European Journal of Medicinal Chemistry 45 (2010) 1648–1653

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

New dioxazole derivatives: Synthesis and effects on the growth of *Entamoeba histolytica* and *Giardia intestinalis*

Iram Irfan [a], Nongyao Sawangjaroen [b], Abdul R. Bhat [a], Amir Azam [a,*]

[a] Department of Chemistry, Jamia Millia Islamia, Jamia Nagar, New Delhi-110025, India
[b] Natural Products Research Center and Department of Microbiology, Faculty of Science, Prince of Songkla University, 90112 Thailand

ARTICLE INFO

Article history:
Received 22 January 2009
Received in revised form
19 December 2009
Accepted 23 December 2009
Available online 13 January 2010

Keywords:
Dioxazoles derivatives
G. intestinalis
E. histolytica
Toxicity studies

ABSTRACT

Cyclization of oxime with different aldehydes and ketones under basic condition led to the formation of new dioxazole derivatives and the structure was elucidated by spectral data. The effects of diaoxazoles on the inhibition of growth of *Entamoeba histolytica* and *Giardia intestinalis in vitro* have been determined, and selected compounds further investigated for their toxicity. SAR showed that the compounds with 5-nitrothiophene group at the 3-postion of the diaoxazole ring were more active than those with the *p*-toluene group at the same position. It is interesting to note that the compounds found active against *E. histolytica* were not found active against *G. intestinalis*. Toxicity studies showed that the compound **8** and **9** were non-toxic against Vero cell line ATCC CCL-81.

© 2010 Elsevier Masson SAS. All rights reserved.

1. Introduction

Intestinal parasitic infections are among the major public health problems in the developing countries [1]. Especially amoebiasis (*Entamoeba histolytica*) and giardiasis (*Giardia intestinalis*) have high morbidity and mortality indexes due to the effects of severe diarrhea and invasive infections. *E. histolytica* causes approximately 50 million cases of infection resulting approximately 100,000 deaths annually [2–5]. *G. intestinalis* is the most prevalent protozoan parasite worldwide with about 200 million people being currently infected [6,7]. Metronidazole is the drug of choice for the treatment of amoebiasis and giardiasis. Recent studies have shown that this drug have several toxic effects such as genotoxicity, gastric mucus irritation, and spermatozoid damage [8,9]. Furthermore, failures in the treatment of several intestinal protozoan parasites may result from drug resistant to parasites [10,11]. Considering these problems, there is a pressing need for new effective drug to treat these infections.

The study of Azoles has become of much interest on account of their diverse biological properties. Azoles as antifungal agents prevent synthesis of ergosterol, a major component of fungal membranes, by inhibiting the cytochrome P-450-dependent enzymes 14-α lanosterol demethylase [12]. The oxazole nucleus is present in wide variety of natural and unnatural biologically active compounds and is useful reagent in the synthesis of a range of biologically active scaffold [13–15]. Isoxazole had been screened for antiviral agents because of their good tolerability and potent antiviral activity [16]. As part of our search for basic information about the requirements for antiprotozoal activity [17], we have synthesized 3,5-Substituted-1,4,2-dioxazole derivatives (**1**–**10**). The *in vitro* antiparasitic activities of these compounds on two intestinal protozoa (*E. histolytica* and *G. intestinalis*) were screened to develop an effective drug than metronidazole, a member of azole family.

2. Chemistry

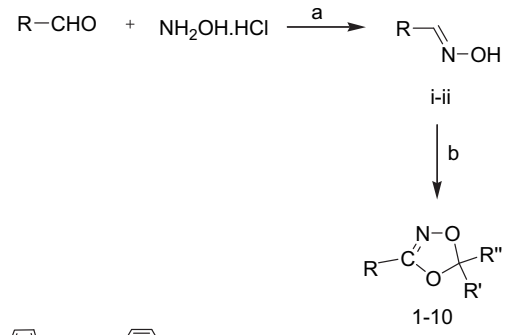
The synthesis of the dioxazole derivatives (**1**–**10**) was performed in a manner as out lined in Scheme 1 [18]. The reaction of aldo-oxime in the presence of sodium hypochlorite and triethylamine in dichloromethane gave the dioxazole deriatives. All the compounds showed sharp melting points and the elemental analysis was found in accordance with ±0.3%. These compounds were stable in the solid state. The compounds are soluble in DMSO, methanol and ethanol.

3. Pharmacology

All the dioxazole derivatives (**1**–**10**) were screened *in vitro* against *HM1:IMSS* strain of *E. histolytica* by microdilution method [19] and *G. intestinalis* on local Thai strain as described previously [20]. All the experiments were carried out in triplicate at each

* Corresponding author. Tel.: +91 11 26981717/3250; fax: +91 11 26980229.
E-mail address: amir_sumbul@yahoo.co.in (A. Azam).

