Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 44 (2009) 426–431
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Short communication

Bis-pyrazolines: Synthesis, characterization and antiamoebic activity as inhibitors of growth of *Entamoeba histolytica*

Abdul R. Bhat, Fareeda Athar[1], Amir Azam*

Department of Chemistry, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025, India

Received 18 June 2007; received in revised form 30 October 2007; accepted 12 November 2007
Available online 19 November 2007

Abstract

The cyclization of chalcone with N-4 substituted thiosemicarbazides under basic condition led to the formation of new compounds, thiocarbamoyl bis-pyrazoline derivatives. The structure of the compounds were elucidated by UV, IR, ^{1}H NMR, ^{13}C NMR and ESI-MS spectral data and thermogravimetric analysis, and their purities were confirmed by elemental analyses. The antiamoebic activity of these complexes was evaluated by microdilution method against *HM1:IMSS* strain of *Entamoeba histolytica* and the results were compared with the standard drug, metronidazole. Structure–activity relationship shows that the compound with aromatic substituents at the thiocarbamoyl group was more active than those with the cyclic groups. However, it was clear from the IC_{50} values that the compounds **15** and **20** are more active and both showed a structural resemblance having an electron withdrawing groups attached to the phenyl ring. MTT assay showed that all the compounds are non-toxic to human kidney epithelial cell line.

Keywords: Bischalcone; Bis-Pyrazolines; Thiocarbamoyl; Antiamoebic activity

1. Introduction

Amoebiasis is the second leading cause of death from parasitic disease worldwide [1]. The causative amoeboid eukaryote parasite, *Entamoeba histolytica*, is a potent pathogen. *E. histolytica* causes approximately 50 million cases and approximately 100,000 deaths annually [2,3]. This parasite acts as a macrophage on steroids with pumped-up phagocytic, proteolytic, and cytolytic capabilities, invading human colonic mucosa, and occasionally penetrating through to the portal circulation, reaching the liver and causing fatal liver abscesses [4]. Brain abscess is the dreadful complication of this disease [5]. The cornerstone of treatment for amoebiasis remains the nitroimidazole derivatives (metronidazole, tinidazole, ornidazole). Metronidazole is the mainstay and used in combination with other drugs such as iodoquinol, paromomycin and diloxanide furoate [6]. Tissue amoebicides such as metronidazole and tinidazole kill amoeba in host tissue and organ whereas iodoquinol and paromomycin are active only in the intestinal lumen [7]. Despite these, there is lack of ideal drug, and immunity acquired to already available drugs and the side effects are a major hurdle in eradicating these diseases [8–11].

Five-membered heterocyclic compounds natural as well as synthetic are important for their biological activities. Compounds with pyrazole ring are of interest due to their broad spectrum of biological activities against monoamine oxidase inhibitor [12], bacterial [13], depression [14], hypotensive [15], pyretic and inflammatory diseases [16]. Some bis-pyrazoline derivatives were also found with antimicrobial activity [17]. The discovery of this class of drugs provides an outstanding case of history of modern drug development and also points out the unpredictability of biological activity from structural modification. In view of the number of

* Corresponding author. Tel.: +91 11 26981717/3253; fax: +91 11 26980229/1232.
E-mail address: amir_sumbul@yahoo.co.in (A. Azam).
[1] Present address: Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi 110025, India.

0223-5234/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2007.11.005

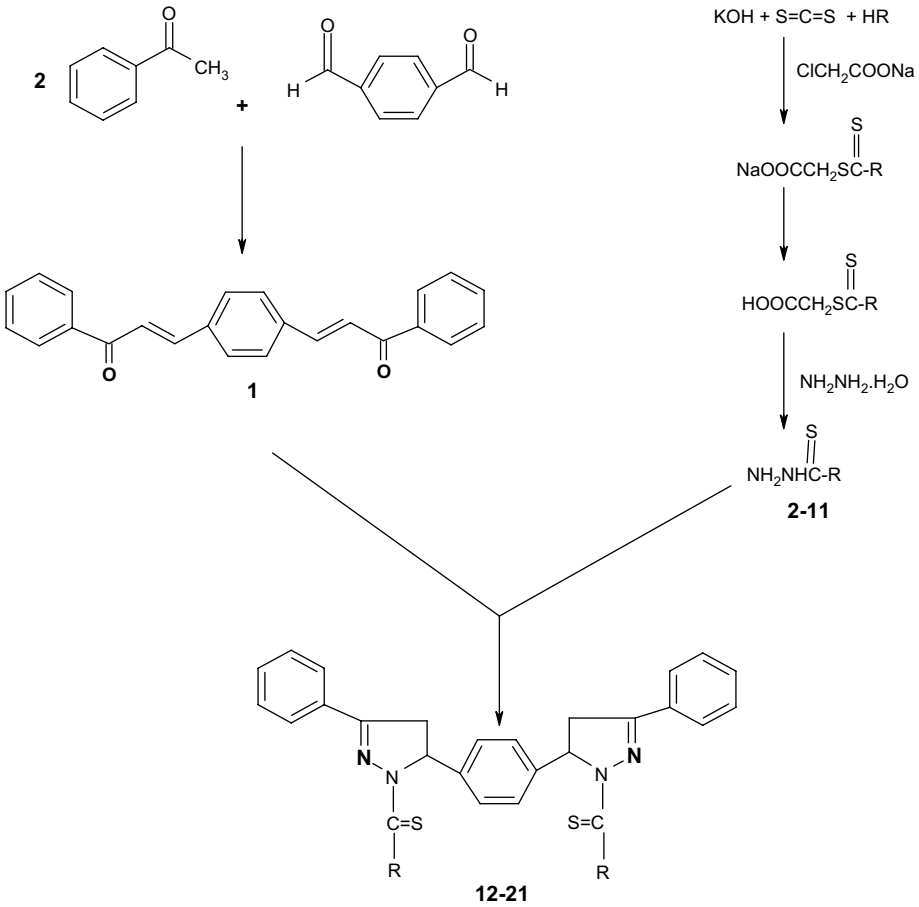
pharmacological significance of pyrazolines, we have synthesized a new series of organic molecules containing two pyrazoline rings and have evaluated the same against the HM1:IMSS strain of the *E. histolytica*, a protozoa responsible for amoebiasis. The ongoing research in our laboratory showed that pyrazoline derivatives [18–20] are good candidates for these studies. On the basis of their activity and favorable therapeutic indexes, these compounds were identified as viable leads for further studies.

