis-imidazole C1, led to >50% elevation in ROS level compared with control (Fig. 3a) while H_2O_2 treatment caused a >100% increase in ROS production (data not shown) in the same assay thus validating the ROS detection. However, 24 h after treatment removal, ROS level decreased for all treatments by >30% (Fig. 3b). When Trolox was added to the anti-parasite assay medium, ROS level decreased for the imidazole derivatives by >50% compared with control (Fig. 3c). In addition, H_2O_2 caused a reduction in parasite growth by >90% while Trolox relieved imidazole-induced parasite growth inhibition at varying level (Fig. 3d).

Having established the likelihood that parasite growth inhibition caused by the imidazole derivatives involved ROS and oxidative stress, we proceeded to determine if the parasite mitochondria were impacted. Oxidative assault may affect mitochondria; therefore, we used a rhodamine-based dye (MitoRed) to measure the parasite mitochondria integrity so as to determine if imidazole treatments impact parasite mitochondria. MitoRed dye is cell permeable dye and usually sequesters in mitochondria where it fluoresces. The dye sequestration is dependent on the intact mitochondria. Initially parasite-infested cells were exposed to imidazole treatments for 24 h, thereafter parasites were harvested and measurement of fluorescein localization in the mitochondria was determined. Our results showed a reduced mitochondrial fluorescence intensity suggesting that parasite mitochondria might be impacted by imidazole treatments (Fig. 4a). A reduction in the fluorescence intensity of the treated parasites, suggests low accumulation of dye in the parasite mitochondria following imidazole treatment. Furthermore, we sought to ascertain if ROS was involved in the impairment of parasite mitochondria, therefore, we added an antioxidant Trolox and found that parasite mitochondria fluorescence intensity improved and was restored to normal level. The finding suggests the restoration of the parasite mitochondria in the presence of Trolox (Fig. 4b). On the other hand, parasite mitochondria failed to improve 24 h after removal of imidazole treatments (Fig. 4c). To validate assay, cells were treated with FCCP for 1 h and as expected, the fluorescence intensity appreciably reduced when compared with control (Fig. 4d).

3.2. L-tryptophan supplementation reverses anti-parasitic action of imidazoles

Furthermore, we tested to determine if L-tryptophan supplementation could relieve the anti-parasite action by the imidazole derivatives. Since *T. gondii* are tryptophan auxotrophs. In this light, we added L-tryptophan to the screening medium. Interestingly, presence of L-tryptophan in the medium reversed inhibition of parasite growth caused by the imidazole derivatives as well as raised the IC_{50} values (Fig. 5a–d, Table 1). Though, we are yet not sure of the reason for this, it is plausible

