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Original article

Probing the antiamoebic and cytotoxicity potency of novel tetrazole and triazine derivatives

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ABSTRACT

A series of compounds bearing a Tetrazole and Triazine ring motif conjugated with a SO_2NH function were synthesized and investigated for their antiamoebic potency. Cytotoxicity of the compounds was checked on human hepatocellular carcinoma cell line HepG2. Incorporation of Triazine ring in place of tetrazole resulted in a precipitous increase in the antiamoebic activity of the compounds. Antiamoebic activity of the investigated compounds was found to be position and substituent dependent. *In vitro* cytotoxicity results revealed noncytotoxic nature of all the tested compounds up to a concentration of 25 μM. Compound **5c** and **5d** were obtained as least cytotoxic ($IC_{50} > 100$ μM) and excellent *Entamoeba histolytica* inhibitors with IC_{50} values of 1.05 μM and 1.02 μM respectively.

1. Introduction

A real advance in chemotherapy of protozoan infections was made more than four decades ago with the introduction of metronidazole. It was the first orally effective treatment for amoebiasis [1,2], one of the most dreadful disease among the parasitic diseases [3,4]. More than 50 million people are estimated to suffer from the symptoms of amoebiasis such as hemorrhagic colitis and amoebic liver abscess resulting in 100,000 deaths annually [5,6]. Metronidazole is still the drug of choice to treat this disease, however, it is associated with serious side effects [7–10], such as neurological alterations and impairment of cardiac rhythm due to the chelation of Metronidazole with calcium ions [11,12]. It is carcinogenic to man and animals [13]. There are occasional reports of failure, recurrence of amoebic liver abscesses even after treatment with metronidazole and survival of parasites in spite of adequate treatment [14–16]. Such serious global health problem demands a renewed effort seeking the development of new antiamoebic agents effective against the pathogenic parasite and non toxic to the human cells.

Considering the need of a new molecule for the treatment of amoebiasis, we had synthesized a series of tetrazole and triazine based sulfonamide derivatives. The ability of tetrazole ring to serve as the bioequivalent (bioisostere) of the carboxylic acid group, often results in a dramatic enhancement in the *in vitro* and/or *in vivo* activity [17,18], and had been extensively studied in pharmaceutical industry [19,20]. Tetrazoles exhibit potential biological activities and the core ring is an important constituent of number of modern drugs [21–26]. Similarly 1,3,5-Triazines show diverse biological activities, including antifungal, antibacterial, antimalarial, anticancerous [27–30] and more importantly antiprotozoal activity [31,32].

$SO_2N <$ moiety is an important function incorporated in many chemotherapeutically important sulpha drugs, like sulphadiazine, sulphathiazole, sulphamerazine, and sulfonamides [33]. Compounds possessing this moiety have been reported to show antibacterial activity [34,35], some of them are HIV protease inhibitors [36], carbonic anhydrase inhibitors [37–39], antiepileptic agents [40], and anticonvulsant agents [41,42], and are used as ETA, being a selective antagonist [43,44]. These observations prompted the incorporation of a sulfonamide moiety into the tetrazole and triazine ring to make use of both functionalities in the potentiation of pharmacological activities. Herein, the new sulfonamide derivatives of piperonal based tetrazole and triazines were evaluated for antiamoebic activity and their toxicity profile was checked on human hepatocellular carcinoma (HepG2) cell line. This work is an additional effort to the development of new chemotherapeutic agents which are antiamoebic and non toxic to human cells.

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2. Results and discussion

2.1. Chemistry

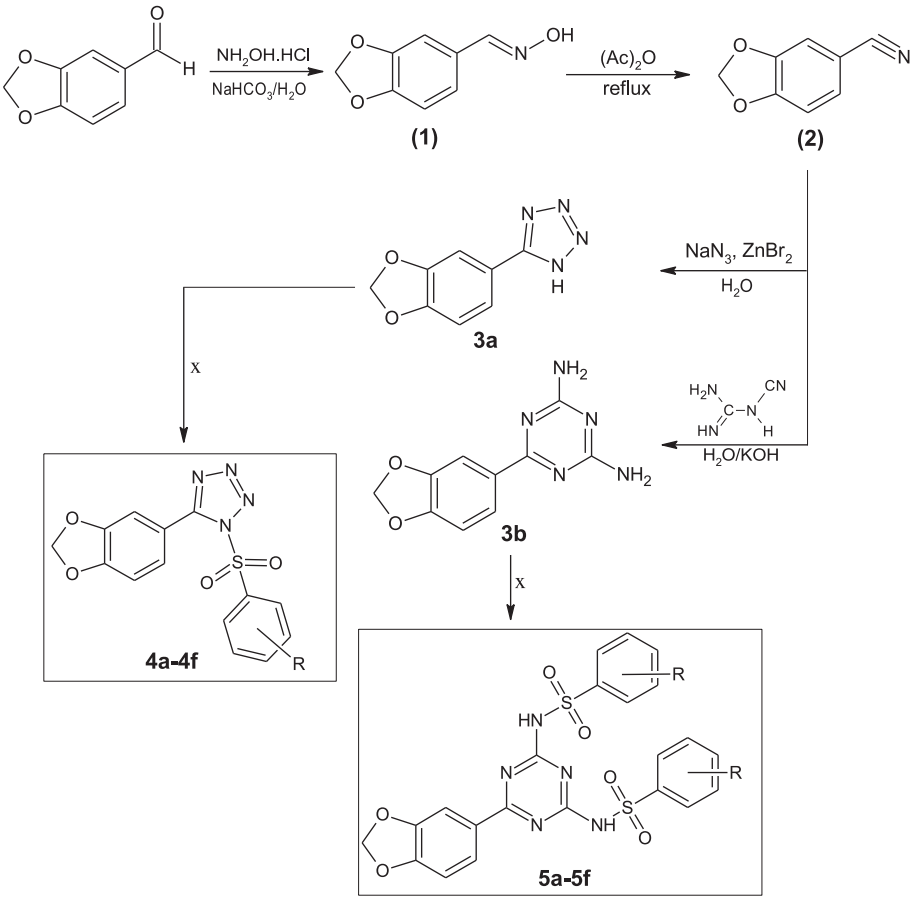
Present study was undertaken to synthesize some novel Sulfonamide derivatives of tetrazole and triazine and investigate their probable antiamoebic effects. Target compounds were obtained in a four step reaction procedure as outlined in Scheme 1. Piperonal (1,3-benzodioxole-5-carbaldehyde) was converted into 1,3-benzodioxole-5-carbonitrile (**2**) in a two step reaction procedure *via* an oxime intermediate (**1**) using a standard procedure [45]. The nitrile group of 1,3-benzodioxole-5-carbonitrile (**2**) was cyclized into 5-(1,3-benzodioxol-5-yl)-1*H*-tetrazole (**3a**) and 6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine (**3b**) [46,47]. The compounds (**4a**–**4f**) and (**5a**–**5f**) were obtained by (**3a**) and (**3b**) with different arylsulfonylchlorides in presence of triethylamine using dry CH_2Cl_2 as a solvent. All the synthesized compounds were characterized by elemental analysis, IR, ^1H, ^{13}C NMR and ESI-MS studies and their data are presented in experimental section.

