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Graphical Abstract

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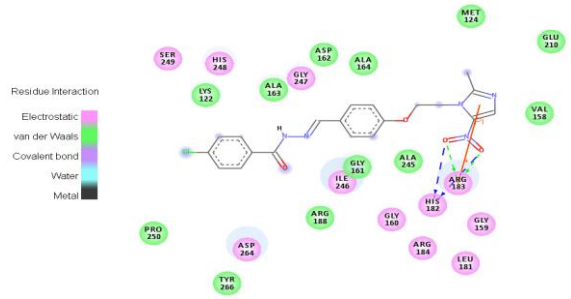
Mohammad Fawad Ansari[a], Shadab Miyan Siddiqui[a], Subhash M. Agarwal[b], Kunwar Somesh Vikramdeo[c], Neelima Mondal[c] and Amir Azam[a] *

[a]*Department of Chemistry, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025 India.*
[b]*Bioinformatics Division, Institute of Cytology and Preventive Oncology (ICPO) I-7, Sector - 39, Noida – 201301, Uttar Pradesh, India.*
[c]*School of Life Sciences, Jawaharlal Nehru University, New Delhi 110062, India.*



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[c] *School of Life Sciences, Jawaharlal Nehru University, New Delhi 110062, India.*
* Corresponding author. Tel.: +91-11-26981717/3254; fax: 91-11-26980229; e-mail: amir_sumbul@yahoo.co.in (A. Azam)

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ABSTRACT

Metronidazole hydrazone conjugates (**2-13**) were synthesized and screened *in vitro* for antiamoebic activity against HM1: IMSS strain of *Entamoeba histolytica.* Six compounds were found to be better inhibitors of *E. histolytica* than the reference drug metronidazole. These compounds showed greater than 50-60% viability against HeLa cervical cancer cell line after 72hr treatment. Also, molecular docking study was undertaken on *E. histolytica* thioredoxin reductase (EhTHRase) protein which showed significant binding affinity in the active site. Out of the six actives, some of the compounds showed lipophilic characteristics.

Amoebiasis, a contagious disease of the human gastrointestinal tract caused by parasitic protozoa *Entamoeba histolytica (E. histolytica*).[1-2] The parasite causes invasive infections and induces tissue destruction, producing amoebic colitis, dysentery and liver abscesses that affects 50 million people and causes 100,000 death per annum world wide.[3-4] Moreover, relapses of intestinal and hepatic amoebiasis have been reported.[5] Metronidazole (MNZ), tinidazole (TZ) and ornidazole (OZ) (Figure 1) are the widely used medicament for the treatment of protozoal infections, in which MNZ is the drug of choice for the treatment of amoebiasis, but long term use causes several side effects.[6] Although, it is mutagenic in bacteria, carcinogenic to rodents and genotoxic to human cells.[7] However, due to inadequate epidemiological evidence, it is not considered as a risk factor of cancer in humans.

The side chains attached to MNZ provide an opportunity to carry out modifications to derive novel molecules which might exhibit better antiamoebic activity and lesser toxicity for the host. In our previous studies some metronidazole conjugates have been found to exhibit promising antiamoebic activities[8-10] (Figure 2). Also, hydrazone derivatives are one of the widely studied pharmacophores showing a vast range of biological activities.[11] Hydrazone bearing pyridyl[12] or quinoline moiety[13] showing promising antiamoebic activity have been reported by us (Figure 3). Considering this perspective, it was envisaged to modify the metronidazole framework to synthesize novel metronidazole hydrazone conjugates. In this paper, we herein report the synthesis, antiamoebic activity, molecular docking and lipophilic studies of metronidazole hydrazone conjugates (Figure 4).

