Synthesis, characterization, cytotoxicity and antimycobacterial screening of some p-substituted benzyl thiosemicarbazones

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The aim of present study is to synthesize a series of different p-substituted benzyl thiosemicarbazone and evaluate their cytotoxicity as well as their antimycobacterial activity. The chemical structures have been assigned by micro analysis and spectral data (UV-Vis, IR and 1H NMR). All the compounds have been tested for their in vitro cytotoxic activity against leukaemia cancer cell line K562 and cell viability was evaluated by MTT assay. All the synthesized compounds indicate significant dose-dependent cytotoxicity in very low micro-molar range (IC50 3.01-5.67 µM). The antimycobacterial drug susceptibility testing (DST) have been performed against M. smegmatis using microtiter plate by resazurin reduction assay (REMA) with glycerol and acetate as two different carbon sources. Some of the compounds show promising (>95%) result with acetate as carbon source and are being considered for further studies.

Keywords: Substituted thiosemicarbazone, cytotoxicity, MTT assay, antimycobacterial, resazurin reduction assay

Thiosemicarbazones constitute an interesting class of compounds with wide pharmacological versatility1. Thiosemicarbazones are of considerable interest and continue to be a class of compounds with broad spectrum of therapeutic activities. Several members of this class have antimicrobial2, antineoplastic3, anti-inflammatory4, anti-convulsant, tuberculostatic5,6 and antiviral including anti-HIV activities7,8. Cytotoxic activity against cancer cells has also been investigated9. Cytotoxic activity of thiosemicarbazones and their complexes against a variety of human solid tumor cell lines as well as against leukemia has been demonstrated by various authors10-14.

In order to correlate biological activity and their pharmacological application, with presence of different functional group, substituted thiosemicarbazones, 1a-f was prepared by condensation of thiosemicarbazide with six different p-substituted benzaldehydes in ethanol (Figure 1, Scheme I).

In this short communication, we are reporting the synthesis and in vitro biological activities of some p-substituted benzyl thiosemicarbazones. The compounds synthesized were evaluated for their cytotoxic activity against K 562 cell line (Human erythromyroblastoic leukemia) and were also screened for their antimycobacterial activity.

Results and Discussion

The synthesis of the target compounds 1a-f was accomplished by condensation of thiosemicarbazide with various p-substituted aromatic aldehydes, in ethanol as shown in Scheme I. The overall yield of all compounds exceeded 80%. The IR, spectra of compounds provide valuable information about functional groups present in compounds. The infrared spectra of all compounds showed characteristic absorption in the region 3495-3280 cm⁻¹ which is assigned to NH2 and NH stretching and absorption in region 1605-1580 cm⁻¹ is due to NH bending. The

![Figure 1 — Generic structure of p-substituted benzyl thiosemicarbazones](image-url)
presence of absorption band between 1380-1340 cm\(^{-1}\) has been assigned to \(\nu(C=S)\) stretching. Absorption band in region 1507-1450 cm\(^{-1}\) was assigned to \(\nu(C=N)\) stretching. I.R. spectral data of all compounds are given in their tentative assignments.

In the \(^1\)H NMR spectra, the presence of aromatic moiety was clearly identified by presence of two doublets in region \(\delta\) 8.12-6.55, except in compound \(1e\) where three doublets and one multiplet is present in this region. The presence of a singlet in all spectra at \(\delta\) 11.12-11.37 is due to NH proton. Peak near \(\delta\) 3.21-3.60 in spectra of compound \(1b\) and \(1d\) is due to methyl (CH\(_3\)) group. Presence of a one hydrogen singlet at \(\delta\) 9.8 and two hydrogen singlet at \(\delta\) 5.04 in spectra of compound \(1f\) and \(1e\) is due to phenolic-OH and methylene (-CH\(_2\)-) respectively.

All synthesized compounds were found to be in E-configuration, which was confirmed using \(^1\)H NMR spectroscopy, showing a singlet, 1H-signal of NH group in range of \(\delta\) 11.0-11.5 (Ref 15-17).This indicates that N-H hydrogen is bonded to solvent.