2.2. Pharmacology

The compounds (**4a**–**4f**) and (**5a**–**5f**) and their key intermediates **3a** and **3b** were screened *in vitro* against HM1:IMSS strain of *Entamoeba histolytica* by microdilution method [48]. All the experiments were carried out in triplicate at each concentration level and repeated thrice. Cytotoxicity of all the compounds has been studied by MTT assay on human hepatocellular carcinoma cell line (HepG2). The results of biological activity and cytotoxicity are summarized in Tables 1a and b and Fig. 1a and b respectively.

2.2.1. Antiamoebic activity

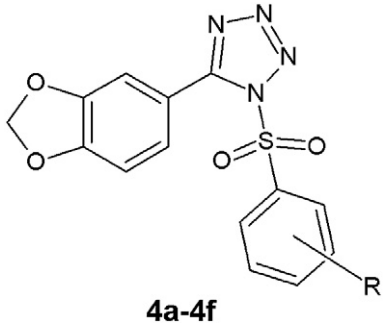
Preliminary experiments were carried out to determine the *in vitro* antiamoebic activity of all the compounds (**4a**–**4f**) and (**5a**–**5f**) by microdilution method using HM1:IMSS strain of *E. histolytica*. The results are summarized in Tables 1a and 1b. The data is presented in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The antiamoebic activity of the synthesized compounds was compared with widely used antiamoebic medication, metronidazole with 50% inhibitory concentration (IC_{50}) of 1.80 μM in our experiments. The target compounds under investigation were synthesized from a simple terpene molecule; piperonal and two different pharmacophore groups were incorporated to study their probable antiamoebic effect. The compounds **4a**–**4f** contain a tetrazole ring while as in compounds **5a**–**5f** a triazine ring was incorporated in the place of tetrazole ring. Replacement of the tetrazole ring with a triazine ring resulted in a 4 fold improvement in the activity of the key intermediate **3b** ($IC_{50} = 3.54$ μM) against *Entamoeba histolytica* as compared to **3a** ($IC_{50} = 14.24$ μM). It is interesting to mention that none of the tetrazole ring bearing derivatives **4a**–**4f** showed any significant activity ($IC_{50} = 3.75$–7.56 μM) against the test organism where as all the triazine ring bearing derivatives **5a**–**5f** showed moderate to excellent activity ($IC_{50} = 1.02$–2.85 μM). This significant change in



Compound	4a, 5a	4b, 5b	4c, 5c	4d, 5d	4e, 5e	4f, 5f
R	H	4-Me	4-Cl	4-NO_2	2,4-diCl	4-Isopropyl

Scheme 1. Schematic representation of synthesis of different substituted tetrazole and Triazine derivatives.

Table 1a
In vitro antiamoebic activity of compounds (**4a**–**4f**) against HM1:IMSS strain of *Entamoeba histolytica* and toxicity profile.



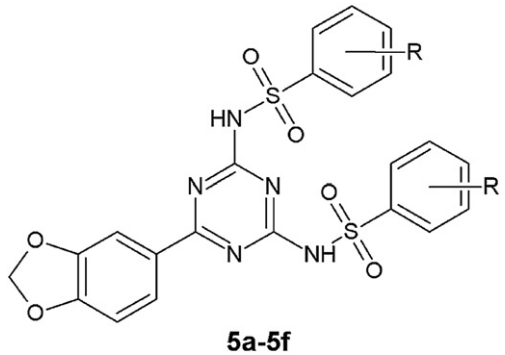
4a-4f

Compound	R	Antiamoebic activity		Toxicity profile	
		IC50 (μM)	S.D.[a] (±)	IC50 (μM)	Safety Index (SI)
4a	H	7.56	0.14	>100	>13.22
4b	4-Me	6.10	0.20	>100	>16.39
4c	4-Cl	4.85	0.18	>100	>20.61
4d	4-NO_2	3.75	0.23	≈84.7	22.58
4e	2,4-diCl	6.90	0.16	≈85	12.31
4f	4-Isopropyl	6.28	0.14	>100	>15.92
MNZ		1.80	0.20	>100	>55.55

[a] The value obtained in at least three separate assay done in triplicate, S.D. (±) Standard deviation.

activity of these compounds can be speculated with either the individual effect of triazine ring and sulfonamide moieties or their conjugated effect. The substituted phenyl groups of the sulfonamide fragment also have a marked effect on the activity of the compounds.

Table 1b
In vitro antiamoebic activity of compounds (**5a**–**5f**) against HM1:IMSS strain of *Entamoeba histolytica* and toxicity profile.