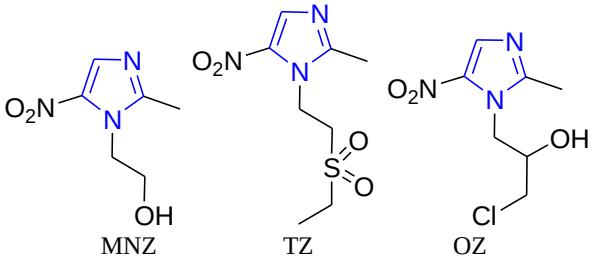
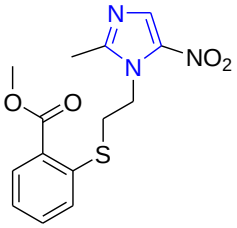


Figure 1: Antiamoebic drugs having imidazole ring

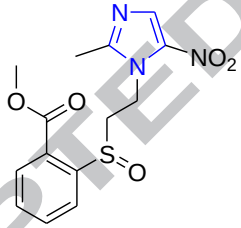
The synthetic pathway leading to target compounds (**2-13**) is depicted in Scheme 1. The key intermediate 4-[2-(2-methyl-5-nitro-1*H*-imidazole-1-yl) ethoxy]benzaldehyde (**1**), was synthesized by a reported method.[14] Further, the condensation reaction of intermediate (**1**) with various aryl hydrazides furnished the final compounds (**2-13**). The structures of all the compounds were elucidated on the basis of FT-IR, ^{1}H NMR, ^{13}C NMR and ESI-MS. The purity of the compounds was confirmed by the elemental analyses.

In order to explore the possible antiamoebic potential of newly synthesized metronidazole-hydrazone conjugates (**2-13**), all the compounds were screened against HM1: IMSS strain of *E. histolytica* by microdilution method[15] and the results were compared with the most widely used antiamoebic drug MNZ that had 50% inhibitory concentration (IC_{50}) 1.81 µM in our experiments. All the title compounds (**2-13**) exhibited better IC_{50} values (0.20-7.12 µM) than the compound **1** (IC_{50} = 11.48 µM). Compound **3** having chloro group at *para* position of phenyl ring exhibited most promising antiamoebic activity (IC_{50} = 0.20 µM) followed by compound **5** (IC_{50} = 0.36 µM) with nitro group at *para* position which can be attributed to electron withdrawing effect. Incorporation of hydroxy (**4**, IC_{50} = 0.38 µM), amino (**8**, IC_{50} = 0.43 µM) and methyl (**7**, IC_{50} = 0.49 µM) group at *para* position, exerted significant inhibitory effect whereas the introduction of methoxy (**6**, IC_{50} = 7.12 µM) and tertiary butyl group (**9**, IC_{50} = 1.98 µM) at the same position did not affect the antiamoebic activity. Compounds **4** and **12** had mono-hydroxy and di-hydroxy substitution on the phenyl group respectively but their antiamoebic activities were almost same. However compounds having mono-methoxy (**6**), di-methoxy (**11**) and tri-methoxy (**13**) group vary in their antiamoebic activities with the increase in number of methoxy groups. Therefore, it can be concluded that the antiamoebic activity varied with the nature as well as the position of the substituents. However, a comparison between precursor (**1**) and final compounds revealed that the better antiamoebic activities of metronidazole-hydrzone conjugates are due to presence of hydrazone moiety.

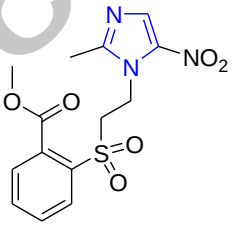
Therefore, it can be concluded that the combination of 5-nitroimidazole, hydrazone and the nature as well as the position of substitution on the phenyl group was responsible for antiamoebic activity.



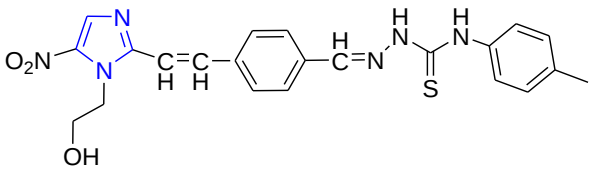
(IC_{50} = 0.028 µM)



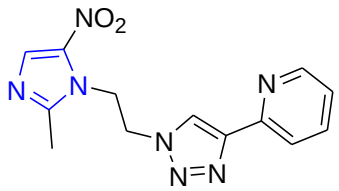
(IC_{50} = 0.015 µM)



(IC_{50} = 0.021 µM)

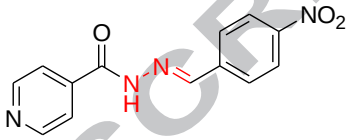


(IC_{50} = 0.560 µM)

