SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NOVEL PYRAZOLINE ANALOGUE

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ABSTRACT

A novel 5-ferrocenyl-3-(4-methylthio phenyl)-4,5-dihydropyrazole-1-carbothioamide (2) has been synthesized by the cyclization of 3-Ferrocenyl-1-(4-methylthio phenyl)-2-propen-1-one (1). The novel compound was screened for in vitro antiamoebic activity against HM1:IMSS strain of *Entamoeba histolytica* and the result revealed that it exhibited higher antiamoebic activity (IC$_{50}$ = 0.77 μM) than the reference drug Metronidazole (IC$_{50}$ = 1.78 μM), concluding that this compounds hold immense potential to be employed as new antiamoebic agent. Also, being novel it can be a solution to the increasing resistance that has posed a major problem globally.

KEYWORDS: Pyrazoline analogue, Amoebiasis, Antiamoebic activity, Entamoeba histolytica.

1. INTRODUCTION

Amoebiasis is the most aggressive protozoal disease and considered to be the second or third leading cause of death amongst the parasitic diseases\(^1\). *Entamoeba histolytica*, a protozoan parasite, is the causative agent of amoebiasis and amoebic dysentery. Though ubiquitous in distribution, this parasite is more prevalent in tropical and subtropical regions\(^2\). Metronidazole is known to be highly effective amoebicide and is considered to be the drug of choice for the treatment of amoebiasis, but this drug has been shown to be mutagenic in a microbiological system and carcinogenic to rodents\(^3\)-\(^5\). Repeated treatment of *Entamoeba histolytica* infection with commonly used antiamoebic drugs results in not only increasing the toxicity potential but also leads to the development of clinical resistance. Therefore, new effective agents with less toxicity against amoebiasis are urgently required. Since the discovery of ferrocene\(^6\),\(^7\) its derivatives have been immensely used for their potential applications in diverse fields such as homogeneous catalysis\(^8\), organic synthesis, supramolecular chemistry\(^9\), biosensors\(^10\), medicinal chemistry\(^11\)-\(^13\) and material science\(^14\). It is now well established that ferrocene functionalized organic compounds often exhibit unexpected biological activity owing to different membrane permeation properties and
anomalous metabolism. Moreover, the stability and non-toxicity of the ferrocenyl moiety is of particular interest rendering such drugs compatible with other treatments. In this sense, the integration of one or more ferrocene units into a heterocyclic molecule has long been recognized as an attractive way to endow a novel molecule functionality. Many ferrocenyl compounds display interesting cytotoxic, anti-tumor, antimalarial, antifungal and DNA-cleaving activity. Recently, some new ferrocenyl-substituted heterocyclic compounds have been reported as potential pharmaceuticals.

Pyrazolines are an important class of heterocyclic compounds containing two nitrogen atoms in the five-membered ring. The current literature is enriched with progressive findings about the synthesis and pharmacological action of pyrazolines and related heterocyclic compounds.

In view of these observations, we have synthesized a novel pyrazoline analogue as useful lead towards the development of potent antiamoebic agents.

2. Results and discussion

2.1. Chemistry. The novel pyrazoline was prepared according to the synthetic sequence illustrated in Scheme-1. The ferrocenyl chalcone 3-Ferrocenyl-1-(4-methylthio-phenyl)-2-propen-1-one (1) was prepared by a classic Claisen–Schmidt condensation of 4-methylthio benzaldehyde with acetyl ferrocene in the presence of KOH and absolute ethanol. The cyclization of ferrocenyl chalcone (1), with thiosemicarbazide under basic condition in the presence of absolute ethanol yielded their corresponding pyrazoline analogues, 3-ferrocenyl-1(-4-methylthiophenyl)-4,5-dihydro-1H-pyrazole-1-carbothioamide (2). According to the mechanism, the formation of pyrazoline analogue is favoured via thiosemicarbazone formation, which undergoes cyclization under basic condition to form desired pyrazoline ring in compound. The synthesized compound was characterized by spectroscopic methods such as IR, 1H-NMR 13C-NMR, Mass and elemental analysis and the data is presented in the experimental section.