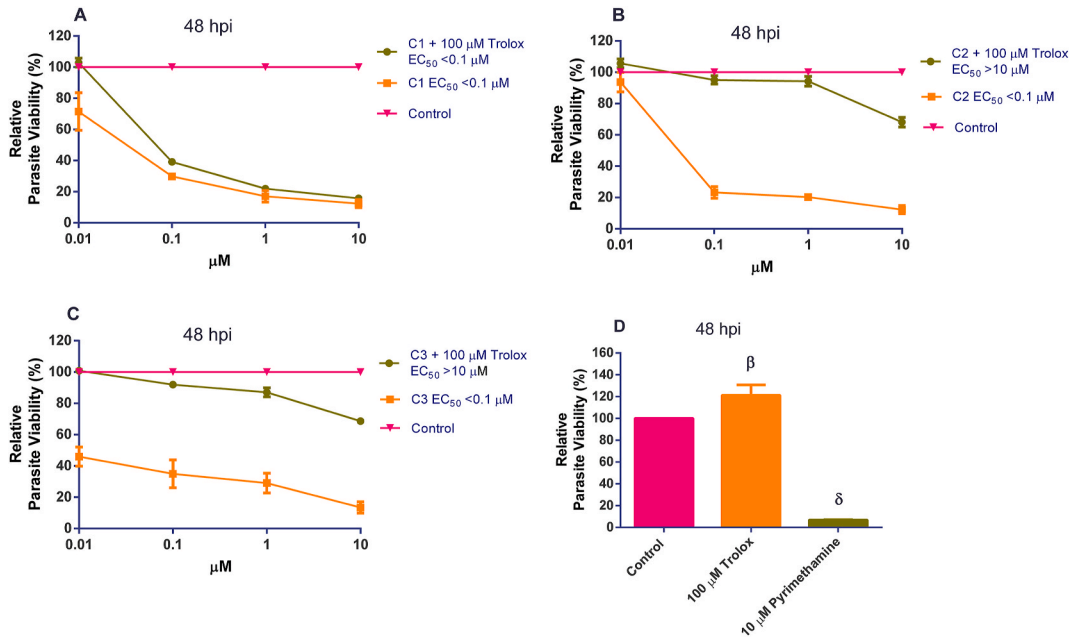

Fig. 2. Viability of parasite. HFF cells infested with *Toxoplasma gondii* were treated with imidazoles singly or in combination with Trolox at the concentrations indicated. [A] C1; [B] C2; [C] C3; [D] Pyrimethamine and Trolox. After 48-h incubation, viability of parasite was assessed. Data are provided as an average of three independent repetitions plus/minus the corresponding error of mean (SEM). β is significant at $p < 0.001$ and δ at $p < 0.0001$ compared with the control while, 'hpi' is hour post-infection.

Table 1
IC_{50} values of imidazole under various treatment conditions.

Treatment	IC_{50} (μM)	Fold change
C1	0.04	–
C1 + Trolox	0.09	2.25
C1 + L-tryptophan	4.45	111.25
C1 + $CoCl_2$	5.39	134.75
C2	0.06	–
C2 + Trolox	21.70	361.67
C2 + L-tryptophan	16.26	271.00
C2 + $CoCl_2$	128.30	2138.33
C3	0.01	–
C3 + Trolox	20.34	2034.00
C3 + L-tryptophan	17.93	1793.00
C3 + $CoCl_2$	30.31	3031.00

that augmentation of local pool of host L-tryptophan dampens parasite sensitivity to the imidazole derivatives. It is possible that the imidazole-based compounds limit L-tryptophan availability to invading parasite, thus restricting their growth.

3.3. Hypoxia augmentation by $CoCl_2$ blocked parasite growth restriction caused by imidazoles

In this study, we attempted to determine if host hypoxia might be involved in the parasite growth restricting potential of the imidazole derivatives. To determine if the imidazole-based compounds restrict parasite growth by limiting hypoxia, we initially added $CoCl_2$ (chemical inducer of hypoxia) to the anti-parasite assay medium. Results showed that $CoCl_2$ ameliorated inhibition of *T. gondii* growth caused by the imidazole derivatives, while raising the IC_{50} values (Fig. 6a–d, Table 1). That $CoCl_2$ addition abated inhibition of parasite growth caused by the imidazole derivatives may be attributable to the fact that it is a chemical inducer of hypoxia and as such may be aiding parasite growth by augmenting hypoxia. In light of this, level of HIF-1α was assayed with or without *T. gondii* infection after treatment with imidazole only or in combination with $CoCl_2$. Without *T. gondii* infection, only bis-imidazoles appreciably increased HIF-1α level compared with control (Fig. 7a). Meantime, with *T. gondii* infection, the imidazole-based compounds did not appreciably affect the HIF-1α level differently from control (Fig. 7b). In the presence of *T. gondii* infection, the imidazole derivatives had no appreciable impact on HIF-1α expression, even though $CoCl_2$-induced hypoxia averted restriction of parasite growth. This fact suggests no clear role for HIF-1α signaling pathway in the growth restricting capacity of the imidazole-based compounds. That $CoCl_2$ restored parasite growth in the presence of imidazoles could be due to increasing hypoxia pressure. More so, $CoCl_2$ enhanced parasite growth, while YC-1 repressed it (Fig. 6d). Taken together, the findings may indicate exclusion of the HIF-1α signaling pathway in the anti-parasite action by imidazoles. That parasite growth inhibition by imidazoles was relieved in the presence of $CoCl_2$ might be as a result of increasing hypoxia pressure. As expected, YC-1 (HIF-1α inhibitor) reduced level of HIF-1α irrespective of whether infection was present or not, while $CoCl_2$ elevated level of HIF-1α. The finding validates our assay. Based on data presented in this study, we proposed a likely mechanism for the *T. gondii* growth inhibitory action of imidazoles (Fig. 8).