2. Results and discussion

Bischalcone was prepared according to Claisen–Schmidt reaction, i.e., by treating acetophenone with NaOH (60%) and terephthalaldehyde gave above 80% yield. All the thiosemicarbazides were prepared by reported method [21]. The cyclization of chalcone with N-4 substituted thiosemicarbazides under basic condition led to the formation of new compounds thiocarbamoyl bis-pyrazoline derivatives, Scheme 1. According to the currently accepted mechanism, formation of the cyclized pyrazoline analogues is favored via thiosemicarbazone formation, which undergo cyclization under basic conditions to form desired pyrazoline ring in all the compounds (**12–21**), (Table 1) [22,23]. The compounds recrystallized by methanol gave crystalline solid compounds in low yield. The yield of cyclized compounds was in the range of 12–32%. All the compounds are insoluble in water but soluble in most of the organic solvents. The structures of the compounds were established by means of their IR, UV, ^{1}H NMR, ^{13}C NMR and mass spectral studies.

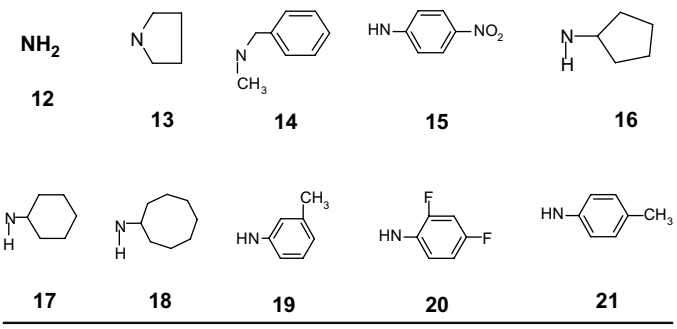
Assignment of selected characteristic IR bands provides significant indications for the formation of the cyclized pyrazoline analogues of the thiosemicarbazones (**12–21**). The absence of the band at/or around 1658 cm^{-1} showed the absence of the carbonyl group in all the compounds (**12–21**). The appearance of an intense band between 1357 and 1393 cm^{-1} regions showed ν (C=S) stretch of the thiocarbamoyl group. The cyclization of the product was confirmed by the appearance of the absorption bands in the region 1583–1618 cm^{-1} and 1055–1096 cm^{-1}. The former band is attributed to ν (C=N) stretch and the latter band to ν (C–N) stretch vibrations. The compounds **12** and **16–21** showed additional bands in the region 3322–3396 cm^{-1} due to ν (N–H) stretch.

The electronic spectra of the cyclized pyrazoline analogue studies in the UV region in methanol, exhibited three absorption bands at 371–338 nm, 269–231 nm and 229–210 nm assigned to $n \rightarrow \pi^*$, $\pi \rightarrow \pi^*$ and $n \rightarrow \sigma^*$ transitions, respectively. The band at 371–338 nm was assigned to the



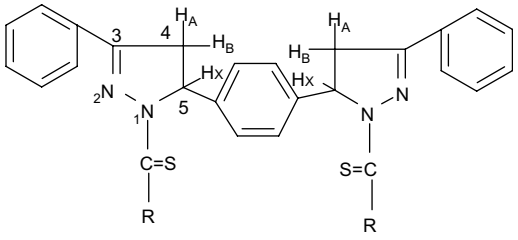
Scheme 1. General representation for the synthesis of bis-pyrazoline derivatives.

Table 1
Different substituent groups 'R'



transition involving the thione portions (C═S) of thiocarbamoyl group. The two other absorption bands at 269–231 nm and 229–210 nm were due to $\pi \rightarrow \pi^*$ transition of phenyl ring and $n \rightarrow \pi^*$ transition of azomethine nitrogen.