0223-5234/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2009.12.051



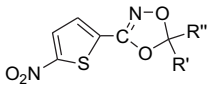
Scheme 1. General method for the synthesis of dioxazole derivatives.

concentration level and repeated thrice. Toxicity of active compounds have been studied against the Vero cell line ATCC CCL-81 (kidney fibroblast of an African green monkey) [21]. The observed data of biological activities of the compounds and the control drug are given in Table 1.

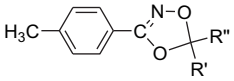
4. Result and discussion

A series of 10 new compounds were synthesized. Scheme 1 illustrated the way used for the preparation of target compounds. The structure of the dioxazole derivatives (**1**–**10**) was elucidated by

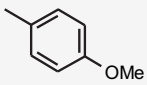
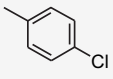
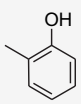
Table 1
In vitro activity of Dioxazole derivatives against *E. histolytica*, *G. intestinalis* and cytotoxicity profile.



(**1-5**)

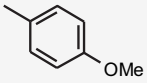
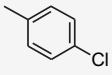


(**6-10**)

Comp. No.	R′	R″	Antiamoebic		Antigiardia	Toxicity profile	Safety Index
			IC_{50} (μM)[a]	S.D.[b]	MIC(μM)[a]	IC_{50} (μM)	
1.	H		9.97	0.19	5.65	<180	nd[c]
2.	H		2.64	0.01	5.33	<180	nd[c]
3.	H		1.60	0.08	20.40	<180	nd[c]
4.	CH_3		3.77	0.03	10.78	<180	nd[c]
5.	CH_3		6.10	0.03	4.55	<180	nd[c]
6.	H		7.82	0.13	>1500	nd[c]	nd[c]
7.	H		8.61	0.07	>1500	nd[c]	nd[c]

(*continued on next page*)

Table 1 (*continued*)

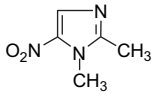
Comp. No.	R′	R″	Antiamoebic		Antigiardia	Toxicity profile	Safety Index
			IC_{50} (μM)[a]	S.D.[b]	MIC(μM)[a]	IC_{50} (μM)	
8.	H		1.80	0.02	>1500	>180	>100
9.	CH_3		1.80	0.01	>1500	>180	>100
10.	CH_3		7.30	0.03	>1500	nd[c]	nd[c]
(MNZ)	H		1.80	0.04	14.61	nd[c]	nd[c]

[a] The values obtained in at least three separate assays done in triplicate.
[b] Standard deviation.
[c] nd not determined.

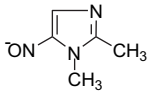
IR, ^{1}H NMR, ^{13}C NMR, electronic spectra and was further supported by FAB MAS.

All the compounds were evaluated for their antiamoebic and anti-giardial properties. The oxime of 5-nitrothiophenecarboxaldehyde and *p*-tolualdehyde (**i**–**ii**) were found inactive on both strains. The oxime of 5-nitrothiophenecarboxaldehyde (**i**) and *p*-tolualdehyde (**ii**) were found inactive (**i**, IC_{50} = 10.28 μM, **ii**, IC_{50} = 2.57 μM) against *E. histolytica* and *G. intestinalis* (**i**, MIC = 48.44 μM, **ii**, MIC = >1500 μM). Two classes of 3,5 subsituted dioxazoles were synthesized and their structural activity relationship (SAR) established on the basis of substituted molecules at position-3 and 5 of the dioxazole ring. The dioxazole derivatives with substituted and unsubstituted phenyl ring attached at carbon-5 of the dioxazole ring showed IC_{50} for antiamoebic activity in the range of 1.80–9.97 μM and MIC for anti-giardial in the range of 4.55–≥15 μM. The compound **3**(IC_{50} = 1.60 μM), in this series showed better antiamoebic activity, vs. IC_{50} value of MNZ (IC_{50} = 1.80 μM), but the compounds **8**(IC_{50} = 1.80 μM) and **9**(IC_{50} = 1.80 μM) showed as active as metronidazole. The compounds **3** and **8** showed activity due to the presence of methoxy substituted phenyl ring at the posistion-5 of the dioxazole ring, but **3** showed better activity because of the presence on nitro group in dioxazole ring [22]. With respect to the activity against *G. intestinalis* four compounds were found more active than metronidazole. Compound **1** (MIC = 5.65 μM), **2** (MIC = 9.45 μM), **4** (MIC = 10.78 μM) and **5** (MIC = 4.55 μM) vs. metronidazole (MIC = 14.61 μM) with a 5-nitrothiophene group at position-3 of the dioxazole ring. The activity of these compounds (**1**, **2**, **4** and **5**) may be due to nitro group at position-5 of the thiophene ring as in the case of metronidazole, the activity is due to the nitro group present at position-5 of the imidazole ring, indicating that toluene group at 3-position drastically decrease the activity against this parasite [22]. Therefore, it is concluded that good anti-giardial activity is limited to those compounds in which 5-nitothiophene group is attached at 3-position of the dioxazole ring. It was further interesting to note that compound found active against G. *intestinalis* did not show activity against *E. histolytica*. The null hypothesis was tested using *t*-test and the significance of the difference between the IC_{50} value of metronidazole vs. **3** and the MIC value of metronidazole vs. **1**, **2**, **4** and **5** was evaluted. The values of the calculated *t* were found higher than the table value of *t* at 4% level, thus concluding that the character under study is said to be influenced by the treatment [23].