4. Discussion

Recently, we reported the promising anti-parasite prospects of new series of imidazole derivatives. In the present study, we attempted to unravel likely mechanistic action of some of these imidazole-based compounds. Consistent with our earlier report, the imidazole derivatives showed remarkable parasite growth inhibitory potential [24]. In this study, findings support a role for ROS and by extension oxidative stress in the anti-parasite efficacy of the imidazole-based compounds. This line of thought could be supported by the fact that Trolox reversed the parasite growth inhibitory action of the imidazole derivatives. Meantime the fluorescent measurement of ROS level directly provides of imidazole-induced oxidative killing of parasites. Earlier studies have shown that compounds with imidazole rings could promote ROS production leading to oxidative stress [30,31].

Together, contribution of ROS and/or oxidative stress in imidazole-induced parasite growth inhibition was reinforced by the amelioration provided Trolox addition. Oxidative assault might have resulted in

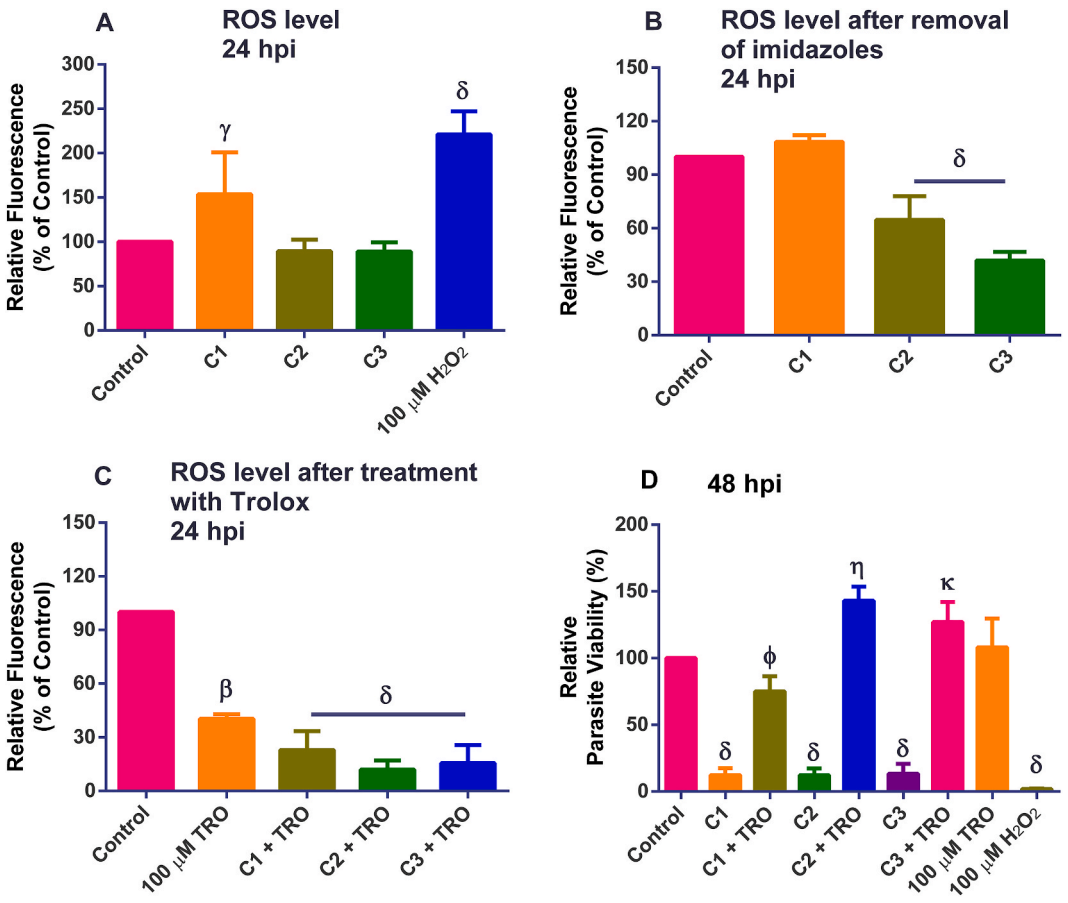

Fig. 3. Intracellular reactive oxygen species (ROS) level and viability of parasite following exposure to imidazoles. [A] ROS level in parasite at single dose treatment 24 hpi; [B] ROS level in parasite 24 h after treatment removal; [C] ROS level in parasite after treatment with 100 μM Trolox; [D] Viability of parasite at single dose treatment 48 hpi. Data are provided as an average of three independent repetitions plus/minus the corresponding error of mean (SEM). β at $p < 0.01$, γ at $p < 0.001$ and δ at $p < 0.0001$ are significant compared with the control. For parasite viability IN [D]; φ, η, and κ are significant at $p < 0.0001$ respectively versus C1, C2 and C3. 'hpi' is hour post-infection.

parasite death and/or growth arrest. Impairment of redox homeostasis could be lethal with oxidative cellular damage [28,32]. In addition, that imidazole treatments compromised parasite mitochondria might be due to overwhelming ROS production and resultant oxidative assault. Mitochondrion is the power house of a cell and intact mitochondria is a requirement for ATP production. Hence impairment of mitochondria integrity could compromise ATP production leading to cellular arrest or death [28]. A reduction in the fluorescence intensity of the treated parasites, suggests low accumulation of dye in the parasite mitochondria following imidazole treatment. The evidence may indicate that imidazole treatments impacted the parasite mitochondrial membrane, thereby impairing the interaction with MitoRed. Interestingly, parasite mitochondria fluorescence intensity improved