The structure of the compounds was further confirmed by ^{1}H NMR and ^{13}C NMR. The pyrazoline protons H_a and H_b (**A**) are geminal protons, appear in the region of 3.05–3.82 ppm and 2.91–2.16 ppm as doublet of doublets in all the compounds.



(A)

The CH proton appeared as doublet of doublets in the region 5.73–6.52 ppm due to vicinal coupling with two non-equivalent geminal protons of adjacent carbon atom. The NH proton of different substituted thiocarbamoyl compounds **12**, and **16–21** showed singlet at 8.36–10.89 ppm. The protons belonging to the aromatic ring were observed within the expected chemical shift region along with integral values.

The ^{13}C NMR spectra of all the compounds were taken in $CDCl_3$. The C_4 and C_5 carbons of the pyrazoline ring resonate at 40.3–48.5 ppm and 62.3–67.9 ppm, respectively. All the compounds showed a signal at 153.2–158.2 ppm assigned to azomethine carbon of pyrazoline ring. Thiocarboxamide carbon (C═S) displayed a signal at 175.0–179.1 ppm in all the compounds. The signals due to the aromatic carbons and the carbons at 1-*N*-substituted aliphatic groups resonate at their usual positions shown in the experimental section.

Table 2
In vitro antiamoebic activity against *E. histolytica* HM1:IMSS strain and cytotoxicity profile of **12–21**

Sample code	Compound	Antiamoebic activity		Toxicity profile (IC_{50} (μM))	Safety Index (S.I.)
		IC_{50} (μM)	S.D.		
12	$C_{26}H_{24}N_6S_2$	1.57	0.51	>90	>57.3
13	$C_{34}H_{36}N_6S_2$	>1.8	0.61	>90	>50.0
14	$C_{42}H_{40}N_6S_2$	>1.8	0.47	>90	>50.0
15	$C_{38}H_{30}N_8S_2O_4$	0.42	0.15	>90	>214
16	$C_{36}H_{40}N_6S_2$	>1.8	0.61	>90	>50.0
17	$C_{38}H_{44}N_6S_2$	1.16	0.39	>90	>77.6
18	$C_{42}H_{52}N_6S_2$	0.92	0.41	>90	>97.8
19	$C_{40}H_{36}N_6S_2$	>1.8	0.73	>90	>50.0
20	$C_{38}H_{28}N_6S_2F_4$	0.62	0.24	>90	>145
21	$C_{40}H_{36}N_6S_2$	>1.8	0.62	>90	>50.0
MNZ	$C_6H_9N_3O_3$	1.8	0.39	>100	>55.55

S.D. = standard deviation. S.I. = toxicity IC_{50}/protozoal IC_{50}.

2.1. In vitro antiamoebic activity

Preliminary experiments were carried out to determine the antiamoebic activity *in vitro* of all the compounds (**12–21**) by microdilution method using HM1:IMSS strain of *E. histolytica*. The results of the bioassays are summarized in Table 2. The data are present in terms of percent growth inhibition relative to untreated controls, and plotted as probit values as a function of drug concentration. The IC_{50} values were interpolated in the corresponding dose response curves. The IC_{50} values for compounds **15** (0.42 μM), **20** (0.62 μM), and **18** (0.920 μM), correspond to 1.95–4.28 fold increase in activity, than the standard drug metronidazole (1.8 μM), thus proved to be better inhibitors of *E. histolytica* growth. The compounds **17** (1.16 μM) and **12** (1.57 μM) showed moderate activity than metronidazole. The results were statistically evaluated by analyses of variance. The null hypothesis was tested using *T*-Test and the significance of the differences between the IC_{50} value(s) of metronidazole vs. **12–21** was evaluated. The calculated *T*-values were higher than the table values at the 4% level. Hence, the character under study was significantly influenced by the treatment. Structure–activity relationship shows that the compound with aromatic substituents at the thiocarbamoyl group was more active than those with the cyclic groups. However, it was clear from the IC_{50} values that the compounds **15** and **20** are more active and both showed a structural resemblance having an electron withdrawing groups attached to the phenyl ring. Out of the cyclic substituent, compound **18** with cyclooctyl ring was found to be more active than cyclohexyl and/or cyclopentyl rings.

2.2. Toxicity profile

To ensure that the bis-pyrazolines were not toxic to human cells these compounds were tested against a human kidney epithelial cell line. None of the compounds inhibited cell growth at a concentration of 90 μM (Table 2). To investigate the selectivity of the compounds, the "safety index" (S.I.) was calculated and defined as: toxicity IC_{50}/protozoal IC_{50}; where toxicity IC_{50} is defined as the concentration of compound that kills 50% of the human (kidney epithelial) cell line and protozoal IC_{50} is the concentration that kills 50% of amoeba protozoa. This allows an estimate of which compounds might be efficacious or toxic against human cells and potentially *in vivo*. The numerical results for each compound are given in

Table 2. These results show that the compound **15** has lowest cytotoxicity and highest antiamoebic activity and overall compounds show more favorable safety profile along with the most promising antiamoebic activity. Thus the accumulation of the bis-pyrazoline derivatives will remain toxic to the parasite, whilst in the human host there will be a decreased likelihood of toxicity.