Selected diagnostic bands of the IR spectrum of pyrazoline analogue (2) of ferrocenyl chalcone (1) showed useful information about the structure of the compound. It showed intense bands in the region 1065 cm⁻¹ due to ν(C=S) stretch of the thiocarboxamide group. The IR spectra of the compound showed ν(C=N) stretch at 1557 cm⁻¹ because of the ring closure. In addition, the absorption band at 1261 cm⁻¹ was attributed to the ν(C-N) stretch vibrations, which also confirmed the formation of the desired pyrazoline ring in the
compound. The structure of the pyrazoline analogue was further supported by its $^1$H-NMR spectra which provided diagnostic tools for the positional elucidation of the protons. Assignments of the signals are based on the chemical shifts and intensity patterns. The pyrazoline protons $H_a$ and $H_b$ (Figure-1) are germinal protons at C-4 carbon and appeared in the region of 3.52 and 3.81 ppm respectively, as doublet of doublets in the compound. The C-H proton ($H_c$) of the pyrazoline ring also appeared as doublet of doublets in the region of 6.36 ppm due to vicinal coupling with two non equivalent germinal protons of C-4 carbon. In addition, the four protons in monosubstituted Cp of ferrocene moiety appeared as three singlet peaks in the region of 4.61, 4.55 and 4.49 ppm and five protons of unsubstituted Cp also appeared as a singlet in the region of 4.26 ppm. The protons belonging to the aromatic ring were observed within the expected chemical shift region along with the integral values and are shown in the data given in the experimental section.

2.2. In vitro antiamoebic activity:

Preliminary experiments were carried out to determine the in vitro antiamoebic activity of the newly synthesized compound (2) by microdilution method using HM1:IMSS strain of *E. histolytica* and the IC$_{50}$ value is reported in Table 1. The metronidazole was used as reference drug with IC$_{50}$ = 1.78 $\mu$M. The result was estimated as the percentage of growth inhibition compared with the untreated controls and plotted as probit values as a function of the drug concentration. IC$_{50}$ and 95% confidence limits were interpolated in the corresponding dose response curve. The pyrazoline derivative (2) showed IC$_{50}$ values in the range 0.34 $\mu$M and emerged as excellent antiamoebic agent as it was found much more active than the standard drug metronidazole, which exhibited IC$_{50}$ value 1.78 $\mu$M in these experiments. The results of antibacterial activity are summarized in Table 1.

3. Conclusion

A novel sulphur and nitrogen containing ferrocenyl linked heterocyclic compound was synthesized and well characterized by spectroscopic methods such as IR, $^1$H-NMR $^{13}$C-NMR, Mass and elemental analysis. The newly synthesized compound (2) was screened for in vitro antiamoebic activity against HM1:IMSS strain of *Entamoeba histolytica*. The result revealed that the compound showed encouraging result with the IC$_{50}$ value in the range of 0.77 $\mu$M, than the reference drug metronidazole ($IC_{50} = 1.78 \mu$M), concluding that this compounds hold immense potential to be employed as new antiamoebic agent. Also, being
novel it can be a solution to the increasing resistance that has posed a major problem globally.

4. Experimental Protocol:

4.1. Materials and Methods.

All the chemicals were purchased from Aldrich Chemical Company (USA). Precoated aluminum sheets (silica gel 60 F254, Merck Germany) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. The results were within ± 0.3% of the theoretical values. Melting points were determined on Stuart SMP10 melting point apparatus and are uncorrected. IR spectra were recorded on Perkin-Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs. 1H NMR and 13C NMR spectra were recorded on Bruker DPX-600 FT NMR spectrometer using CDCl3 as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, double doublet; m, multiplet. Chemical shift values are given in ppm. Elemental analyses were performed on a 2400 Perkin Elmer Series II analyzer. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer.

4.2. General procedure for the preparation of ferrocenyl chalcones (1).

The acetyl ferrocene (3 mmol) and KOH (0.2 g) was dissolved in ethanol (5 mL) in a round bottomed flask and stirred at room temperature (25ºC) for 10 minutes. An ethanolic solution of the 4-(methylthio) benzaldehyde (3 mmol, 5 mL) was added drop wise to the reaction mixture and stirred at room temperature. The progress of the reaction was monitored by TLC on silica gel sheets. The reaction was stopped by neutralizing the stirred solution with 2M HCl. In most cases the product was obtained as a dark red precipitate after neutralization. It was then removed by filtration, washed with water. In the absence of a precipitate on neutralization, the solution was extracted with ethyl acetate (20 mLx3). The organic layer was dried over anhydrous sodium sulphate and removed by evaporation under reduced pressure to give a liquid residue. The latter was passed through a column of silica gel (230-400 mesh) and eluted with THF-hexane (1:4) to yield pure compound.

4.2.2. 3-Ferrocenyl-1-(4-methylsulfanyl-phenyl)-2-propen-1-one (1): Yield 74%; m.p: 140 ºC; deep red solid; Anal. calc. for C21H20FeOS : C 66.31, H 5.01 %. Found: C 66.28, H 5.03 %. IR νmax (cm⁻¹): 3068 (Ar–H), 2950 (C–H), 1655 (C=O), 1564 (C=C); 1H NMR (CDCl3) δ
(ppm): 7.86-7.84 (m, 2H, Ar-H), 7.78 (d, 1H, J=15.2 Hz, H$_\beta$), 7.58 -7.43 (m, 2H, Ar-H), 7.38 (d, 1H, J=15.3 Hz, H$_\alpha$), 4.62 (s, 2H, ferrocene), 4.47 (s, 2H, ferrocene), 4.18 (s, 5H, ferrocene), 2.42 (s, 3H, CH$_3$). $^{13}$C NMR (CDCl$_3$) $\delta$ (ppm): 189.87 (C=O), 145.56 (C-$\beta$), (137.51, 133.72, 128.99, 127.43, aromatic), 122.67 (C-$\alpha$). 16.7 (SCH$_3$). ESI-MS $m/z$: [M$^{+}$+1] 376.29.