and was restored to normal level following addition of Trolox. This further underscores that parasite growth restriction by the imidazole derivatives might likely involve oxidative stress and/or ROS. On the other hand, parasite mitochondria failed to improve 24 h after removal of imidazole treatments. This finding further implicates ROS production and oxidative stress as contributors to the parasite growth restricting action by imidazole compounds.

Together, data are consistent with earlier investigations that linked parasite death with mitochondria impairment and oxidative assault [25,33,34]. Nevertheless, it should be noted that oxidative stress is a complex process involving several reactive species and as such our findings in this respect is limited in that we used a DCFH-DA which is non-specific probe for ROS detection. In addition, impairment of parasite mitochondria following treatment with imidazole-based compounds might also be due to reduced mitochondria density rather than membrane depolarization.

Furthermore, our study revealed that imidazole-induced parasite growth inhibition was averted by L-tryptophan. Reason for this is yet unknown, but it is likely that imidazole treatments decreased host L-tryptophan level by promoting oxidative degradation to kynurenine and metabolites. Hence, an augmentation of the local pool of host L-tryptophan may be expected to dampen parasite sensitivity to the imidazole derivatives. If imidazole-based compounds limit L-tryptophan availability to invading parasite, this may likely restrict their growth. While our explanation here is tentative, earlier reports [15,28] showed that anti-parasite agents may activate the kynurenine pathway by upregulating expression of indoleamine 2,3-dioxygenase (IDO1), thus L-tryptophan is oxidatively degraded by action of IDO1 to kynurenine, thereby limiting local availability of this amino acid required for parasite growth. In addition, oxidative stress has been shown to have led to degradation of L-tryptophan to kynurenine [15,28,35,36]. Therefore, L-tryptophan addition would be expected to augment the nutrient provision thereby allowing for unrestricted parasite growth by shielding imidazole anti-parasitic effect. More so, this revelation conforms with, and underscores the auxotrophic nature of *T. gondii* for L-tryptophan [37]. Thus, declining free access to this amino acid may arrest parasite growth.

Host hypoxia has been identified as one of the survival mechanisms and hallmarks of pathogen infections including that of *T. gondii* [16]. During hypoxia, there is upregulation and stabilization of HIF-1α [38]. HIF-1 is a transcription factor used by cells to survive stress imposed by