3. Experimental

Reactions were conducted in oven dried glass wear. All the chemicals were purchased from Aldrich chemical company (USA). Analytical thin-layer chromatography was performed on precoated silica gel 60 F_{254} plates and column chromatography was accomplished using Silica gel, 60 Å (200–400 mesh) and basic alumina. Elemental analyses were performed by Central Drug Research Institute, Lucknow, using Heraeus Vario EL III analyzer, and the results were within 0.3% of the theoretical values. Electronic spectra were recorded in methanol 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 taken in $CDCl_3$ at ambient temperature using a Bruker Spectrospin DPX-300 MHz spectrophotometer with TMS as internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Chemical shift values are given in ppm.

3.1. Chemistry

3.1.1. General procedure for the synthesis of bischalcone (*1*)

A solution of acetophenone (2 equiv) and terapthalaldehyde (1 equiv) in methanolic solution of NaOH (60%) was stirred for 20 h at room temperature. The solution was poured into ice cold water of pH ~ 2 (pH adjusted by HCl). The solid was separated and dissolved in CH_2Cl_2, washed with saturated solution of $NaHCO_3$ and evaporated to dryness. The residual was purified by column chromatography using CH_2Cl_2 as eluent. The compound was recrystallized in ethanol.

Pale yellow crystal (chloroform); yield 83%; m.p. 158; Anal. Calc. for $C_{24}H_{18}O_2$: C 85.17, H 5.36, found: C 85.5, H 5.73%; UV/vis λ_{max} (nm) 374, 288, 239, 219; IR ν (cm^{-1}) 3060 (Ar–H), 2924 (CH), 1653 (C=O), 1610 (CH=CH), 1541 (C=C); ^{1}H NMR ($CDCl_3$): δ/ppm 6.9 (d, 2H $J = 15$ Hz, C=CH), 7.25–7.49 (m, 10H, Ar–H), 7.6 (d, 4H, Ar–H), 7.92 (d, 2H $J = 15$ Hz, CO–CH); ^{13}C NMR (DMSO): δ/ppm 190.2 (C=O), 143.5 (C=C), 138.0 (C=C), 136.8, 132.9, 128.9, 128.6, 128.5, 123.0 (Ar–C). FABMS (M + 1): *m/z* 339.1, calc. 338.

3.1.2. Synthesis of thiosemicarbazides (*2–11*)

Substituted thiosemicarbazides (**2–11**) were prepared by a reported method [21].

3.1.3. Synthesis of thiocarbamoyl bis-pyrazoline derivatives (*12–21*)

A mixture of bischalcone **1** (1 equiv), thiosemicarbazides **2–11** (4 equiv) and NaOH (2.5 equiv) was refluxed in ethanol for 3 days. The solution was poured into ice water. The precipitate was filtered and recrystallized from methanol to yield **12–21**.

*3.1.3.1. Amino[5-(4-{1-(aminothioxomethyl)-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**12**).* Brownish yellow crystal (chloroform); yield 16%; m.p. 231 °C; Anal. Calc. for $C_{26}H_{24}N_6S_2$: C 64.43, H 4.99, N 17.34, found C 64.71, H 5.21, N 17.61%; UV/vis λ_{max} (nm) 356, 247, 223; IR ν (cm^{-1}) 3318 (NH), 3096 (Ar–H), 2925 (CH), 1590 (CH=N), 1373 (C=S), 1096 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 8.36 (s, 4H, NH_2), 7.86 (s, 4H, Ar–H), 7.10–7.28 (m, 10H, Ar–H), 5.91 (dd, 2H, H_x, J_{xA} 8.93 Hz, J_{xB} 16.81 Hz), 3.82 (dd, 2H, H_A, J_{AB} 6.41 Hz, J_{Ax} 8.93 Hz), 2.9 (dd, 2H, H_B, J_{BA} 6.41 Hz, J_{Bx} 16.81 Hz); ^{13}C NMR (DMSO): δ/ppm 176.4 (C=S), 154.9 (C=N), 141.1, 130.5, 130.1, 128.2, 126.6, 125.2 (Ar–C), 62.3 (CH), 42.0 (CH_2). FABMS (M + 1): *m/z* 485.32, calc. 484.20.