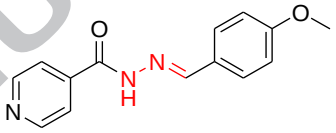


(IC_{50} = 0.008 µM)

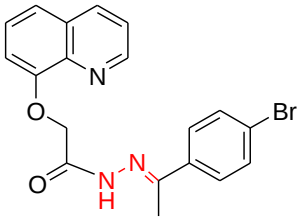
Figure 2: Metronidazole based compounds having antiamoebic activity



(IC_{50} = 0.370 µM)



(IC_{50} = 0.390 µM)



(IC_{50} = 0.040 µM)

Figure 3: Hydrazones with antiamoebic activity

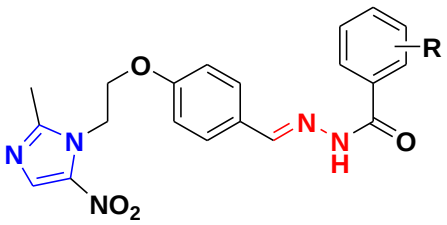
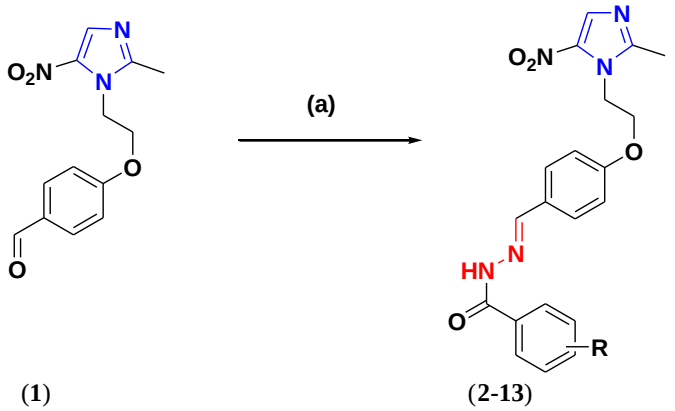


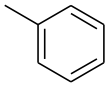
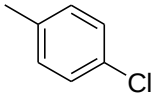
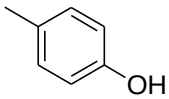
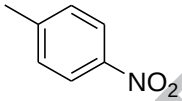
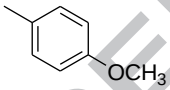
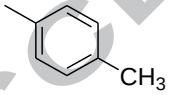
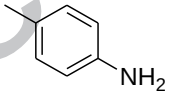
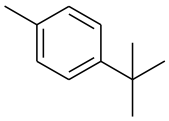
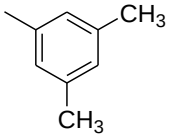
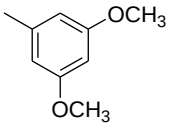
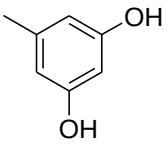
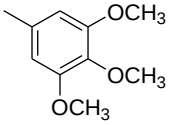
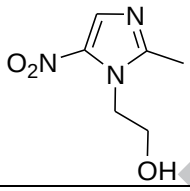
Figure 4: General structure of metronidazole hydrazone conjugates (blue and red colour depicts imidazole ring and hydrazone linkage respectively)

Further to assess the effect of the compounds (**3, 4, 5, 7, 8** and **12**) on cervical cancer cell line, HeLa, cells (4000cells/well) were plated in 96 well plate in triplicate. Cells were treated with compounds (**3, 4, 5, 7, 8** and **12**) as indicated in the Figure 5, the value correspond to the IC_{50} value observed for these compounds for *E. histolytica* (Table 2). In case of **7** and **4** these values resulted in reduction of viability to 50.8 and 54.4 percent respectively, whereas 60 % or more of the cells were viable in response to compounds **12, 8, 5** and **3**.



Scheme 1: Synthesis of Metronidazole hydrazone conjugates (**2—13**): Reagents and condition: (a) Different aryl hydrazides, ethanol, reflux.