5a-5f

Compound	R	Antiamoebic activity		Toxicity profile	
		IC50 (μM)	S.D.[a] (±)	IC50 (μM)	Safety Index(SI)
5a	H	2.60	0.22	>100	>38.46
5b	4-Me	2.15	0.16	>100	>46.51
5c	4-Cl	**1.05**	0.17	>100	>**95.23**
5d	4-NO_2	**1.02**	0.14	>100	>**98.03**
5e	2,4-diCl	2.56	0.24	>100	>39.06
5f	4-Isopropyl	2.85	0.17	>100	>35.08
MNZ		1.80	0.20	>100	>55.55

The compounds with bold font IC_{50} values are more active than metronidazole.
[a] The value obtained in at least three separate assay done in triplicate, S.D. (±) Standard deviation.

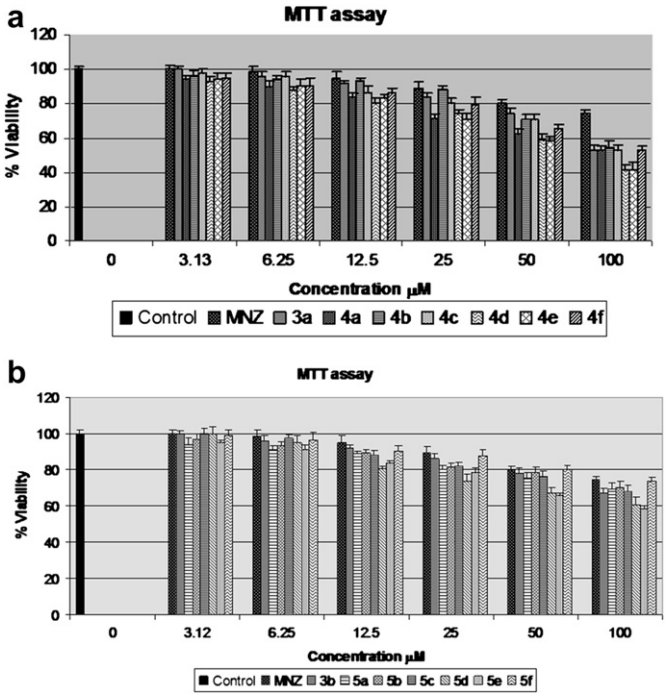


Fig. 1. (a): Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with Metronidazole, key intermediate **3a** and compounds **4a**–**4f** evaluated by MTT assay. (b): Percentage of viable cells after 48 h pre-treatment of HepG2 cell line with Metronidazole, key intermediate **3b** and compounds **5a**–**5f** evaluated by MTT assay.

It was observed that the compounds bearing a chloro or nitro group at the *para* position of the phenyl ring showed better activity. The presence of methyl group at *para* position decreased the activity (**5b**; $IC_{50} = 2.15$ μM) and was further decreased with insertion of isopropyl group (**5f**; $IC_{50} = 2.85$ μM). The presence of chloro group at the ortho and *para* position of the phenyl ring in compound **5e** showed moderate activity similar to the unsubstituted phenyl ring containing compound **5a**. It will be interesting to speculate the effect with drastic conformational and steric changes at the ortho and *para* position of the phenyl ring of the sulfonamide fragment. These findings indicate that the presence of electron withdrawing groups like chloro and nitro group at the *para* position of the phenyl ring of the sulfonamide fragment of the triazine ring incorporated compounds, generally increase the antiamoebic activity of the compounds under study than the compounds bearing electron releasing groups. Tetrazole ring bearing derivatives (**4a**–**4f**) also adopted a similar behaviour but not that significant. The poor antiamoebic activity of the key intermediates and the enhancement of activity with the introduction of SO_2NH group suggest that the sulfonamide moiety was important to activity. Based upon the results it will also be necessary to optimize the lead compound by substitution in C-4 position of phenyl ring of the sulfonamide fragment of the triazine ring incorporated compounds by more polar groups, which seem to be very important for antiamoebic effect, besides the position of the substituents seems to be an important factor behind the antiamoebic activity of the tested compounds. From the results it can be inferred that the compound **5c** *(N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzene sulfonamide)* $IC_{50} = 1.05$ μM, and **5d** *(N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzene sulfonamide)* $IC_{50} = 1.02$ μM is a promising amoebicidal with high antiamoebic efficacy and least cytotoxicity on human cell line. The results were statistically evaluated by analysis of variance. The null hypothesis was tested using *t*-test. The significance of the difference between the IC_{50} values of metronidazole and the compound **5c** and **5d** was

evaluated by *t*-test. The values of the calculated *t* were found higher than the Table value of *t* at 5% level, thus concluding that the character under study is said to be significantly influenced by the treatment.

2.2.2. In vitro cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by the succinate dehydrogenase system of mitochondrial living cells to produce water insoluble purple formazan crystals [49,50] which, after solubilization, can be measured spectrophotometrically. Since the amount of formazan produced is directly proportional to the number of active cells in the culture, MTT has long been used to assess the cell viability in cell proliferation and cytotoxicity [51–53].

In the present study, some newly synthesized compounds were screened for their antiamoebic activity and then evaluated for their cytotoxicity against *Human hepatocellular carcinoma cell line* (HepG2) to ensure their toxic effect. Metronidazole was used as a reference drug. A sub-confluent population of HepG2 cells was treated with increasing concentration of these compounds and the number of viable cells was measured after 48 h by MTT cell viability assay. The concentration range of all the compounds was 3.13–100 μM. The cell viability (%) obtained with continuous exposure for 48 h are depicted in Fig. 1a and b. The cytotoxicity of all the compounds was found to be concentration-dependent. Fig. 1a and b depicts that all the compounds including the reference compound metronidazole showed viability ranging from 92.5 to 100% at the concentration range of 3.13 μM and up to a concentration of 25 μM all the compounds showed a viability of ≥70%. On increasing the concentration range up to 50 and 100 μM the compounds showed moderate to high cytotoxicity against the HepG2 cell line. Except for compounds **4d** and **4e** all the compounds showed least cytotoxicity and have IC_{50} values greater than 100 μM as given in Tables 1a and 1b. To further investigate the selectivity of the compounds, the "safety index" (SI), defined as the toxicity IC_{50}/protozoal IC_{50}, was calculated. This allows estimating the efficacy of compounds. The results are summarized in Tables 1a and 1b. Compound **5c** and **5d** showed higher safety index values, better than metronidazole. From the results of antiamoebic activity and cytotoxicity it can be inferred that compound **5c** and **5d** are least cytotoxic and excellent *E. histolytica* inhibitors as compared to the reference drug metronidazole (Fig. 2). These results also showed that the compounds **5c** and **5d** despite of being highly antiamoebic do not show any marked toxicity on human cell line and have safety index values of ≥95 which is better than metronidazole.