*3.1.3.2. (3-Phenyl-5-{4-3-phenyl-1-(pyrrolidinylthioxomethyl)(2-pyrazoline-5-yl-phenyl}(2-pyrazolinyl))pyrrolidinylmethane-1-thione (**13**).* Brownish yellow crystal (chloroform); yield 12%; m.p. 271 °C; Anal. Calc. for $C_{34}H_{36}N_6S_2$: C 68.88, H 6.12, N 14.18, found: C 69.05, H 6.42, N 14.41%; UV/vis λ_{max} (nm) 342, 297, 228; IR ν (cm^{-1}) 3041 (Ar–H), 2961 (CH), 1615 (CH=N), 1386 (C=S), 1064 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 7.53 (s, 4H, Ar–H), 7.18–7.46 (m, 10H, Ar–H), 6.52 (dd, 2H, H_x, J_{xA} 8.72 Hz, J_{xB} 16.21 Hz), 3.94–4.19 (16H, m, $-CH_2$), 3.58 (dd, 2H, H_A, J_{AB} 6.32 Hz, J_{Ax} 8.72 Hz), 2.16 (dd, 2H, H_B, J_{BA} 6.32 Hz, J_{Bx} 16.21 Hz); ^{13}C NMR (DMSO): δ/ppm 175.8 (C=S), 153.4 (C=N), 142.4, 130.1, 129.9, 128.6, 125.7, 124.2 (Ar–C), 65.3 (CH), 63.1, 61.4, 60.9, 43.1 (CH_2); FABMS (M + 1): *m/z* 593.69, calc. 592.29.

*3.1.3.3. (Methylphenylamino)[5-(4-{1[(methylphenylamino)-thioxomethyl]-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**14**).* Brownish yellow crystal (chloroform); yield 26%; m.p. 235 °C; Anal. calc. for $C_{42}H_{40}N_6S_2$: C 72.8, H 5.82, N 12.13, found: C 72.97, H 5.61, N 12.38%; UV/vis λ_{max} (nm), 366, 247, 216; IR ν (cm^{-1}) 3071 (Ar–H), 2914 (CH), 1588 (CH=N), 1357 (C=S), 1083 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 7.25–7.61 (m, 22H, Ar–H), 7.82 (s, 4H, Ar–H), 6.13 (dd, 2H, H_x, J_{xA} 9.71 Hz, J_{xB} 17.31 Hz), 5.02 (2H, s, $-NCH_2-$), 3.38 (3H, s, $-N-CH_3$), 3.15 (dd, 2H, H_A, J_{AB} 5.91 Hz, J_{Ax} 9.71 Hz), 2.46 (dd, 2H, H_B, J_{BA} 5.91 Hz, J_{Bx} 17.31 Hz); ^{13}C NMR (DMSO): δ/ppm 177.2 (C=S), 155.0 (C=N), 132.2, 131.2, 128.4, 128.1, 126.9, 125.3 (Ar–C), 65.8 (CH_2), 62.3 (CH), 43.8 (CH_2), 40.6 (CH_3). FABMS (M + 1): *m/z* 693.52, calc. 692.32.

*3.1.3.4. (4-Nitrophenylamino)[5-(4-{1-[(4-nitrophenylamino) thioxomethyl]-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**15**).* Brownish yellow crystal (chloroform); yield 19%; m.p. 274 °C; Anal. Calc. for

$C_{38}H_{30}N_8O_4S_2$: C 62.78, H 4.16, N 15.42, found: C 62.39, H 4.32, N 15.69%; UV/vis λ_{max} (nm) 338, 239, 215; IR ν (cm^{-1}) 3034 (Ar–H), 2989 (CH), 1583 (CH=N), 1393 (C=S), 1055 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 9.62 (s, 2H, NH), 7.82 (s, 4H, Ar–H), 7.41–7.79 (m, 18H, Ar–H), 5.85 (dd, 2H, H_x, J_{xA} 9.68 Hz, J_{xB} 17.39 Hz), 3.49 (dd, 2H, H_A, J_{AB} 6.71 Hz, J_{Ax} 9.68 Hz), 2.36 (dd, 2H, H_B, J_{BA} 6.71 Hz, J_{Bx} 17.39 Hz); ^{13}C NMR (DMSO): δ/ppm 176.7 (C=S), 157.8 (C=N), 143.4, 130.5, 130.1, 128.2, 127.5, 126.6, 125.9, 125.2 (Ar–C), 67.9 (CH), 46.1 (CH_2); FABMS (M + 1): *m/z* 727.50, calc. 726.24.

*3.1.3.5. (Cyclopentylamino)[5-(4-{1-[(cyclopentylamino)thioxomethyl]-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**16**).* Brownish yellow crystal (chloroform); yield 32%; m.p. 246 °C; Anal. calc. for $C_{36}H_{40}N_6S_2$: C 69.64, H 6.49, N 13.54, found: C 69.79, H 6.73, N 13.81%; UV/vis λ_{max} (nm) 363, 245, 211; IR ν (cm^{-1}) 3349 (NH), 3012 (Ar–H), 2947 (CH), 1595 (CH=N), 1371 (C=S), 1076 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 9.15 (s, 2H, NH), 7.61 (s, 4H, Ar–H), 7.03–7.41 (m, 10H, Ar–H), 5.73 (dd, 2H, H_x, J_{xA} 8.83 Hz, J_{xB} 17.46 Hz), 4.55–4.63 (2H, m, –N–CH– (cyclopentyl ring)), 3.64 (dd, 2H, H_A, J_{AB} 6.23 Hz, J_{Ax} 8.83 Hz), 2.13 (dd, 2H, H_B, J_{BA} 6.23 Hz, J_{Bx} 17.46 Hz), 1.43–1.69 (16 H, m, cyclopentyl ring); ^{13}C NMR (DMSO): δ/ppm 178.0 (C=S), 155.3 (C=N), 142.2, 132.1, 131.9, 128.4, 127.3, 124.2 (Ar–C), 63.8 (CH), 62.8, 61.2, 59.0, 46.3 (CH_2); FABMS (M + 1) *m/z* 621.81, calc. 620.32.