The compounds with less (IC_{50}) value than metronidazole were evaluated for their toxicity against Vero cell line ATCC CCL-81 (kidney fibroblast of an African green monkey) and the results are shown in Table 1. It was found that only compounds **8** and **9** are non cytotoxic ($IC_{50} \geq 180$ μM). The safety index: Toxicity IC_{50}/Protozoal IC_{50} was therefore calculated for compounds **8** and **9**. It is remarkable that all compounds that were active against *G. intestinalis* were toxic to normal cell line ($IC_{50} \leq 180$ μM). This is because that the nitro group of 5-nitrothiophene converted into it's reduced form to nitroso group and this reduced form is toxic as it was in the case of metronidazole [22].



Metronidazole



Nitroso free radical

Based on this experiment, compounds **8** and **9** may consider to be the greatest potential for future use as an antiamoebic drug, since they were non cytotoxic and their IC_{50} was similar to that of metronidazole. Further study to pursue more experiments trying to find potential compounds for use as therapeutic agents against both *E. histolytica* and *G. intestinalis* infections may carry out. The numerical results of each compound are given in Table 1.

5. Conclusion

The antiamoebic and anti-giardial activities of new dioxazole derivatives having different aldehyde and ketone substitutions were examined. The biological behavior revealed that the compounds containing nitrothiophene ring are more active than the compounds containing simple toluene ring. Moreover, the compounds **1**, **2**, **4** and **5** were found to be more active than the reference drug, metronidazole against *G. intestinalis in vitro*. The

compound **3**, **8** and **9** showed significant less/at par IC_{50} value than metronidazole against HM1:1MSS strain of *E. histolytica*. Toxicity assay showed that compound **8** and **9** are non-toxic against Vero cell line ATCC CCL-81.

6. Experimental protocols

Reaction was monitored on Merck pre-coated aluminum plate silica gel $^{60}F_{254}$ thin layer plates. All chemicals were purchased from Sigma–Aldrich Chemical Company (USA). Melting points were recorded on KSW melting point apparatus. Elemental analysis (C, H, N) was carried out on Heraeus Vario EL III analyzer by Central Drug Research Instituted, Lucknow, India and the results were within ±0.3 of the theoretical values. The electronic spectra were recorded in DMSO on a Shimadzu UV-1601 PC UV–Visible spectrophotometer. IR spectra on KBR disks were recorded on a Perkin Elmer model 1620 FT-IR spectrophotometer. ^{1}H NMR spectra were obtained at ambient temperature using a Brucker spectroscopic DPX-300 MHZ spectrometer in DMSO using Tetramethylsilane (TMS) as an internal standard. Splitting pattern are designated as; s, singlet; d, doublet; m, multiplet, coupling constant *J* is given in hertz. Chemical shift value is given in (ppm). The FAB mass spectra of the compounds were recorded on JEOL SX 102/DA-6000 mass spectrometer using Argon/Xenon 6 kV, 10 mA as the FAB gas and *m*-nitro benzyl alcohol (NBA) was used as the matrix.

6.1. Chemistry

6.1.1. General procedure for the synthesis of oxime ***i*** and ***ii***

A solution of 5-nitro–2-thiophenecarboxyldehyde or *p*-tolualdehyde (1 equiv.) and hydroxylamine hydrochloride (1.08 equiv.) in a solution of ethanol and pyridine (2:1) was refluxed with stirring for 24 h. After cooling the mixture was concentrated and then poured into 600 mL of ice water. The precipitated solid was collected and recrystallized from methanol, which gave corresponding oxime.