Thus, on the basis of above discussion, the tentative structure of the synthesized compounds could be assigned.

**Biological evaluation**

Experimental data were subjected to computer assisted statistical analysis by Graph pad prism 5 software using analysis of variance (ANOVA). The statistical significance was considered only when \(p<0.05\). The IC\(_{50}\) value was determined from sigmoidal dose-response curve by non-linear fitting of data using graph pad software\(^{18}\).

**Anti-proliferative activity**

In vitro cytotoxic activity was evaluated by 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT assay), as this technique is a reliable method to determine bioactivity of compounds\(^9\). MTT assay was also conducted in well containing 0.01% DMSO and it showed 100% cell viability suggesting no toxicity of DMSO on K562 cell lines\(^{20}\). All synthesized compounds were tested against human chronic myelogenous Leukemia cancer line K562 and showed different responses on K562 cells in culture when incubation was restricted to 48 h. The Table I shows effects on % cell viability (as % of control) of in vitro cultured human cancer cells incubated at 2, 4, 6 and 8 \(\mu\)M compound concentration.

The results show that cell viability decreased with increase in concentration from 2 to 8 \(\mu\)M. All compounds showed significant concentration dependent cytotoxicity. These compounds are found to be active in very low micro molar concentration with IC\(_{50}\) value ranging from 3.01-5.67 \(\mu\)M. Both methoxy and benzyloxy substituted benzyl thiosemicarbazone, \(1d\) and \(1e\) was found to have maximum cytotoxic potential of 3.0 \(\mu\)M. The cytotoxic potency data IC\(_{50}\) of compounds are given in Table II. In order to know if their leukemia cancer inhibition property is exclusive of any non-specific cytotoxic activity against cell, their cell inhibitory effect was evaluated in normal epithelial kidney cell line HEK-293. All compounds were found to be almost inactive in inhibiting growth and proliferation towards the normal non-cancerous cells. Results of cytotoxic activity provide only information on the restriction of the population of viable cells but not the
mechanism by which restriction is observed, which is a matter of further investigation.

**Antimicrobial screening**

The drug susceptibility studies (DST) using colorimetric methods are low cost alternatives to MGIT and BACTEC systems with good correlation (Martin et al. 2005; Martin et al. 2007; Palomino et al. 2002) (Ref 21). The colorimetric indicators such as Alamar blue, MTT and resazurin have been found to have good specificity and hence resazurin was used in this study. The resazurin reduction assays using *M. smegmatis* offers a rapid and safe screening system for antimycobacterial compounds. Mycobacterium is responsible for various diseases and tuberculosis caused by *M. tuberculosis* is one of them. Despite being a preventable disease, tuberculosis accounts for 2 million deaths per year. The addition of rifampin in 1971 enabled much shorter and more effective therapy. The result from the present study shows that % inhibition (at 50 µM test compound concentration) is less than 30% with glycerol as carbon source, which increases to 76-99% with acetate as carbon source (Table III). Among all the tested compounds, 1f was found to show maximum inhibition of about 99%. This compound is being considered for further studies on animals and work is still under progress.

**Experimental Section**

Melting points (°C) were determined in an open capillary on electro thermal melting point apparatus and are uncorrected. Elemental analysis (C, H, N and S) was performed on Varian Elementar-III instrument. Infra-red spectra were recorded in KBr pellets on Perkin-Elmer RX-1 spectrophotometer. Wave numbers in the IR spectra are given in cm⁻¹. ¹H NMR spectra of synthesized compounds were recorded in DMSO- d₆ on Bruker Avance 500 instrument. Chemical shift values are given on δ-scale relative to TMS as internal reference. Coupling constants J are expressed in Hertz. UV-Vis spectra were obtained on Labtronics LT2900 spectrophotometer operating between 200-900 nm in 1.0 cm quartz cells.