3. Conclusion

This study has achieved the efficient synthesis of novel Tetrazole and Triazine ring containing derivatives and examined their *in vitro* antiamoebic and cytotoxic activity. Incorporation of triazine ring in place of tetrazole results in enhancement of antiamoebic activity. These results also clearly documented that modification at position-4 of the phenyl ring of the sulfonamide fragment of the triazine ring incorporated compounds by some electron withdrawing substituents allows an optimization of these compounds for an effective and probably selective antiamoebic therapy. More importantly, antiamoebic and cytotoxicity studies of these compounds resulted in the binding of two compounds **5c** *(N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-4-chlorobenzene sulfonamide)* IC_{50} = 1.05 μM, and **5d** *(N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzene sulfonamide)* IC_{50} = 1.02 μM as stronger *E. histolytica* inhibitors with least cytotoxicity to Human cells (HepG2) than the standard drug metronidazole IC_{50} = 1.80 μM, which gains some insights into the synthesis or structure modifications of triazine pharmacophore bearing derivatives for the purpose of discovering new antiamoebic drug candidates.

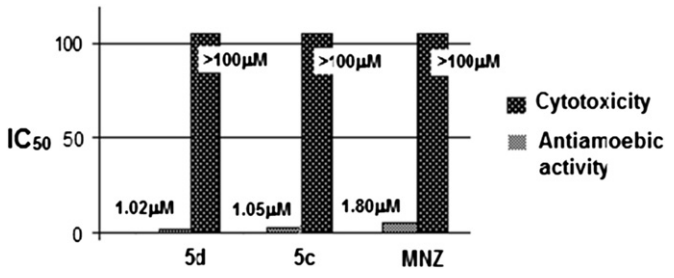


Fig. 2. Comparison of antiamoebic activity and cytotoxicity profile of compounds **5d**, **5c** and reference drug metronidazole. Compound **3d** has almost 100 times more antiamoebic potency than its cytotoxicity which is better than Metronidazole. Schemes.

4. Experimental Protocol

Solvents and organic reagents were purchased from Sigma Aldrich, Merck (Germany) and Loba Chemie (India) and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and the results are uncorrected. Elemental analyses were performed on HeraeusVario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ±0.4% of the theoretical values. IR spectra were recorded on Perkin–Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs/ATR mode. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 (300.13) MHz spectrometer using DMSO-d_6/$CDCl_3$ as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; br s for broad singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica). Visualization was achieved with UV light at 254 nm or I_2 vapour staining.

4.1. Synthesis of 5-(1,3-benzodioxol-5-yl)-1H-tetrazole (*3a*)

5-(1,3-benzodioxol-5-yl)-1H-tetrazole was synthesized from 1,3-benzodioxole-5-carbonitrile by following a reported procedure [46]. The requisite nitrile (2) was prepared from Piperonal (1,3-benzodioxole-5-carbaldehyde) *via* oxime formation, followed by dehydration with acetic anhydride, following a reported procedure [45]. Nitrile (2.95 g, 20 mmol), sodium azide (1.43 g, 22 mmol) and zinc bromide (4.50 g, 20 mmol), were put in 60 mL of water. 5 mL of isopropanol was also added to stop the formation of clumps. The reaction mixture was refluxed for 24 h and monitored by TLC; vigorous stirring is essential. After 24 h HCl (3 N, 30 mL) and ethyl acetate (100 mL) were added, and vigorous stirring was continued until no solid was present and the aqueous layer had a pH of 1. If necessary, additional ethyl acetate was added. The organic layer was isolated and the aqueous layer extracted with 2 × 100 mL of ethyl acetate. The combined organic layers were evaporated, 200 mL of 0.25 N NaOH was added, and the mixture was stirred for 30 min, until the original precipitate was dissolved and a suspension of zinc hydroxide was formed. The suspension was filtered, and the solid washed with 20 mL of 1 N NaOH. To the filtrate was added 50 mL of 3 N HCl with vigorous stirring causing the tetrazole to precipitate. The tetrazole was filtered and washed with 2 × 50 mL of 3 N HCl and dried in a drying oven to furnish the tetrazole as a white powder.

*4.1.1. (E)-1-(1,3-benzodioxol-5-yl)-N-hydroxymethanimine (**1**)*

White; Yield 95%; mp. 100–105 °C; IR ν_{max} cm^{-1}: 3225 (NO–H), 2921 (C–H), 1660 (C=N), 1608, 1500 (C=C, Ar), 937 (N–O stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 10.85 (broad s, 1H, N–OH), 7.90 (s, 1H, CH = N–OH), 7.30–6.50 (m, 3H, Ar–H), 5.90 (s, –O–CH_2–O–); ^{13}C NMR (DMSO-d_6) d(ppm): 152.3, 148.1, 146.0, 132.6, 123.8, 115.8, 101.6 (–O–CH_2–O–); ESI-MS m/z: [M^++1] 166.04.

*4.1.2. 1,3-Benzodioxole-5-carbonitrile (**2**)*

White; Yield 92%; mp. 95–98 °C; IR ν_{max} cm^{-1}: 2928 (C–H, Ar), 2204 (CN), 1592 (C=C, Ar); ^{1}H NMR (DMSO-d_6) (ppm): 7.18–6.90 (m, 3H, Ar–H), 6.03 (s, 2H, –O–CH_2–O–); ^{13}C NMR (DMSO-d_6) δ (ppm): 153.1, 147.9, 123.1, 123.6, 118.2, 116.1, 113.8, 103.4 (–O–CH_2–O–); ESI-MS m/z: [M^++1] 148.02.