*3.1.3.6. (Cyclohexylamino)[5-(4-{1-[(cyclohexylamino)thioxomethyl]-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**17**).* Brownish yellow crystal (chloroform); yield 23%; m.p. 261 °C; Anal. Calc. for $C_{38}H_{44}N_6S_2$: C 70.33, H 6.84, N 12.27, found: C 70.94, H 6.43, N 12.56%; UV/vis λ_{max} (nm) 363, 251, 229; IR ν (cm^{-1}) 3316 (NH), 3019 (Ar–H), 2947 (CH), 1609 (CH=N), 1369 (C=S), 1086 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 8.81 (s, 2H, NH), 7.42–7.81 (m, 10H, Ar–H), 7.31 (s, 4H, Ar–H), 6.03 (dd, 2H, H_x, J_{xA} 9.61 Hz, J_{xB} 16.81 Hz), 4.14–4.29 (2H, m, –N–CH (cyclohexyl ring)), 3.28 (dd, 2H, H_A, J_{AB} 5.81 Hz, J_{Ax} 9.61 Hz), 2.95 (dd, 2H, H_B, J_{BA} 5.81 Hz, J_{Bx} 16.81 Hz), 1.23–1.97 (20H, m, cyclohexyl ring); ^{13}C NMR (DMSO): δ/ppm 177.3 (C=S), 154.5 (C=N), 134.2, 132.6, 130.4, 128.3, 126.2 (Ar–C), 65.9 (CH), 63.3, 62.1, 61.6, 61.0, 59.9, 41.8 (CH_2). FABMS (M – 1): *m/z* 647.2, calc. 648.35.

*3.1.3.7. (Cyclooctylamino)[5-(4-{1-[(cyclooctylamino)thioxomethyl]-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**18**).* Brownish yellow crystal (chloroform); yield 17%; m.p. 268 °C Anal. Calc. for $C_{42}H_{52}N_6S_2$: C 71.54, H 7.44, N 11.92, found: C 71.76, H 7.13, N 12.14%; UV/vis λ_{max} (nm) 369, 231, 220; IR ν (cm^{-1}) 3312 (NH), 3059 (Ar–H), 2893 (CH), 1618 (CH=N), 1368 (C=S), 1063 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 8.56 (s, 2H, NH), 7.56 (s, 4H, Ar–H), 7.21–7.39 (m, 10H, Ar–H), 6.42 (dd, 2H, H_x, J_{xA} 9.92 Hz, J_{xB} 17.35 Hz), 4.65–5.01 (2H, m, –N–CH– (cyclooctyl ring)), 4.31–4.43 (28H, m, cyclooctyl ring), 3.32 (dd, 2H, H_A, J_{AB} 5.52 Hz, J_{Ax} 9.92 Hz), 2.71 (dd, 2H, H_B, J_{BA} 5.52 Hz, J_{Bx} 17.35 Hz); ^{13}C NMR (DMSO): δ/ppm 175.0 (C=S), 153.2 (C=N), 143.2, 133.1, 132.3, 130.6, 130.2, 128.4 (Ar–C), 67.8 (CH), 66.3, 65.9, 62.9, 61.3, 61.1, 60.2, 40.3 (CH_2). FABMS (M + 1): *m/z* 705.93, calc. 704.41.

*3.1.3.8. [(3-Methylphenyl)amino][5-(4-{1-{[(3-methylphenyl)amino]thioxomethyl}-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**19**).* Brownish yellow crystal (chloroform); yield 26%; m.p. 249 °C; Anal. calc. for $C_{40}H_{36}N_6S_2$: C 72.25, H 5.46, N 12.64, found: C 72.68, H 5.76, N 12.91%; UV/vis λ_{max} (nm) 371, 246, 210; IR ν (cm^{-1}) 3376 (NH), 3073 (Ar–H), 2921 (CH), 1593 (CH=N), 1377 (C=S), 1064 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 10.83 (s, 2H, NH), 7.62 (s, 4H, Ar–H), 7.19–7.53 (m, 18H, Ar–H), 5.93 (dd, 2H, H_x, J_{xA} 9.54 Hz, J_{xB} 16.29 Hz), 3.47 (dd, 2H, H_A, J_{AB} 5.97 Hz, J_{Ax} 9.54 Hz), 2.56 (dd, 2H, H_B, J_{BA} 5.97 Hz, J_{Bx} 16.29 Hz), 2.15 (3H, s, $-CH_3$); ^{13}C NMR (DMSO): δ/ppm 179.1 (C=S), 154.8 (C=N), 141.6, 132.5, 131.6, 131.1, 130.8, 128.3, 128.1, 126.4, 126.0, 125.9 (Ar–C), 63.1 (CH), 47.1 (CH_2), 46.9 (CH_3). FABMS (M + 1): *m/z* 665.86 calc. 664.28.