Synthetic materials and reagents were purchased from HIMEDIA chemicals. All solvents were distilled prior to use following standard procedures. RPMI-1640 medium, Middlebrook 7H9 broth medium and fetal bovine serum was purchased from Sigma-Aldrich.

**General method for preparation of benzyl substituted thiosemicarbazone 1a-f**

In a 50 mL round bottom flask fitted with a reflux condenser, *p*-substituted benzaldehyde (0.001 mol) and thiosemicarbazide (0.001 mol, 0.091 g) were added. The mixture was refluxed in ethanol with catalytic amount of acetic acid for 4 hr and left overnight. Progress of reaction was monitored by TLC. The solid that separated out was filtered and dried. The crude solid was purified by recrystallization from ethanol to give purified compound. All the compounds were confirmed for purity from their melting point, elemental analysis and other spectral studies.

(E)-2-(4-nitrobenzylidene) hydrazine-carbothioamide, 1a. Bright yellow powder; Yield: 210 mg, 87%; m.p. 196-98°C; UV-Vis (ethanol): 302, 363 and 419 nm; IR (KBr): 3489-3364 (NH₂, NH str.), 2992

### Table I — Mean % Cell viability (as % of control) data at different concentration of test compound, after 48 hr of incubation against K562 cell line

<table>
<thead>
<tr>
<th>Compd</th>
<th>Conc. (µM)</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td></td>
<td>71.94</td>
<td>52.53</td>
<td>33.12</td>
<td>18.81</td>
</tr>
<tr>
<td>1b</td>
<td></td>
<td>81.80</td>
<td>53.60</td>
<td>40.67</td>
<td>27.14</td>
</tr>
<tr>
<td>1c</td>
<td></td>
<td>69.59</td>
<td>47.12</td>
<td>38.21</td>
<td>26.76</td>
</tr>
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<td>1d</td>
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<td>67.09</td>
<td>51.12</td>
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<tr>
<td>1e</td>
<td></td>
<td>63.48</td>
<td>41.83</td>
<td>24.74</td>
<td>16.48</td>
</tr>
<tr>
<td>1f</td>
<td></td>
<td>85.12</td>
<td>48.62</td>
<td>30.56</td>
<td>22.44</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table II — IC₅₀ value of compounds against K562 leukaemia cell line after 48 hr incubation, along their standard error(±)

<table>
<thead>
<tr>
<th>Compd</th>
<th><em>IC₅₀ (±)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>5.88(±0.32)</td>
</tr>
<tr>
<td>1b</td>
<td>5.67(±0.26)</td>
</tr>
<tr>
<td>1c</td>
<td>3.14(±0.18)</td>
</tr>
<tr>
<td>1d</td>
<td>3.03(±0.26)</td>
</tr>
<tr>
<td>1e</td>
<td>3.01(±0.15)</td>
</tr>
<tr>
<td>1f</td>
<td>5.67(±0.04)</td>
</tr>
</tbody>
</table>

*50% inhibitory concentration, required to inhibit cancer cell proliferation by 50%*

### Table III — Mean % Growth inhibition against *Mycobacterium smegmatis*, in MB7H9 media with glycerol and acetate as carbon source, at an inhibitor concentration of 50 µM

<table>
<thead>
<tr>
<th>Compd</th>
<th>Carbon source</th>
<th>Glycerol</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td></td>
<td>25.36</td>
<td>86.95</td>
</tr>
<tr>
<td>1b</td>
<td></td>
<td>8.61</td>
<td>76.34</td>
</tr>
<tr>
<td>1c</td>
<td></td>
<td>13.40</td>
<td>84.19</td>
</tr>
<tr>
<td>1d</td>
<td></td>
<td>7.66</td>
<td>95.53</td>
</tr>
<tr>
<td>1e</td>
<td></td>
<td>26.32</td>
<td>96.06</td>
</tr>
<tr>
<td>1f</td>
<td></td>
<td>15.31</td>
<td>98.87</td>
</tr>
</tbody>
</table>