*4.1.3. 5-(1,3-Benzodioxol-5-yl)-1H-tetrazole (**3a**)*

White solid; Yield 84%; mp. 190–193 °C; Anal. Calc. for $C_8H_6N_4O_2$: C 50.53, H 3.18, N 29.46%, found: C 50.43, H 3.08, N 29.58%; IR ν_{max} cm^{-1}: 3280 (N–H br stretch), 2864 (C–H, Ar), 1632 (C=N), 1595 (C=C, Ar); ^{1}H NMR (DMSO) δ(ppm): 7.62 (1H, d, J = 7.2 Hz), 7.54 (1H, s), 7.16 (1H, d, J = 8.1 Hz), 6.15 (2H, s, O–CH_2–O), 4.45 (1H, NH, br s); ^{13}C NMR (DMSO) δ(ppm): 154.8 (C=N), 149.2, 147.6, 121.2, 118.5, 108.2, 106.7, 101.6 (–O–CH_2–O); ESI-MS m/z [M^++1] 191.07.

*4.2. General procedure for the synthesis of 5-(1,3-benzodioxol-5-yl)-1-(phenyl/substituted phenyl sulfonyl)-2H-tetrazoles (**4a**–**4f**)*

To a solution of 5-(1,3-benzodioxol-5-yl)-1H-tetrazole (1 eq.) and triethylamine (3 eq.) in dry CH_2Cl_2 at 0 °C was added aryl sulfonyl chlorides (1.2 eq.). The reaction mixture was stirred at 0 °C for about 2 h and stirring was continued at room temperature for about 4–5 h (completion of reaction was monitored by TLC). After the completion of reaction the reaction mass was quenched with distilled water and extracted with dichloromethane. Finally the combined organic layer was washed with distilled water again and dried over anhydrous Na_2SO_4. After removal of the solvent in vacuum, the residue was purified by recrystallization.

*4.2.1. 5-(1,3-Benzodioxol-5-yl)-1-(phenylsulfonyl)-1H-tetrazole (**4a**)*

Cream solid; Yield 65%; mp. 182–185 °C; Anal. Calc. for $C_{14}H_{10}N_4O_4S$: C 50.91, H 3.05, N 17.03; found: 50.82, H, 3.01, N, 17.18%; IR ν_{max} cm^{-1}: 3068 (C–H, Ar), 1595 (C=C, Ar), 1690 (C=N), 1174 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.93 (2H, d, J = 7.2 Hz) 7.73 (1H, t, J = 7.2 Hz), 7.62 (2H, m), 7.05 (1H, dd, J = 1.8 Hz; ^2J = 4.8 Hz), 6.93 (1H, d, J = 1.5 Hz), 6.81(1H, d, J = 8.1 Hz), 6.04 (2H, s, O–CH_2–O); ^{13}C NMR (DMSO-d_6) δ(ppm): 154.8 (C=N), 150.6, 147.3, 137.1, 134.1, 128.9, 128.0, 123.6, 122.6, 115.7, 113.0, 108.2, 107.8, (Ar–C), 101.5 (O–CH_2–O); ESI-MS m/z: [M^++1] 331.05.

*4.2.2. 5-(1,3-Benzodioxol-5-yl)-1-[(4-methylphenyl)sulfonyl]-1H-tetrazole (**4b**)*

Yellow solid; Yield 60%; mp. 220–223 °C; Anal. Calc. for $C_{15}H_{12}N_4O_4S$: C 52.32, H 3.51, N 16.27; found: C 52.26, H, 3.48, N, 16.35%. IR ν_{max} cm^{-1}: 3068 (C–H, Ar), 1598 (C=C, Ar), 1648 (C=N), 1158 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.90 (2H, d, J = 7.8 Hz), 7.65–7.60 (2H, m), 7.02 (1H, dd, J = 1.2 Hz; ^2J = 5.2 Hz), 6.90 (1H, s), 6.83 (1H, d, J = 8.1 Hz), 6.07 (2H, s, O–CH_2–O), 2.83 (3H, s); ^{13}C NMR (DMSO-d_6) δ(ppm): ^{13}C NMR (DMSO-d_6) δ(ppm): 156.4 (C=N), 152.5, 146.3, 143.9, 136.1, 128.6, 127.8, 122.4, 116.0, 112.9 (Ar–C), 101.5 (O–CH_2–O), 16.4 (CH_3); ESI-MS m/z: [M^++1] 345.06.

*4.2.3. 5-(1,3-Benzodioxol-5-yl)-1-[(4-chlorophenyl)sulfonyl]-1H-tetrazole (**4c**)*

White solid; Yield 67%; mp. 228–230 °C; Anal. Calc. for $C_{14}H_9N_4ClO_4S$: C 46.10, H 2.49, N 15.36; found: 46.24, H, 2.38, N, 15.47%. IR ν_{max} cm^{-1}: 3065 (C–H, Ar), 1595 (C=C, Ar), 1645 (C=N), 1328,1147 (S=O), 730 (C–Cl); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.98 (2H, d, J = 7.2 Hz), 7.77 (2H, m), 7.08 (1H, dd, J = 2.2 Hz; ^2J = 4.8 Hz), 6.90 (1H, d, J = 2.5 Hz), 6.85 (1H, d, J = 8.5 Hz), 6.01 (2H, s, O–CH_2–O); ^{13}C NMR (DMSO-d_6) δ(ppm): 154.6 (C=N), 149.8, 147.7, 139.2, 134.1, 128.9, 128.0, 123.6, 115.7, 113.0, 108.2 (Ar–C), 101.5 (O–CH_2–O); ESI-MS m/z: [M^++1] 366.00.