*6.1.1.1. 5-Nitrohiophene-2-carbaldehyde oxime (**i**): Yellow solid (dichloromethane).* Yield 85%, Mp. 130 °C, Anal ($C_5H_4N_2O_3S$) calc. C 34.87, H 2.26, N 16.21, found C 34.88, H 2.34, N 16.27; IR: ν_{max}(cm^{-1}): 1624.31 (C═N), 2817 (C–H), 1500 (NO_2); ^{1}H NMR($CDCl_3$) (δ, ppm)11.28 (s, 1H, N–OH), 6.81 (s, 1H, CH═N), 7.80(d, 1H, 2.91 Hz, Ar-H), 7.70(d, 1H, 2.91 Hz, Ar-H).

*6.1.1.2. 4-Methylbenzaldehyde oxime (**ii**): White solid (dichloromethane).* Yield 76%, Mp. 80 °C, Anal (C_8H_9NO) calc. C 71.06, H 6.62, N 10.41, found C 71.09, H 6.71, N 10.36; ^{1}H NMR ($CDCl_3$) (δ, ppm) 11.31 (s, 1H, N–OH), 7.40 (s, 1H, CH═N), 2.40 (s, 3H, CH_3), 7.94 (d, 2H, J = 2.60 Hz, Ar-H), 7.98 (d, 2H, J = 2.60 Hz, Ar-H).

6.1.2. General procedure for the preparation of 3, 5-substituted-1,4,2-dioxazole (***1***–***10***)

The 4% aqueous solution of NaOCl (1.6 equiv.) was added to a solution of dipolarophile (respective aldehydes and ketones, 1 equiv.) and triethylamine (0.1 equiv.) in dichloromethane under argon atmosphere. The oxime (1 equiv.) in dichloromethane was added drop wise (over a period of 1 h) at 0 °C, to an above solution. After stirred at room temperature, water was added to the reaction mixture and the aqueous layer was extracted with dichloromethane. The combined organic layer were washed with water, brine, dried over ($MgSO_4$), filtered and concentrated under *vacuo*. The compounds were recrystallized using dichloromethane/hexane solution.

*6.1.2.1. 3-(5-Nitro-2-thienyl)-5-phenyl-1,4,2-dioxazole (**1**): Black solid(DMSO).* Yield 52%, Mp. 276 °C, Anal ($C_{12}H_8N_2O_4S$) calc. C 52.17, H 2.80, N 10.10, found C 52.19, H 2.72, N 10.00; UV:λ_{max} (nm) 311, 318, 290. IR: ν_{max}(cm^{-1}): 1656.41 (C═N), 2879 (CH), 1167 (C–O–C); ^{1}H NMR (DMSO-d_6), (δ, ppm) 5.94 (s, 1H, CH), 7.76 (d, 1H, J = 2.92 Hz, Ar-H), 8.10 (d, 1H, J = 2.92 Hz, Ar-H), 7.72–8.12 (m, 5H, Ar-H); ^{13}C NMR (DMSO-d_6): (δ, ppm) 159.73 (C═N), 116.80 (O–C–O), 152, 127, 128, 138, 141, 127, 129 (Ar-C); FAB MAS: m/z (M^++1)277.13.

*6.1.2.2. 2-[3-(5-nitro-2-thienyl)-1,4,2dioxazol-5-yl]phenol (**2**): Brown solid (methanol:DMSO).* Yield 40%, Mp. 165 °C, Anal ($C_{12}H_8N_2O_5S$) calc. C 49.31, H 2.76, N 10.14, found C 49.32, H 2.76, N 10.14; UV:λ_{max} (nm) 253, 299, 367; IR: ν_{max}(cm^{-1}): 1611 (C═N), 2845 (CH), 1191 (C–O–C); ^{1}H NMR (DMSO-d_6), (δ, ppm) 5.91 (s, 1H, CH), 7.83 (d, 1H, J = 2.70 Hz, Ar-H), 8.10 (d, 1H, J = 2.70 Hz, Ar-H), 8.40–7.71 (m, 4H, Ar-H). ^{13}C NMR (DMSO-d_6): (δ, ppm) 161.72 (C═N), 113.60 (O–C–O), 151, 121, 128.81, 136, 142.21, 128.60, 121.62, 129.11 (Ar-C), 158 (C–OH); FAB MAS: m/z (M^+ + 1) 293.64.