*3.1.3.9. [(2,4-Difluorophenyl)amino[5-(4-{1-{[(2,4-difluorophenyl)amino]thioxomethyl}-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**20**).* Brownish yellow crystal (chloroform); yield 16%; m.p. 253 °C; Anal. Calc. for $C_{38}H_{28}N_6S_2F_4$: C 64.38, H 3.98, N 11.86, found: C 64.73, H 3.86, N 11.93%; UV/vis λ_{max} (nm) 358, 269, 218; IR ν (cm^{-1}) 3365 (NH), 3065 (Ar–H), 2892 (CH), 1587 (CH=N), 1358 (C=S), 1073 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 10.05 (s, 2H, NH), 7.69 (s, 4H, Ar–H), 7.12–7.44 (m, 16H, Ar–H), 6.41 (dd, 2H, H_x, J_{xA} 9.32 Hz, J_{xB} 17.45 Hz), 3.05 (dd, 2H, H_A, J_{AB} 5.63 Hz, J_{Ax} 9.32 Hz), 2.37 (dd, 2H, H_B, J_{BA} 5.63 Hz, J_{Bx} 17.45 Hz); ^{13}C NMR (DMSO): δ/ppm 176.2 (C=S), 155.1 (C=N), 133.4, 133.1, 131.8, 130.3, 128.4, 128.1, 126.9, 126.4, 125.1 (Ar–C), 64.2 (CH), 42.3 (CH_2). FABMS (M + 1): *m/z* 709.49, calc. 708.22.

*3.1.3.10. [(4-Methylphenyl)amino][5-(4-{1-{[(4-methylphenyl)amino]thioxomethyl}-3-phenyl(2-pyrazoline-5-yl-phenyl)}-3-phenyl(2-pyrazolinyl)]methane-1-thione (**21**).* Brownish yellow crystal (chloroform); yield 18%; m.p. 236 °C; Anal. Calc. for $C_{40}H_{36}N_6S_2$: C 72.25, H 5.46, N 12.64, found: C 72.61, H 5.71, N 12.87%; UV/vis λ_{max} (nm) 368, 251, 317; IR ν (cm^{-1}) 3396 (NH), 3058 (Ar–H), 2929 (CH), 1605 (CH=N), 1368 (C=S), 1072 (C–N); ^{1}H NMR ($CDCl_3$): δ/ppm 10.65 (s, 2H, NH), 7.81 (s, 4H, Ar–H), 7.31–7.57 (m, 18H, Ar–H), 5.96 (dd, 2H, H_x, J_{xA} 8.68 Hz, J_{xB} 16.52 Hz), 3.18 (dd, 2H, H_A, J_{AB} 5.99 Hz, J_{Ax} 8.68 Hz), 2.91 (dd, 2H, H_B, J_{BA} 5.99 Hz, J_{Bx} 16.52 Hz), 2.33 (3H, s, $-CH_3$); ^{13}C NMR (DMSO): δ/ppm 178.3 (C=S), 158.2 (C=N), 132.8, 131.8, 131.4, 130.5, 128.2, 128.0, 126.9, 126.2, 125.3 (Ar–C), 65.2 (CH), 48.5 (CH_2), 47.2 (CH_3). FABMS (M + 1): *m/z* 665.50, calc. 664.28.

4. Antiamoebic activity

The compounds **12–21** were screened *in vitro* for antiamoebic activity against the HM1:IMSS strain of *E. histolytica* by the microdilution method [24]. *E. histolytica* trophozoites were cultured in TYIS-33 growth medium as described previously, in wells of 96 microtiter plate (Costar) [25]. The test compounds were dissolved in DMSO (40 µl), at which level no inhibition of the amoeba occurs [26,27]. Then, culture medium was added to obtain a concentration of 1 mg/ml. Two fold serial dilutions were then made. Each test includes metronidazole as a standard amoebic drug, control wells (culture medium plus amoeba), and a blank (culture medium only). The number of amoeba per millimeter was estimated with a hemocytometer, and trypan blue exclusion was used to confirm viability. The cell suspension used was diluted to 10^5 organisms/ml by adding fresh medium, and 170 µl of this suspension was added to the test and control wells in the plate. An inoculum of 1.7×10^4 organisms/well was chosen, so that confluent, but not excessive, growth took place. The plates were sealed, and gassed for 10 min with N_2, and then incubated at 37 °C for 72 h. After incubation the growth of the amoeba was checked with a low power microscope. The culture medium was removed by inverting the plate and shaking gently. The plates were immediately washed with 0.9% aq. NaCl solution at 37 °C. This procedure was performed quickly, and the plate was not allowed to cool, to prevent the detachment of amoebae. The plate was allowed to dry at room temperature, and the amoebas were fixed with chilled MeOH by keeping it in an ice bath for 15 min, dried, and stained with 0.5% aq. eosin for 15 min. The stained plate was washed once with tap H_2O, and then twice with distilled H_2O, and allowed to dry. Then 0.1 N aq. NaOH solution (200 µl) was added to each well to dissolve the protein and to release the dye (eosin). The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader. The inhibition (in %) 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 straight line, from which IC_{50} values were determined.