*4.2.4. 5-(1,3-Benzodioxol-5-yl)-1-[(4-nitrophenyl)sulfonyl]-1H-tetrazole (**4d**)*

Yellowish solid; Yield 62%; mp. 210–213 °C; Anal. Calc. for $C_{14}H_9N_5O_6S$: C 44.80, H 2.42, N 18.66; found: 44.92, H, 2.36, N, 18.74%; IR ν_{max} cm^{-1}: 3068 (C–H, Ar), 1645 (C=N), 1590 (C=C, Ar), 1545, 1350 (NO_2), 1321, 1128 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 8.13 (2H, d, J = 7.8 Hz), 7.76 (2H, m), 7.12 (1H, dd, J = 2.8 Hz; ^2J = 5.8 Hz), 6.96 (1H, s), 6.93 (1H, d, J = 8.7 Hz), 6.07 (2H, s, O–CH_2–O); ^{13}C NMR (DMSO-d_6) δ(ppm): ^{13}C NMR (DMSO-d_6) δ(ppm): 156.4 (C=N), 152.5, 146.3, 143.9, 136.1, 128.6, 127.8, 122.4, 112.9 (Ar–C), 101.5 (O–CH_2–O); ESI-MS m/z: [M^++1] 376.03.

*4.2.5. 5-(1,3-Benzodioxol-5-yl)-1-[(2,4-dichlorophenyl)sulfonyl]-1H-tetrazole (**4e**)*

White solid; Yield 58%; mp. 228–230 °C; Anal. Calc. for $C_{14}H_8Cl_2N_4O_4S$: C 44.80, H 2.42, N 18.66; found: 44.68, H 2.47, N 18.73%; IR ν_{max} cm^{-1}: 3062 (C–H Ar), 1590 (C=C, Ar), 1640 (C=N), 1321, 1128 (S=O), 742 (C–Cl), ^{1}H NMR (DMSO-d_6) δ(ppm): 7.89 (1H, d, J = 7.5 Hz) 7.58 (1H, s), 7.42 (1H, d, J = 7.8 Hz), 7.10 (1H, dd, J = 2.5 Hz; ^2J = 4.5 Hz), 6.94 (1H, d, J = 2.4 Hz), 6.86 (1H, d, J = 8.4 Hz), 6.02 (2H, s, O–CH_2–O); ^{13}C NMR (DMSO-d_6) δ(ppm): 154.8 (C=N), 148.2, 147.5, 138.4, 136.0, 128.6, 128.0, 122.6, 118.3, 112.0, 105.5, (Ar–C), 101.8 (O–CH_2–O); ESI-MS m/z : [M^++1] 399.94, [M^++2] 400.96.

*4.2.6. 5-(1,3-Benzodioxol-5-yl)-1-[(4-isopropylphenyl)sulfonyl]-1H-tetrazole (**4f**)*

White solid; Yield 60%; mp. 242–245 °C; Anal. Calc. for $C_{17}H_{16}N_4O_4S$: C 54.83, H 4.33, N 15.04%; found: 54.76, H 4.10, N 15.26%; IR ν_{max} cm^{-1}: 3032 (C–H, Ar), 1595 (C=C, Ar), 1645 (C=N), 1545, 1350 (NO_2) 1321, 1128 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.95 (2H, d, J = 7.8 Hz), 7.54 (2H, d, J = 7.4 Hz), 7.11 (1H, dd, J = 1.8 Hz; ^2J = 4.8 Hz), 6.97 (1H, s), 6.85 (1H, d, J = 8.1 Hz), 6.01 (2H, s, O–CH_2–O), 3.83–3.68 (1H, m), 1.89 (6H, s); ^{13}C NMR (DMSO-d_6) δ(ppm): 156.8 (C=N), 152.4, 146.8, 143.5, 135.1, 128.5, 128.0, 126.7, 125.2, 120.6, 115.0 (Ar–C), 101.5 (O–CH_2–O), 42.6, 26.5 (Isopropyl); ESI-MS m/z: [M^++1] 373.06.

*4.3. Synthesis of 6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine (**3b**)*

6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine (**3b**) was synthesized from 1,3-benzodioxole-5-carbonitrile (**2**) by following a reported procedure [47]. The requisite nitrile (2) was prepared from Piperonal (1,3-benzodioxole-5-carbaldehyde), *via* oxime formation, followed by dehydration with acetic anhydride, following a reported procedure [45]. Nitrile (5 m mol), dicyandiamide (5.5 m mol) and KOH (10 m mol) were put in 50 ml water. The reaction mixture was heated at reflux for 12–48 h. The suspended solid products were filtered and rinsed with Et_2O to give a pure diaminotriazine.

4.3.1. 6-(1,3-Benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine (**3b**)

Cream solid; Yield: 80%; mp. 220–223 °C; Anal. Calc. for $C_{10}H_9N_5O_2$: C 51.95, H 3.92, N 30.29%; found: C 51.82, H 4.12, N 30.37%; IR ν_{max} cm^{-1}: 3408 (NH_2), 2865 (C–H, Ar), 1662 (C=N), 1587 (C=C, Ar); ^1H NMR ($CDCl_3$) δ(ppm): 7.26–6.77 (3H, m), 5.87 (2H, s, O–CH_2–O), 4.70 (4H, NH_2 br s); ^{13}C NMR ($CDCl_3$) δ(ppm): 169.8, 167.9 (C=N), 149.5, 148.6, 125.2, 121.0, 115.8, 113.2 (Ar–C), 102.2 (O–CH_2–O); ESI-MS m/z: [M^++1] 232.07.