Table 1: *In vitro* antiamoebic activity of metronidazole – hydrazone conjugates (**2-13**) against HM1: IMSS strain of *E. histolytica*

Compound No.	R	Antiamoebic Activity IC_{50}(μM)[a]	SD[b]
1	-	11.48	±0.003
2		2.02	±0.017
3		0.20	±0.007
4		0.38	±0.004
5		0.36	±0.002
6		7.12	±0.005
7		0.49	±0.007
8		0.43	±0.005
9		1.98	±0.004
10		5.96	±0.005
11		4.39	±0.003
12		0.37	±0.004
13		2.60	±0.004
MNZ		1.81	±0.029

[a] The values obtained in at least three separate assays done in triplicate
[b] Standard Deviation.

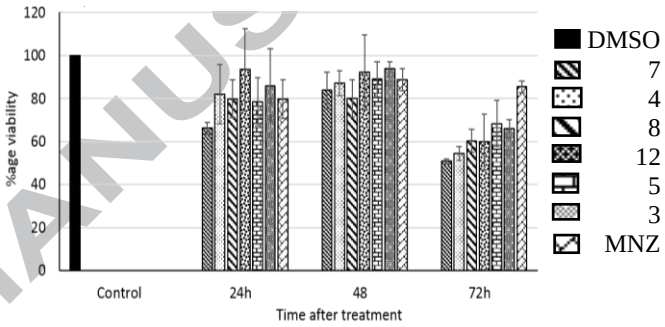


Figure 5: Assessment of viability of HeLa cells in response to compound **7, 4, 8, 12, 5** and **3**. Cells were plated in triplicates for 24h, 48h and 72h and treated with the compounds. Cells treated with DMSO are used as the control. MTT was added after completion of stipulated time intervals and processed. Absorbance was taken at 570 nm. Results were plotted taking control (DMSO) as 100%.

Studies have indicated that *E. histolytica* thioredoxin reductase (EhTHRase) is an important drug target for nitroimidazole based drugs including metronidazole[16-17] making it a suitable candidate for the search of novel leads against amoebiasis. In order to understand the possible mechanism of action of the compounds docking has been extensively exploited. Thus, using the previously published homology model of EhTHRase by our group[8] we docked the most active analogue so as to identify the key interactions between the inhibitor–target complex. It was observed that the compound **3** fits tightly into the active binding site (Figure 6 and 7) and exhibits GOLD score of 54.44, much higher than the metronidazole complex (score: 39.33). Protein-ligand interactions identified that nitrogen atom of His 182 and Arg 183 of the receptor active site forms hydrogen bonds with oxygen atoms of compound **3** (Figure 6 and 7). The two H-bonds could be responsible for enhanced binding affinity and thereby antiamoebic activity. Also, the docking studies demonstrates the possible role of the nitro group present in these compounds which is known to get reduced to highly reactive intermediate which then further reduces the enzyme like thioredoxin reductase.[16] Moreover, the residues Lys 122, Val 158, Gly 159, Gly 160, Gly 161, Ala 163, Glu 210, Ala 245, Ile 246, Gly 247, His 248, Ser 249 and Asp 264 contribute towards the hydrophobic interactions (Figure 6B). Additionally, we observed Pi-interaction (represented in orange) with Arg 183 which further stabilizes the complex (Figure 7). The molecular docking thereby supports the previous observations that 5-nitroimidazole analogues with hydrophobic groups show better antiamoebic activity[8-9, 18] and thus paves the way for identifying new leads

which could be useful for developing novel antiamoebic drugs.[19-20]

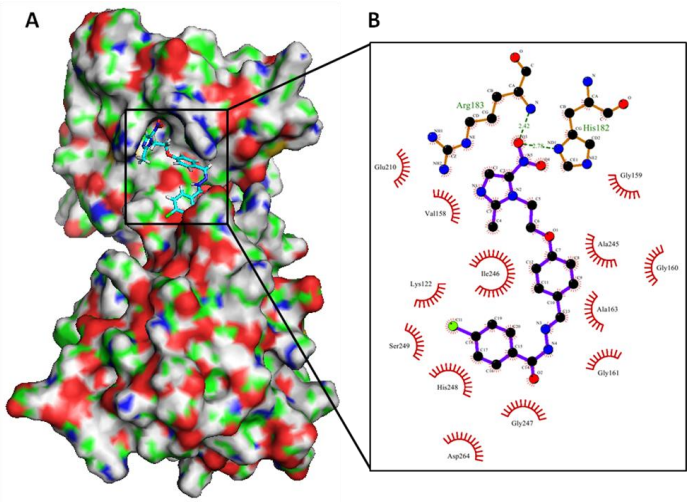


Figure 6: (A) Molecular surface representation of EhTHRase protein with compound 3 (shown in stick) docked in the active site (B) Ligplot showing hydrogen bonding interaction with green dashed lines, and hydrophobic contacts by red arcs with radiating lines.