*6.1.2.3. 5-(4-Methoxy-phenyl)-3-(5-nitro-2-thienyl)-1,4,2-dioxazole (**3**): Brown solid (DMSO).* Yield 45%; Mp. 93 °C; Anal ($C_{12}H_8N_2O_4S$) calc. C 50.90, H 3.26, N 9.15, found C 50.81, H 3.27, N 9.16; UV:Vis λ_{max}(nm): 321, 297, 250; IR: ν_{max}(cm^{-1}): 1675 (C═N), 2870 (CH), 1153 (C–O–C); ^{1}H NMR (DMSO-d_6), (δ, ppm) 5.89 (s, 1H, CH), 8.16 (d, 1H, J = 2.91 Hz, Ar-H), 7.90 (d, 1H, J = 2.90 Hz, Ar-H), 8.12 (d, 2H, J = 6.60 Hz, Ar-H), 8.51 (d, 2H, J = 6.60 Hz, Ar-H), 2.42 (s, 3H, CH_3); ^{13}C NMR(DMSO-d_6) (δ, ppm) 163.23 (C═N), 112.90 (O–C–O) 151, 129, 128, 135, 133, 128, 114, 159.62 (Ar-C), 27.7 (CH_3); FAB MAS: m/z (M^+ + 1) 307.29.

*6.1.2.4. 5-Methyl-3-(5-nitro-2-thienyl)-5-phenyl-[1,4,2]dioxazole (**4**): Black solid (DMSO).* Yield 43%; Mp. 120 °C; Anal ($C_{12}H_8N_2O_4S$) calc. C 53.79, H 3.47, N 9.65, found C 53.78, H 3.47, N 9.66; UV:Vis λ (nm): 337, 296, 261; IR:ν_{max}(cm^{-1}): 1678 (C═N), 2973 (CH_3), 1176 (C–O–C); ^{1}H NMR(DMSO-d_6), (δ, ppm) 7.91 (d, 1H, J 42.51 Hz, Ar-H), 8.12 (d, 1H, J = 2.51 Hz, Ar-H), 2.72 (s, 3H, CH_3), 7.91–8.20 (m, 5H, Ar-H); ^{13}C NMR(DMSO-d_6), (δ, ppm), 159.31 (C═N), 115.74 (O–C–O), 25 (CH_3), 154, 124, 127.80, 134, 141, 127.21, 129, 127 (Ar-C); FAB MAS: m/z (M^++1) 291.11.

*6.1.2.5. 5-(4-Chloro phenyl)-5-methyl-3-(5-nitro-2-thienyl)-1,4,2-dioxazole (**5**): Brown solid (methanol:DMSO).* Yield 39%; Mp. 150 °C; Anal ($C_{13}H_9ClN_2O_4S$) calc. C 48.08, H 2.79, N 8.63, found C 48.09, H 2.77, N 8.64; UV:Vis λ (nm): 317, 274, 318; IR:ν_{max}(cm^{-1}): 1693 (C═N), 3816 (Ar-C–H), 2361 (CH_3), 1191 (C–O–C); ^{1}H NMR (DMSO-d_6), (δ, ppm) 2.51 (s, 1H, CH_3), 7.74 (d, 1H, J = 2.81 Hz, Ar-H), 8.11 (d, 1H, J = 2.81 Hz, Ar-H),7.58 (d, 2H, J = 6.42 Hz, Ar-H), 7.90 (d, 2H, J = 6.42 Hz, Ar-H); ^{13}C NMR(DMS-d_6), (δ, ppm) 160.2 (C═N), 111.92 (O–C–O), 28.13 (CH_3), 151, 126, 128, 135, 139, 128, 129 (Ar-C); FAB MAS: m/z (M^+ + 1) 325.82.

*6.1.2.6. 3-(4-methylphenyl)-5-phenyl-1,4,2-dioxazole (**6**): Light pink (methanol:DMSO).* Yield 33%; Mp. 127 °C; Anal ($C_{15}H_{13}NO_2$) calc. C 75.31, H 5.43, N 5.85, found C 75.32, H 5.44, N 5.87; UV/Vis λ (nm): 315, 291, 240; IR: ν_{max}(cm^{-1}): 1611 (C═N), 3046 (Ar-C–H), 2861 (CH), 1178 (C–O–C); ^{1}H NMR(DMSO-d_6), (δ, ppm) 2.21 (s, 3H, CH_3), 5.90 (s, 1H, CH), 7.53 (d, 2H, J = 6.74 Hz, Ar-H), 7.52 (d, 2H, J = 6.74 Hz, Ar-H), 7.91–8.21 (m, 5H, Ar-H); ^{13}C NMR(DMSO-d_6), (δ, ppm) 161.71(C═N), 116.14 (O–C–O), 21.92 (CH_3), 140, 127, 129, 126, 141, 127.20, 129, 127 (Ar-C); FAB MAS: m/z (M^+ + 1) 239.27.