4.3. General procedure for the synthesis of 5-ferrocenyl-3-substituted aryl-4,5-dihydro-1H-pyrazol-1-carbothioamides (2).

A solution of corresponding chalcone (1) (6.3 mmol), thiosemicarbazide (12.6 mmol) and NaOH (15.8 mmol) in absolute ethanol (50 mL), was refluxed for 6 hours and then cooled to room temperature. After that, water (25 ml) was added to the reaction mixture and the solid obtained, was filtered and dried to give corresponding compounds (2).

4.3.1. 5-Ferrocenyl-3-(4-methylthiophenyl)-4,5-dihydropyrazole-1-carbothioamide (2):

Yield 65 %; m.p: 314 °C; dark orange solid. Anal. calc. for C$_{21}$H$_{21}$FeN$_3$S$_2$: C 62.54, H 5.25, N 10.42%. Found: C 62.47, H 5.22, N 10.37%. IR $\nu$ max (cm$^{-1}$): 3371, 3165 (N-H), 1557 (C=N), 1261 (C-N), 1065 (C=S); $^1$H-NMR (CDCl$_3$) $\delta$ (ppm) 7.54-7.68 (m, 3H, Ar-H), 7.42 (d, 1H, J= 8.7 Hz, Ar-H), 6.84 (s, 2H, -NH$_2$), 6.36 (dd, 1H, Hx, $J_{ax}$= 10.9, $J_{bx}$=12.5 Hz, pyrazoline), 4.61 (s, 1H, ferrocene), 4.55 (s, 1H, ferrocene), 4.49 (s, 2H, ferrocene), 4.26 (s, 5H, ferrocene), 3.81 (dd, 1H, H$_b$, $J_{ab}$=16.8, $J_{ax}$=12.5 Hz, pyrazoline), 3.52 (dd, 1H, H$_a$, $J_{ab}$=16.8, $J_{ax}$=10.9 Hz, pyrazoline), 2.34 (s, 3H, methyl); $^{13}$C-NMR (CDCl$_3$) $\delta$(ppm): 185.40 (C=S) pyrazoline), 161.55 (C=N), 58.4, (C-5, pyrazoline), 44.2 (C-4, pyrazoline), 145.9-124.5(Ar-C), 76.3 (ipso-C$_3$H$_4$), 70.2, 69.8 (meta-C$_3$H$_4$), 69.2, 69.0 (C$_5$H$_5$), 66.6, 68.2 (ortho-C$_3$H$_4$), 35.1 (-CH$_3$ phenyl); ESI-MS $m/z$: [M$^{+}$+1] 436.32.

5. In vitro antiamoebic assay:

The newly synthesized compound (2) was screened in vitro for antiamoebic activity against HM1:IMSS strain of E. histolytica by microdilution method. E. histolytica trophozoites were cultured in wells of 96-well microtiter plate by using Diamond TYIS-33 growth medium. The test compounds (1 mg) were dissolved in DMSO (40 $\mu$L, level at which no inhibition of amoeba occurs). The stock solutions of the compounds were prepared freshly before use at a concentration of 1 mg/mL. 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 at 37 ℃ 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 was estimated with a haemocytometer, using trypan blue exclusion to confirm the viability. The suspension was diluted to 10⁵ organism/mL 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 × 10⁴ 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 37 ℃ for 72 hours. 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 37 ℃. 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 IC₅₀ value was found. The IC₅₀ value in μM is reported in Table 1.

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References:

Caption to Illustration

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Figure 1: Novel pyrazoline analogue (2).

Schemes:
Scheme 1: General method for the synthesis of novel pyrazoline analogue (2).
Figure 1: Novel thiazolyl-pyrazoline derivatives (7-12).
Scheme 1: General method for the synthesis of novel pyrazoline analogue (2).

Table 1: Antiamoebic activity of novel pyrazoline analogue (2) against HM1:IMSS strain of *Entamoeba histolytica*.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>Antiamoebic activity</th>
<th>S.D. ± (μM)</th>
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<tr>
<td>1</td>
<td>2</td>
<td>2.88</td>
<td>0.013114</td>
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<tr>
<td></td>
<td>Metronidazole</td>
<td>1.78</td>
<td>0.003151</td>
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*Standard Deviation. The compounds with bold font IC₅₀ values are more active than metronidazole.

Figure 1: Novel pyrazoline analogue (2).