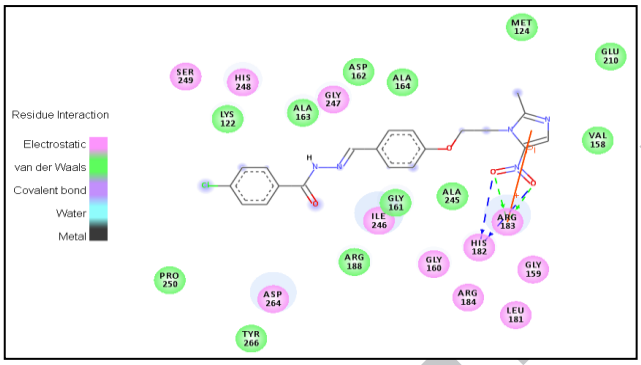


Figure7: 2-D diagrams depicting protein-ligand interactions.

Table 2: *Effect on cell viability of HeLa cells in response to compounds 7, 4, 8, 12, 5 and 3 as assessed by MTT assay*

Compound No.	Conc. in nM	24h	48h	72h
7	490	66.25± 2.7	83.85±8.5	50.80± 1.1
4	387	81.90± 13.8	87.10± 5.7	54.40± 3.2
8	435	79.80± 8.9	80.10± 8.6	60.30±5.3
12	370	93.40±19.0	92.10± 17.4	59.80±13.0
5	360	78.50± 11.2	88.90± 8.2	68.30± 10.7
3	208	85.90± 17.2	93.70±3.3	65.90± 4.3

To evaluate the lipophilicity of the compounds (**3**, **4**, **5**, **7**, **8**, **12**) having IC_{50} values better than MNZ, relative fluorescence intensity was analyzed using the Nile red stain.[21] The compounds **5** and **12** showed significantly higher fluorescence (Figure 8), which was due to the binding of Nile red stain to the hydrophobic groups. Thus, it can be construed that the active compounds **3**, **4**, **7** and **8** are highly less lipophilic than **5** and **12.**

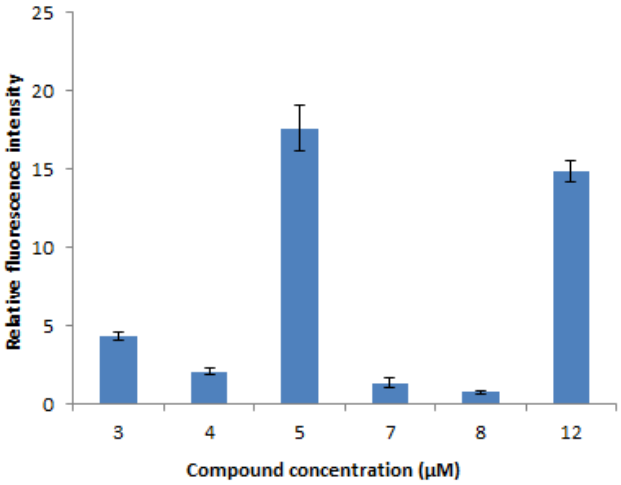


Figure 8: Relative fluorescence intensity for compound **3**, **4**, **5**, **7**, **8** and **12**.

In summary, six metronidazole-hydrazone conjugates showed better antiamoebic activity than metronidazole and the activity was substituent dependent. Molecular docking study of the most promising compound *i.e.* compound **3** demonstrated that the compound **3**-ETHRase complex had significant binding affinity. Compound **5** was most lipophilic compound followed by compound **12**. These promising results suggests the possibility of developing metronidazole hydrazone conjugates as potential drug candidates therefore, it may be hoped that the present study will stimulate efforts towards the development of novel effective antiamoebic agents.

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Supplementary data

Supplementary data (synthetic procedures, spectral data, antiamoebic assay, MTT assay and molecular docking) associated with this article can be found, in the online version, at doi:

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