*6.1.2.7. 2-3(4-methylphenyl)-1,4,2-dioxazole-5-yl-phenol (**7**): Light yellow (DMSO).* Yield 46; Mp. 93 °C; Anal ($C_{15}H_{13}NO_3$) found C 70.59, H 5.09, N 5.47 calc. C 70.58, H 5.09, N 5.49; UV/Vis λ (nm):

310, 285, 312; IR:ν_{max}(cm^{-1}): 1670 (C═N), 3166 (Ar-C–H), 2862 (CH), 1183 (C–O–C); ^{1}H NMR(DMSO-d_6), (δ, ppm) 2.41 (s, 3H, CH_3), 6.19 (s, 1H, CH), 7.50 (d, 1H, J = 6.90 Hz, Ar-H), 7.71 (d, 1H, J = 6.90 Hz, Ar-H),7.40–8.31(m, 1H, Ar-H); ^{13}C NMR(DMSO-d_6), (δ, ppm) 159.95 (C═N), 112.19 (O–C–O) 26.11, (CH_3), 140, 129.21, 125, 126.90, 122, 128.6, 116.11, 121.65, 129.11 (Ar-C); FAB MAS: m/z (M^+ + 1) 256.93.

*6.1.2.8. 5-(4-Methoxy-phenyl)-3-(4-methyl phenyl)-1,4,2-dioxazole (**8**): Pink solid (DMSO).* Yield 40%, Mp. 110 °C, Anal ($C_{16}H_{16}NO_3$) calc. C 71.11, H 5.92, N 7.03, found C 71.11, H 5.91, N 7.02; UV/Vis λ (nm): 317, 270; IR: ν_{max}(cm^{-1}): 1667 (C═N), 3029 (Ar-C–H), 2849 (CH), 1158 (O–C–O); ^{1}H NMR(DMSO-d_6), (δ, ppm) 6.19 (s, 1H, CH), 7.31 (d, 2H, J = 6.91 Hz, Ar-H), 7.39 (d, 2H, J = 6.91 Hz, Ar-H), 2.33 (s, 1H, CH_3), 7.41(d, 2H, J = 6.62 Hz, Ar-H), 7.45 (d, 2H, J = 6.62 Hz, Ar-H), 6.56 (s, 1H, CH_3); ^{13}C NMR (DMSO-d_6), (δ, ppm) 163.29 (C═N), 115.31 (O–C–O), 24.63 (CH_3), 140, 129.21, 129, 126.90, 141, 127.21, 129 (Ar-C); FAB MAS: m/z (M^++1)271.73.

*6.1.2.9. 5-Methyl-3-(4-methly phenyl)-5-phenyl-1,4,2-dioxazole (**9**): Creamy white solid (DMSO).* Yield 37%; Mp. 115 °C; Anal ($C_{16}H_{15}NO_2$) calc. C 75.8, H 5.81, N 5.53, found C75.90, H 5.82, N 5.52; IR:ν_{max}(cm^{-1}): 1669 (C═N), 3163 (Ar-C–H), 1187 (O–C–O), 839.31, 2861 (CH_3), 2981 (CH_3); ^{1}H NMR (DMSO-d_6), (δ, ppm) 2.34 (s, 1H, CH_3), 7.2 (d, 1H, J = 6.81 Hz, Ar-H), 7.75 (d, 1H, J = 6.81 Hz, Ar-H), 7.92–8.21 (m, 5H, Ar-H); ^{13}C NMR (DMSO-d_6), (δ, ppm) 162.13 (C═N), 118.71 (O–C–O), 27.81 (CH_3), 26.90 (CH_3), 143,129.28, 140.75, 126.9, 141.12, 127.22, 129, 127.75 (Ar-C); FAB MAS: m/z (M^+ + 1)254.11.

*6.1.2.10. 5-(4-Chloro-phenyl)-5-methyl-3-(4-methyl)-1,4,2-dioxazole (**10**): Creamy white solid (DMSO).* Yield 31%; Mp. 130 °C; Anal ($C_{16}H_{14}NO_2Cl$) calc. C 66.80, H 4.90, N 4.87, found C 66.80, H 4.88, N 4.86; UV/Vis λ (nm): 355, 318, 297; IR:ν_{max}(cm^{-1}): 1653 (C═N), 3046 (Ar-C–H), 2982 (CH_3), 1189 (O–C–O); ^{1}H NMR(DMSO-d_6), (δ, ppm) 2.46 (s, 3H, CH_3), 7.64 (d, 2H, J = 6.70 Hz, Ar-H), 7.81 (d, 2H, J = 6.70 Hz, Ar-H), 2.27 (s, 3H, CH_3), 8.11 (d, 2H, J = 6.44 Hz, Ar-H), 8.36 (d, 2H, J = 2.44 Hz, Ar-H); ^{13}C NMR (DMSO-d_6) (δ, ppm) 160.3 (C═N),1 11.90 (O–C–O), 28.91 (CH_3), 25.64 (CH_3), 140.40, 129, 124.96, 139.21, 128.63, 129.11, 133.22 (Ar-C); FAB MAS: m/z (M^+ + 1) 288.18.