4.4. General procedure for the synthesis of N, N′-6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diyl di/bisbenzene/substituted benzene sulfonamides (**5a**–**5f**)

To a solution of 6-(1,3-benzodioxol-5-yl)-1,3,5-triazine-2,4-diamine (1.0 eq.) and triethylamine (5.0 eq.) in dry CH_2Cl_2 at 0 °C was added aryl sulfonyl chlorides (2.0 eq.). The reaction mixture was stirred at 0 °C for about 2 h and stirring was continued at room temperature for about 4–5 h (completion of reaction was monitored by TLC). After the completion of reaction the reaction mass was quenched with distilled water and extracted with dichloromethane. Finally the combined organic layer was washed with distilled water again and dried over anhydrous Na_2SO_4. After removal of the solvent in vacuum, the residue was purified by recrystallization.

4.4.1. N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-dibenzenesulfonamide (**5a**)

White solid; Yield 68%; mp. 233–235 °C; Anal. Calc. for $C_{22}H_{17}N_5O_6S_2$: C 51.66, H 3.35, N 13.69%; found: C 51.54, H 3.42, N 13.76%; IR ν_{max} cm^{-1}: 3295 (NH), 3025 (C–H, Ar), 1598 (C=C, Ar), 1322, 1147 (S=O); ^1H NMR ($CDCl_3$) δ(ppm): 7.68 (2H, NH, br s), 7.52 (2H, d, J = 7.8 Hz), 7.36–7.30 (4H, m), 7.02 (1H, d, J = 8.1 Hz), 6.91–6.75 (4H, m), 6.49 (2H, t, J = 7.5 Hz), 5.95 (2H, s, O–CH_2–O); ^{13}C NMR ($CDCl_3$) δ(ppm): 174.8, 171.0 (C=N), 150.2, 149.6, 135.7, 134.4, 130.6, 129.8, 126.3, 115.5, 113.6 (Ar–C), 101.2 (O–CH_2–O); ESI-MS m/z: [M^++1] 512.06.

4.4.2. N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-4-methylbenzene sulfonamide (**5b**)

Yellow solid; Yield 63%; mp. 230–233 °C; Anal. Calc. for $C_{24}H_{21}N_5O_6S_2$: C 53.42, H 3.92, N 12.98%; found: C 53.48, H 3.84, N 13.15%; IR ν_{max} cm^{-1}: 3285 (NH), 3012 (C–H, Ar), 1589 (C=C, Ar), 1329, 1131 (S=O); ^1H NMR ($CDCl_3$) δ(ppm): 8.41 (2H, NH, br s), 7.95 (1H, d, J = 7.8 Hz), 7.58–7.49 (4H, m), 7.10–7.00 (4H, m), 6.95 (1H, d, J = 8.1 Hz), 6.87 (1H, d, J = 8.1 Hz), 6.05 (2H, s, O–CH_2–O), 2.69 (6H, s); ^{13}C NMR ($CDCl_3$) δ(ppm): 176.4, 172.8 (C=N), 150.6, 149.1, 138.2, 130.2, 128.5, 126.3, 124.7, 115.8, 112.4 (Ar–C), 101.2 (O–CH_2–O), 26.8 (CH_3); ESI-MS m/z: [M^++1] 540.08.

4.4.3. N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-4-chlorobenzene sulfonamide (**5c**)

White solid; Yield 67%; mp. 215–218 °C; Anal. Calc. for $C_{22}H_{15}N_5Cl_2O_6S_2$: C 45.52, H 2.60, N 12.07; found: C 45.43, H 2.54, N 12.25%; IR ν_{max} cm^{-1}: 3262 (NH), 3052 (C–H Ar), 1594 (C=C, Ar), 1310, 1115 (S=O), 732 (C–Cl); ^1H NMR ($CDCl_3$) δ(ppm): 8.96 (2H, NH, br s), 8.15–7.89 (4H, m, Ar–H), 7.45–7.28 (4H, m, Ar–H), 7.17 (1H, d, J = 7.8 Hz), 6.72 (1H, d, J = 7.8 Hz), 6.43 (1H, d, J = 8.5 Hz), 6.02 (2H, s, O–CH_2–O); ^{13}C NMR ($CDCl_3$) δ(ppm): 176.8, 172.0 (C=N), 152.5, 148.6, 137.2, 129.1, 128.2, 125.0, 121.7, 118.0, 116.3 (Ar–C), 101.8 (O–CH_2–O); ESI-MS m/z: [M^++1] 580.98.

4.4.4. N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-4-nitrobenzene sulfonamide (**5d**)

White solid; Yield 68%; mp. 205–208 °C; Anal. Calc. for $C_{22}H_{15}N_7O_{10}S_2$: C 43.93, H 2.51, N 16.30%; found: C 44.06, H 2.57, N 16.23%; IR ν_{max} cm^{-1}: 3258 (NH), 3042 (C–H, Ar), 1586 (C=C, Ar), 1545, 1350 (NO_2), 1314, 1127 (S=O); ^1H NMR ($CDCl_3$) δ(ppm): 8.30 (2H, NH, br s), 7.99–7.82 (4H, m, Ar–H), 7.60–7.48 (4H, m, Ar–H), 7.25 (1H, d, J = 7.6 Hz), 6.98 (1H, d, J = 8.2 Hz), 6.20 (1H, d, J = 8.4 Hz), 6.01 (2H, s, O–CH_2–O); ^{13}C NMR ($CDCl_3$) δ(ppm): 178.2, 176.2 (C=N), 152.5, 151.5, 149.4, 148.6, 145.8, 128.2, 124.8, 121.7, 115.6, 112.0 (Ar–C), 100.8 (O–CH_2–O); ESI-MS m/z: [M^++1] 602.05.