4.1. MTT toxicity assay

For the toxicity assay, transformed human kidney epithelium (Graham) cells was continuously maintained in culture at 37 °C in 5% CO_2. The MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide, USB) cellular viability assay was used to determine the toxicity profile of the compounds [28]. The trypsinized cell suspension was adjusted to 0.5 million cells/ml and plated out with the various compounds. After 44 h of incubation, 2 mM MTT was added to the plates and incubated for a further 4 h. DMSO was then added to stop the reaction and dissolve the formazan crystals. The absorbance was read at the test wavelength of 540 nm and reference wavelength of 690 nm and the percentage cellular viability calculated with appropriate controls taken into account. The mean ± S.D. values of IC_{50} values in Table 2 are from three independent experiments.

References

[1] S.L. Stanley Jr., Lancet 361 (2003) 1025–1034.
[2] WHO, Wkly. Epidemiol. Rec. 14 (1997) 97–100.
[3] C.D. Huston, R. Haque, W.A. Petri Jr., Expert Rev. Mol. Med. (1999) 1–11.
[4] J.L. Ravdin, Clin. Infect. Dis. 20 (1995) 1453–1466.
[5] F.D. Rocco, G. Sabatino, G. Tamburrini, O. Ranno, P. Valentini, M. Caldarlli, Acta Neurochir. 146 (2004) 1271–1272.
[6] D.C.K. Ng, S.Y. Kwok, Y. Cheng, C.C. Chung, M.K.W. Li, Hong Kong Med. J. 12 (2006) 71–73.
[7] A. Azam, S.M. Agarwal, Curr. Bioact. Compd. 3 (2007) 121–123.
[8] F. Roe, Surgery 93 (1983) 158–164.
[9] J.C. Samuelson, A. Burke, J.M. Courval, Antimicrob. Agents Chemother. 36 (1992) 2392–2397.
[10] I.S. Adagu, D. Nolder, D.C. Warhurst, J.F. Rossignol, J. Antimicrob. Chemother. 49 (2002) 103–111.
[11] G. Turan-Zitouni, A. Ozdemir, K. Guven, Arch. Pharm. (Weinheim) 338 (2005) 96–104.
[12] N. Gokhan, A. Yesilada, G. Ucar, K. Erol, A.A. Bilgin, Arch. Pharm. Med. Chem. 336 (2003) 362–371.
[13] B.S. Holla, P.M. Akbarali, M.K. Shivanada, IL Farmaco 55 (2000) 256–263.
[14] Y.R. Prassad, A.L. Rao, L. Prasoona, K. Murali, P.R. Kumar, Bioorg. Med. Chem. Lett. 15 (2005) 5030–5034.
[15] J.T. Zitouni, P. Chevallet, F.S. Kilic, K. Erol, Eur. J. Med. Chem. 35 (2000) 635–641.
[16] O. Bruno, A. Ranise, F. Bondavalli, F. Schenone, M. D' Amico, A. Filipelli, W. Filipelli, R. Francesco, IL Farmaco 48 (1993) 949–966.
[17] D.B. Reddy, B. Seenaiah, S. Eswaraiah, T. Seshamma, M.V.R. Reddy, J. Indian Chem. Soc. 66 (1989) 893–896.
[18] M. Abid, A. Azam, Bioorg. Med. Chem. 13 (2005) 2213–2220.
[19] M. Abid, A. Azam, Bioorg. Med. Chem. Lett. 16 (2006) 2812–2816.
[20] M. Abid, A. Azam, Eur. J. Med. Chem. 40 (2005) 935–942.
[21] D.G. O' Sullivan, P.W. Sadler, C. Webley, Chem. Commun. 7 (1) (1963) 17–26.
[22] H. Ferres, W.R. Jackson, J. Chem. Soc., D. Chem. Commun. 6 (1969) 261–262.
[23] R.H. Wiley, C.H. Jarboe, F.N. Hayes, E. Hansbury, J.T. Nielson, P.X. Callahan, M.C. Sellars, J. Org. Chem. 23 (1958) 732–738.
[24] W. Wright, M.J. O'Neill, J.D. Phillipson, D.C. Warhurst, Antimicrob. Agents Chemother. 32 (1988) 1725–1729.
[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. Suffness, Antimicrob. Agents Chemother. 22 (1982) 342–345.
[27] A.T. Keen, A. Harris, J.D. Phillipson, D.C. Warhurst, Planta Med. (1986) 278–284.
[28] T. Mosmann, J. Immunol. Methods 65 (1–2) (1983) 55–63.