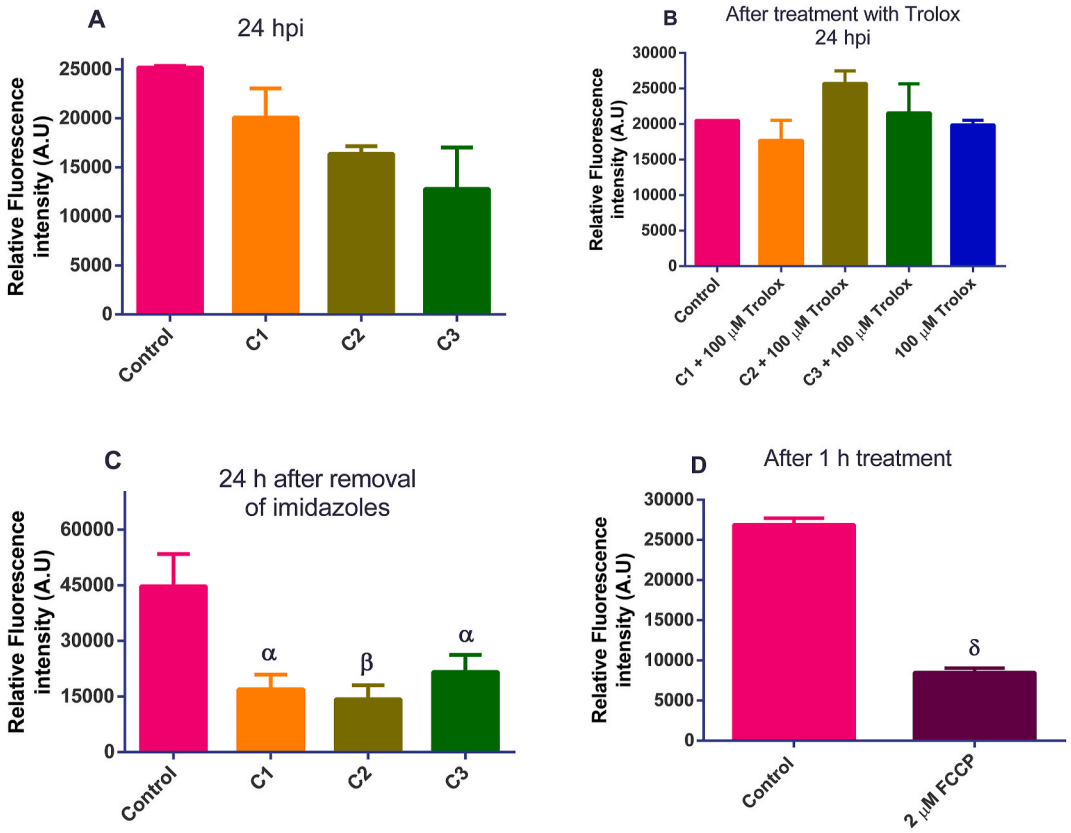

Fig. 4. Effect of imidazoles on parasite mitochondrial membrane potential. A cell-permeable rhodamine-based dye (MitoRed), which localizes to mitochondria and emits fluorescence, was used. The interaction of MitoRed with the mitochondria is dependent on the mitochondrial membrane potential. The relative fluorescence intensity of MitoRed in parasite mitochondria was measured [A] After 24 h treatment with imidazoles 24 hpi; [B] Treatment with imidazoles plus Trolox 24 hpi; [C] 24 h following treatment (imidazole) removal; [D] After 1 h treatment with the ionophore carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). Data are provided as an average of three independent repetitions plus/minus the corresponding error of mean (SEM). α at $p < 0.05$, β at $p < 0.001$, and δ at $p < 0.0001$ are significant relative to the control while, 'hpi' is hour post-infection.