6.2. Antiprotozoal activity

6.2.1. In vitro testing against G. intestinalis

The anti-giardial activity of the compounds **i–ii** and **1–10** were performed according to the standard methods as described elsewhere [20]. Briefly, trophozoites were harvested by chilling the tube on ice for 15 min to detach the monolayer and centrifuged at 300*g* for 5 min. The supernatant was decanted and cells were re-suspended in fresh medium. The numbers of viable cells were calculated using a haemocytometer and 0.4% (w/v) trypan blue. The criteria used for viability were motility and dye exclusion. *G. intestinalis*, at a density of 2 × 10^5 trophozoites per mL of culture medium, were incubated in 96-well tissue culture plates (200 μl/well) in the presence of two fold serial dilutions of compound, with maximum concentration of 1500 μM. Metronidazole with maximum final concentration of 116.88 μM and complete medium with added DMSO were used as negative and positive controls respectively. After 24 h of incubation at 37 °C under anaerobic conditions, the trophozoites from each well were examined and counted using an inverted microscope. The appearance and numbers of trophozoites were scored from 1 to 4 with 1 showing the most inhibition of growth and 4 showing no inhibition according to Upcroft et al. [24] and the minimum inhibitory concentration (MIC) was recorded (the lowest concentration at which >90% of the trophozoites rounded up). Each concentration was tested in duplicate and at least three experiments were performed on separate occasions.

6.2.2. In vitro testing against E. histolytica

All the dioxazole compounds (**1–10**) were screened *in vitro* for antiamoebic activity against *HM1:1MSS* strain of *E. histolytica* by using a microplate method [19]. *E. histolytica* trophozoites were cultured in TYIS-33 growth medium in wells of 96 microtiter plate [25]. DMSO (40 μL) was added to all the samples (1 mg) followed by enough culture medium to obtain concentration of 1 mg/mL. The maximum concentration of DMSO in the test did not exceed 0.1%, at which level no inhibition of amoebal growth occurred [26,27]. Sample were dissolved or suspended by mild sonication in a soniclearner bath for few minutes and then further dilution with medium to concentration of 0.1 mg/mL. Two fold serial dilutions were made in the wells of 96-well microtiter plate (Costar) in 170 μL of the medium. Each test included metronidazole as the standard amoebicidal drug, control wells (culture medium plus amoebae) was prepared from a confluent culture by pouring off the medium, adding 2 mL of medium and chilling the culture on ice to detach the organisms from the side of the flask. The number of the amoeba per mL was estimated with a heamocytometer and trypan blue exclusion was used to confirm viability. The cell suspension used was diluted to 10^5 organism/mL by adding fresh medium and 170 μl of this suspension was added to the test and control well 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. Plate was sealed with expended polystyrene (0.5 thick). Secured with tape, placed in a modular incubating chamber (flow laboratories, High Wycombe, UK), and gassed for 10 min with nitrogen before incubation at 37 °C for 72 h.

6.2.2.1. Assessment of antiamoebic activity. After incubation, the growth of amoebae in the plate was checked with a low power microscope. The culture medium was removed by inverting the plate and shaking gently. The plate was then immediately washed once in sodium chloride solution (0.9%) at 37 °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 the room temperature, and the amoebae was fixed with methanol, when dry, stained with (0.5%) aqueous eosin for 15 min. Stained plate was washed once with tap water and then twice with distilled water and allowed to dry. A 200 μL portion of 0.1 N sodium hydroxide solution was added in 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-fitted straight line from which the IC_{50} value was found.

6.3. Toxicity assay

The cytotoxicity of the active compounds **1–5** and **8–9** were determined calorimetrically as modified against a normal cell lines (Vero cell lines) [27]. The test samples (maximum final concentration 180 μM) were put into wells of microtiter plate together with 10^5 cell/mL of Vero cell line ATCC CCL-81 (kidney fibroblast of an African green monkey), that was cultured in Eagle's minimum essential medium with the addition of 10%/mL heat-inactivation fetal bovine serum and 1 mM sodium pyruvate. The positive control

was an antineoplastic agent, ellipticine (IC_{50} of 2.5 μM). DMSO (0.05%) was used as negative control. After incubation at 37 °C for 72 h in a 5% CO_2 incubator, the viability of the Vero cell was determined by the sulphorhodamine B (SRB) assay [21]. It was define as being cytotoxic when an $IC_{50} \geq 180$ μM.