4.4.5. N, N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-(2,4-dichlorobenzene sulfonamide) (**5e**)

White solid; Yield 60%; mp. 210–213 °C; Anal. Calc. for $C_{22}H_{13}N_5Cl_4O_6S_2$: C 40.69, H 2.02, N 10.79%; found: C 40.58, H 2.08, N 10.68%; IR ν_{max} cm^{-1}: 3283 (NH), 3015 (C–H, Ar), 1595 (C=C, Ar), 1556, 1343 (NO_2), 1322, 1117 (S=O); ^1H NMR ($CDCl_3$) δ(ppm): 8.38 (2H, NH, br s), 7.98–7.83 (2H, m, Ar–H), 7.70–7.58 (2H, m, Ar–H), 7.30 (2H, s), 6.80 (1H, d, J = 5.4 Hz), 6.65 (1H, d, J = 6.5 Hz), 6.56 (1H, d, J = 5.8 Hz), 5.95 (2H, s, O–CH_2–O); ^{13}C NMR ($CDCl_3$) δ(ppm): 176.8, 174.9 (C=N), 149.4, 148.6, 138.8, 137.8, 132.6, 130.6, 128.2, 124.8, 120.7, 115.4, 112.3 (Ar–C), 101.8 (O–CH_2–O); ESI-MS m/z: [M^++1] 647.86, [M^++2] 648.91.

4.4.6. N,N′-6-(1,3-benzodioxol-5-yl)-(1,3,5-triazine-2,4-diyl)-bis-(4-sopropylbenzenesulfonamide) (**5f**)

White solid; Yield 65%; mp. 233–235 °C; Anal. Calc. for $C_{28}H_{29}N_5Cl_4O_6S_2$: C 56.46, H 4.91, N 11.96%; found: C 56.54, H 5.02, N 11.86%; IR ν_{max} cm^{-1}: 3256 (NH), 3020 (C–H, Ar), 1598 (C=C, Ar), 1533, 1320 (NO_2), 1300, 1125 (S=O); ^1H NMR ($CDCl_3$) δ(ppm): 8.80 (2H, NH, br s), 7.85–7.43 (8H, m, Ar–H), 6.95 (1H, d, J = 5.8 Hz), 6.55 (1H, d, J = 7.5 Hz), 6.20 (1H, d, J = 7.2), 5.95 (2H, s, O–CH_2–O), 3.30–3.10 (2H, m), 1.93 (12H, s, CH_3); ^{13}C NMR ($CDCl_3$) δ(ppm): 179.0, 172.6 (C=N), 150.8, 149.8, 148.4, 138.2, 128.2, 128.0, 124.8, 120.7, 115.6, 112.5 (Ar–C), 101.8 (O–CH_2–O), 38.5, 24.0; ESI-MS m/z: [M^++1] 596.18.

4.5. In vitro antiamoebic assay

All the test compounds (**4a**–**4f** and **5a**–**5f**) and the key intermediates **3a** and **3b** were screened *in vitro* for antiamoebic activity against HM1:IMSS strain of *E. histolytica* by microdilution method [48]. *E. histolytica* trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium [54]. The test compounds (1 mg) were dissolved in DMSO (40 μL, level at which no inhibition of amoeba occurs) [55,56]. The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg mL^{-1}. Two-fold serial dilutions were made in the wells of 96-well microtiter plate (costar). Each test includes metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoebae) and a blank (culture medium only). All the experiments were carried out in triplicate at each concentration level and repeated thrice. The amoeba suspension was prepared from a confluent culture by pouring off the medium and adding 5 mL of fresh medium, chilling the culture tube on ice to detach the organisms from the side of the flask. The number of amoeba mL^{-1} was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The suspension was diluted to 10^5 cells mL^{-1} by adding fresh medium and 170 μL of this suspension was added to the test and control wells in the plate so that the wells were completely filled (total volume, 340 μL). An inoculum of 1.7×10^4 organisms/well was chosen so that confluent, but not excessive growth, took place in control wells. Plates were sealed and gassed for 10 min with nitrogen before incubation at 35.5 °C for 72 h. After incubation, the growth of amoeba in the plate was checked with a low power microscope. The culture medium was removed by inverting the plate and shaking gently. Plate was then immediately washed with sodium

chloride solution (0.9%) at 35.5 °C. This procedure was completed quickly and the plate was not allowed to cool in order to prevent the detachment of amoebae. The plate was allowed to dry at room temperature and the amoebae were fixed with methanol and when dried, stained with (0.5%) aqueous eosin for 15 min. The stained plate was washed once with tap water, then twice with distilled water and allowed to dry. A 200 μL portion of 0.1N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader. The % inhibition of amoebal growth was calculated from the optical densities of the control and test wells and plotted against the logarithm of the dose of the drug tested. Linear regression analysis was used to determine the best fitting line from which the IC_{50} value was found. The IC_{50} values in μM are reported in Tables 1a and 1b.

4.6. Cytotoxicity studies (MTT assay)

4.6.1. Cell culture

Human hepatocellular carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle's medium with 10% foetal bovine serum (heat inactivated), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B, at 37 °C in a saturated humidity atmosphere containing 95% air/5% CO_2 [57]. The cell lines were harvested when they reached 80% confluence to maintain exponential growth.

4.6.2. MTT assay

The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only [58]. For viability testing, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, M2128 from Sigma) cell proliferation assay was carried out. The cell monolayers in exponential growth were harvested using 0.25% trypsin and single-cell suspensions were obtained by repeated pipetting. Only viable cells were used in the assay. Exponentially growing cells were plated at 1.2×10^4 cells per well into 96-well plates (Costar, Corning, NY, USA) and incubated for 48 h before the addition of drugs to achieve the maximum confluency of the cells. Stock solutions were prepared by dissolving the compounds in 10% (v/v) DMSO and further diluted with fresh complete medium to achieve 1 M concentration. Cells were incubated with different concentrations of metronidazole and test compounds for 48 h at 37 °C in 5% CO_2 humidified incubator together with untreated control sample. At appropriate time points, cells were washed in PBS, treated with 50 μL MTT solution (5 mg mL^{-1}, tetrazolium salt) and incubated for 4 h at 37 °C. At the end of the incubation period, the medium was removed and pure DMSO 150 μL was added to each well. The metabolized MTT product dissolved in DMSO was quantified by measuring the absorbance at 570 nm on an Microplate reader (iMark, BIORAD, S/N 10321) with a reference wavelength of 655 nm. All assays were performed in triplicate and repeated thrice. Percent viability was defined as the relative absorbance of treated versus untreated control cells.

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