low oxygen concentration. However, HIF-1 expression is also used by infectious agents such as *T. gondii* to boost and sustain their growth and replication [12,20,28]. Our investigation revealed that hypoxia induction in host by $CoCl_2$ blocked restriction of parasite growth caused by imidazole and raised IC_{50} values. One of the growth strategies of *T. gondii* is their ability to cause hypoxia with elevation in level of HIF-1α in host [16]. Chemically-induced hypoxia in host restored parasite growth in the presence of imidazole derivatives, thus suggesting a role for hypoxia in the parasite growth inhibitory action of imidazole. In this light, we assayed for HIF-1α expression. However, in the presence of *T. gondii* infection, level of HIF-1α remained unaffected by the imidazole derivatives but not by $CoCl_2$ and YC-1 which respectively raised and reduced level of HIF-1α when compared with control. It is likely that $CoCl_2$ caused hypoxia augmentation with concomitant elevation of HIF-1α expression, thereby dampening the growth inhibition effects of the imidazole derivatives. In contrast, the imidazole derivatives did not appreciably affect the HIF-1α expression either with and/or without *T. gondii* infection. This fact may mean that parasite growth arrest and/or death caused by imidazole-based compounds precludes HIF-1α modulation. The exclusion of HIF-1α signaling pathway in the parasite growth restriction by imidazole derivatives may indicate that relieve of imidazole-induced growth inhibition might be due to increasing hypoxia augmentation consequent of $CoCl_2$. Moreover, YC-1 blocked growth of parasite whereas $CoCl_2$ promoted it, irrespective of whether infection was present or not. The finding is consistent with earlier studies as reported elsewhere [38–40].

In part, few investigations have attributed the antimicrobial, anti-parasite and anti-cancer properties of imidazole compounds to their capacity to cause and promote oxidative stress [30,31,41]. These reports are in tandem with our current findings. In addition, that L-tryptophan supplementation reversed the parasite growth inhibition caused by imidazole derivatives likely suggests a modulation of host L-tryptophan pool, thereby limiting access to this parasite growth essential nutrient. If imidazole-based compounds caused oxidative stress, this may trigger oxidative degradation of L-tryptophan to kynurenine. Eventually, this reduces the local pool of L-tryptophan making it limiting for parasite growth. The findings and this explanation conform to earlier investigations that demonstrated L-tryptophan as feasible treatment strategy for parasitic infections [17]. Taken together, our findings implicate involvement of oxidative stress and host L-tryptophan metabolic reprogramming in the parasite growth inhibition and/or death caused by imidazole derivatives. If oxidative stress could promote the oxidative degradation of L-tryptophan [15,28,29,35,36,42], then it is plausible that causation of oxidative stress by imidazole-based compounds might facilitate metabolic reprogramming of the host L-tryptophan in such a manner that affects parasite growth. More so, we recently reported that imidazole-based compounds caused cellular toxicity by promoting oxidative stress and impairing mitochondria integrity [41]. In addition, investigations have identified L-tryptophan starvation as a feasible strategy to restrict *T. gondii* growth [12,43].

5. Conclusion

Findings support that parasite death and/or growth inhibition caused by the new series of imidazole derivatives might involve oxidative stress while suggesting no clear role for HIF-1α signaling pathway.