Acknowledgements

The authors are thankful to Prof. Alok Bhattacharya and Prof Sudha Bhattacharya, School of Environmental Science, Jawaharlal Nehru University, New Delhi, respectively for providing Laboratory facilities for antiamoebic activities.

References

[1] V. Mehraj, J. Hatcher, S. Akther, G. Rafique, M.A. Beg, PLoS Biol. 3 (2008) e3680.
[2] World Health Organization, Wkly. Epidemiol. Rec. 72 (1997) 97–99.
[3] D. Leitsch, D. Kolarich, I.B.H. Wilson, F. Altmann, M. Duchene, PLoS Biol. 5 (2007) 1820–1834.
[4] A. Caballero-Salcedo, M. Viverose-Rogel, B. Salvatierra, R. Tapia-Conyer, J. Sepulveda-Amor, G. Gutierrez, L. Ortiz-Ortiz, Am. J. Trop. Med. Hyg. 50 (1994) 412–419.
[5] L.A. Moraes, Estudo clinico de 104 casos de abscesso hepatico amebiano do figado no HJBB. Rev. Bras. Cir. 227 (1999) 54–60.
[6] D.R. Pillai, K.C. Kain, Curr. Treat. Options Infect. Dis. 5 (2003) 207–217.
[7] T. Minenoa, M.A. Avery, Curr. Pharm. 9 (2003) 841–855.
[8] F.A. el-Nahas, M.I. el-Ashmawy, Basic Clin. Pharmacol. Toxicol. 94 (2004) 226–231.
[9] V. Purohit, K.A. Basu, Chem. Res. Toxicol. 13 (2000) 673–692.
[10] P. Abboud, V. Lemee, G. Gargala, P. Brasseur, J.J. Ballet, F. Borsa-Lebas, F. Caron, L. Favennec, Clin. Infect. Dis. 32 (2001) 1792–1794.
[11] W. Petri, Trends Parasitol. 19 (2003) 523–526.
[12] K. Asai, N. Tsuchimori, K. Okonogi, J.R. Perfect, O. Gotoh, Y. Yoshida, Antimicrob. Agents Chemother. 43 (1999) 1163–1169.
[13] Z. Jin, Nat. Prod. Rep. 20 (2003) 584–605.
[14] J.J. Talley, S.R. Bertenshaw, D.L. Brown, J.S. Carter, M.J. Graneto, C.M. Koboldt, J.L. Masferrer, B.H. Norman, D.J. Rogier, B.S. Zweifel, K. Seibert, Med. Res. Rev. 19 (1999) 199–208.
[15] P. Wipf, C.P. Miller, J. Org. Chem. 58 (1993) 3604–3606.
[16] V.A. Makarov, O.B. Riabova, V.G. Granik, P. Wutzler, M.J. Schmidtke, J. Antimicrob. Chemother. 55 (2005) 483–488.
[17] A.R. Bhat, F. Athar, A. Azam, Eur. J. Med. Chem. (2008) Mar 4. [Epub ahead of print].
[18] Julia Kaffy, Renee Pontikis, Daniele Carrez, Alain Croisy, Claude Monneret, Jean-Claude Florent, Bioorg. Med. Chem. 14 (2006) 4067–4077.
[19] C.W. Wright, M.J.O' neill, J.D. Phillipson, D.C. Warhurst, Antimicrob. Agents Chemother. 32 (1988) 1725–1729.
[20] N. Sawangjaroen, S. Subhadhirasakul, S. Phongpaichit, C. Siripanth, K. Jamjaroen, K. Sawangjaroen, Parasitol. Res. 95 (2005) 17–21.
[21] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kanney, M.R. Boyd, J. Natl. Cancer Inst. 82 (1990) 1107–1112.
[22] R. Knight, J. Antimicrob. Chemother. 6 (1980) 577–593.
[23] B.J. Winer, Statistical Principals in Experimental Design, second ed., vol. 22, McGraw-Hill Book Company, 1971, p. 45.
[24] J.A. Upcroft, P. Upcroft, Antimicrob. Agents Chemother. 45 (2001) 1810–1814.
[25] L.S. Diamond, D.R. Harlow, C.C. Cunnick, Trans. R. Soc. Trop. Med. Hyg. 72 (1978) 431–432.
[26] F.D. Gillin, D.S. Reiner, M. Suffnes, Antimicrob. Agents Chemother. 22 (1982) 342–345.
[27] A.T. Keen, A. Harris, J.D. Phillipson, D.C. Warhurst, Planta Med. (1986) 278–284.