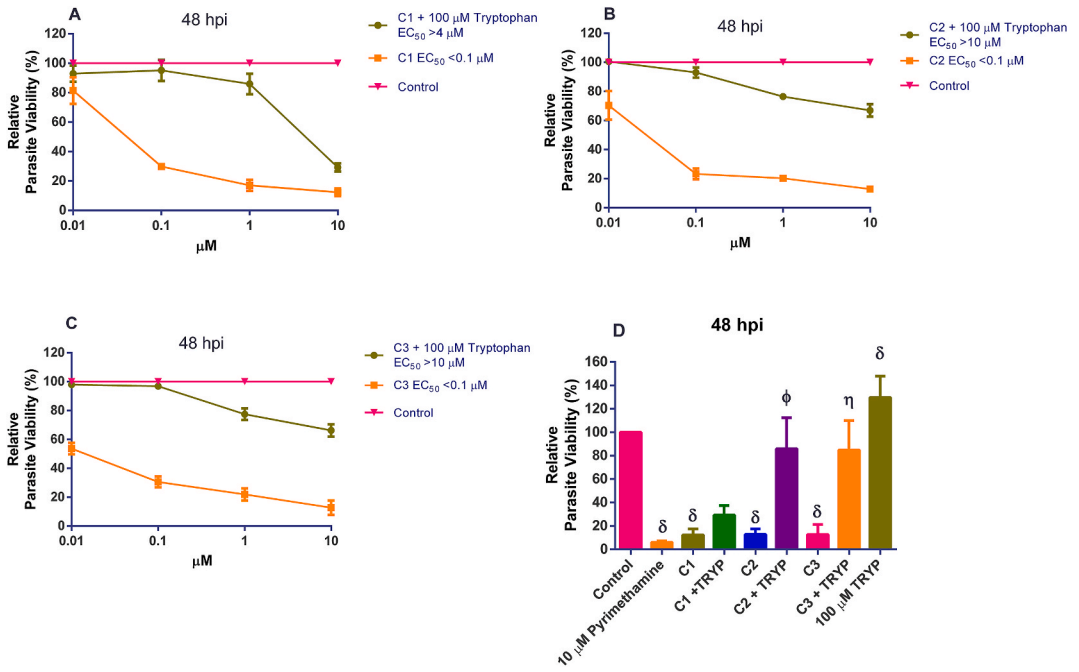

Fig. 5. Viability of parasite. HFF cells infested with *Toxoplasma gondii* were treated with imidazoles singly or in combination with L-tryptophan at the concentrations indicated. [A] C1; [B] C2; [C] C3; [D] At single dose with pyrimethamine and L-tryptophan. After 48-h incubation, viability of parasite was assessed. Data are provided as an average of three independent repetitions plus/minus the corresponding error of mean (SEM). δ is significant at $p < 0.0001$ relative to the control. Φ and η are significant at $p < 0.0001$ respectively compared with C2 and C3 while, 'hpi' is hour post-infection.

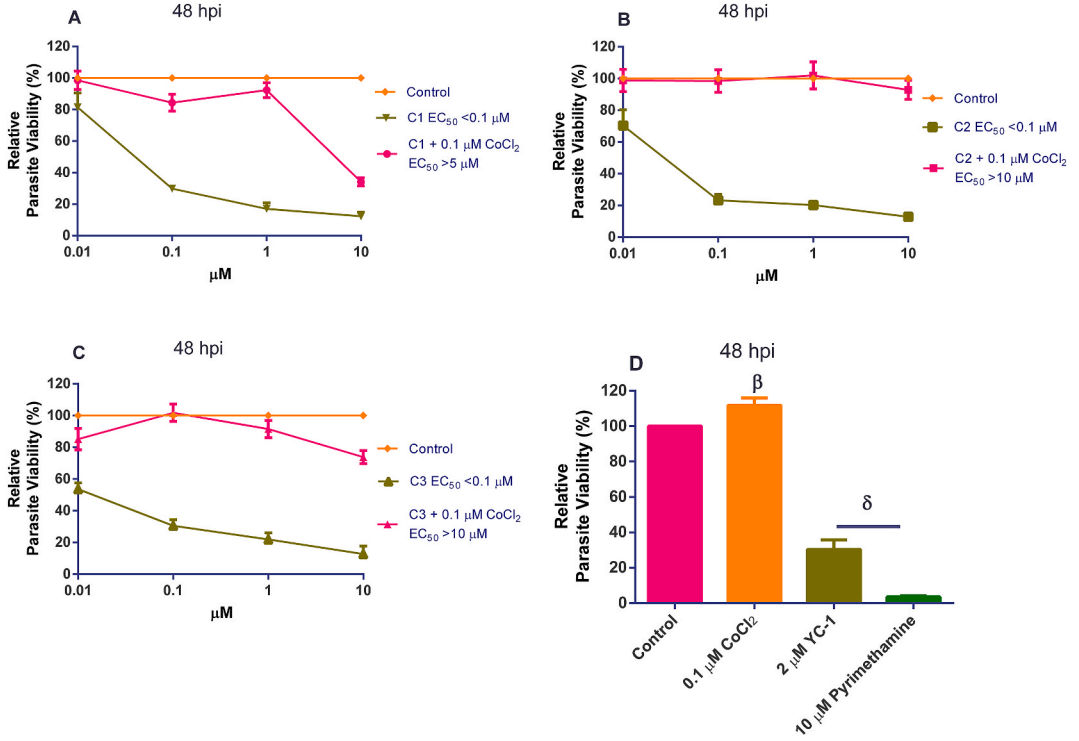

Fig. 6. Viability of parasite. HFF cells infested with *Toxoplasma gondii* were treated with imidazoles singly or in combination with $CoCl_2$ at the concentrations indicated. [A] C1; [B] C2; [C] C3; [D] Pyrimethamine, $CoCl_2$ and YC-1. After 48-h incubation, viability of parasite was assessed. Data are provided as an average of three independent repetitions plus/minus the corresponding error of mean (SEM). β at $p < 0.001$, δ at $p < 0.0001$ are significant compared with the control while, 'hpi' is hour post-infection.

The findings underscore the prospects of imidazole derivatives as new anti-parasitics, noting that anti-parasite therapy with multiple action mechanisms is desirable to reduce risk of parasite resistance. Overall, our findings contribute to deepen our understanding of the mechanistic action of imidazole-based compounds as alternative anti-parasite therapy.

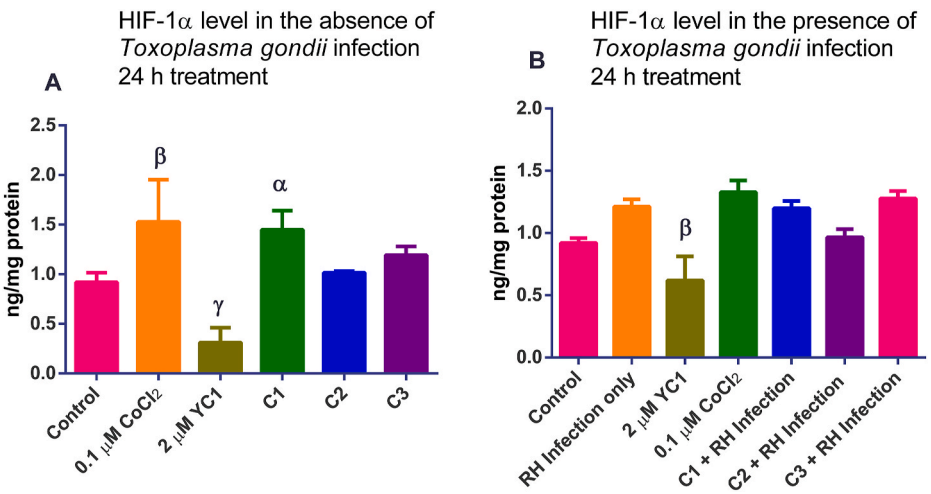

Fig. 7. Viability of parasite and hypoxia inducing factor – 1 alpha (HIF-1α) expression level. [A] In the absence of *Toxoplasma gondii* infection and after 24 h treatment, HIF-1α concentration was assessed; [B] In the presence of *Toxoplasma gondii* infection and after 24 h treatment, HIF-1α concentration was assessed. Data are provided as an average of three independent repetitions plus/minus the corresponding error of mean (SEM). α at $p < 0.05$ and γ at $p < 0.001$ are significant compared with the control but β at $p < 0.01$ relative to the control and/or RH infection only while, 'hpi' is hour post-infection.

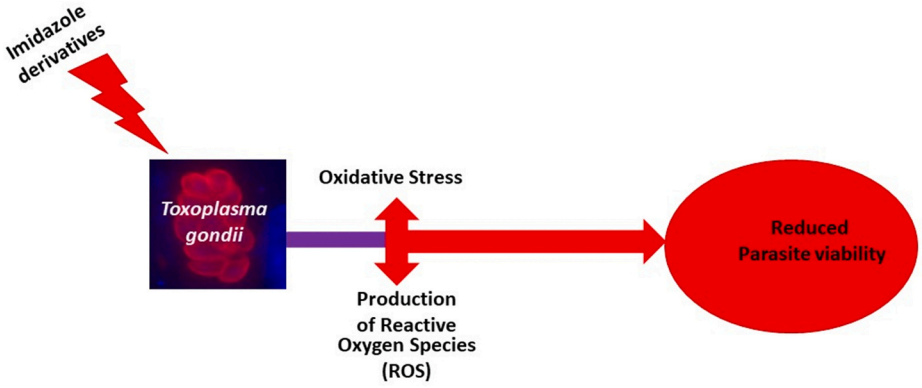

Fig. 8. Proposed mechanism for parasite death and/or growth inhibition by imidazole derivatives.

Availability of data

Data readily available.

Supplementary information

CCDC 850686 and 1897702 contain the supplementary crystallographic data for compounds **C-1** and **C-2,** respectively. These data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 01223 336033; e-mail: deposit@ccdc.cam.ac.uk).

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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