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(CH imines), 1581 (NH, bend.), 1522-1450 (C=N), 1366 (C=S), 1522, 1340 cm⁻¹ (NO₂); 1H NMR (DMSO-d₆): δ 11.57 (1H, s, NH), 7.86 (1H, s, =CH), 7.76 (2H, s, NH₂), 7.97-8.00 (2H, d, C-2, C-6, J = 8.6 Hz), 8.08-8.10 (2H, d, C-3, C-5, J = 8.6 Hz); MS: m/z (%) 224.04 (100.0), 225.04 (9.4), 226.03 (4.3). Anal. Calcd for C₈H₁₀N₂O₃S (Mol. Wt. 224.24): C, 50.02; H, 4.19; N, 21.13; S, 16.07%. Found: C, 62.59; H, 5.16; N, 14.39; S, 11.09%.

(E)-2-(4-(dimethylamino)benzylidene)-hydrazine-carbothioamide, 1b. Light yellowish crystalline solid; Yield: 214 mg, 89%; m.p. 179-81°C; UV-Vis (ethanol): 208, 303, and 366 nm; IR (KBr): 3435-3281 (NH, str.), 2976 (CH imines), 1603 (NH bend.), 1581 (C=N), 1522-1450 (C=N), 1372 (C=S), 1250, 1168 cm⁻¹ (OCH₃); 1H NMR (DMSO-d₆): δ 11.18 (1H, s, NH), 7.96 (1H, s, =CH), 7.96 (2H, s, NH₂), 7.6 (2H, d, C-2, C-6, J=8.7 Hz), 6.9 (2H, d, C-3, C-5, J = 8.7 Hz), 7.3 (2H, d, C-2, C-6, J = 8.7 Hz), 7.25 (1H, m), 5.04 (2H, s,-OCH₃); MS: m/z (%): 285.09 (100.0), 286.10 (16.3), 287.09 (4.8). Anal. Calcd for C₁₃H₁₁N₃O₃S (Mol. Wt. 285.36): C, 63.13; H, 5.30; N, 14.73; S, 11.24. Found: C, 62.59; H, 5.16; N, 14.39; S, 11.09%.

(E)-2-(4-hydroxybenzylidene)hydrazine-carbothioamide, 1f. Creamy white crystalline solid; Yield: 168 mg, 79%; m.p. 183-85°C; UV-Vis (ethanol): 229, 311 and 365 nm; IR (KBr): 3468-3363 (NH, bend.); 1H NMR (DMSO-d₆): δ 11.12 (1H, s, NH), 7.94 (1H, s, =CH), 7.85 (2H, s, NH₂), 7.49 (2H, d, C-2, C-6, J = 8.9 Hz), 6.67 (2H, d, C-3, C-5, J = 8.9 Hz), 9.43 (1H, s,-OH); MS: m/z (%): 195.05 (100.0), 196.05 (9.6), 197.04 (4.6). Anal. Calcd for C₁₃H₁₂N₂OS (Mol. Wt. 195.24): C, 49.21; H, 4.65; N, 21.52; S, 16.42. Found: C, 50.02; H, 4.19; N, 21.13; S, 16.07%.

Cell culture and maintenance

K562 is a human cell chronic myelogenous Leukemia cancer line (Human erythromyeoblastic leukemia) and commonly used cell line for screening of anticancer leukemia agents. This was obtained from NCCS Pune, India (job no 1196). The viable K562 cells, determined by trypan blue exclusion test, and 1×10^⁴ cells were seeded onto 96 well plates in 100 µL complete RPMI-1640 culture media supplemented with 4 mM L-glutamine, 1.5g/lit NaHCO₃, with 10% fetal bovine calf serum and allowed to grow in air in a CO₂ incubator with 5% CO₂ at 37°C.

Cytotoxicity evaluation (MTT assay)

The stock solutions of test compounds were prepared in DMSO. After 24 hr incubation, different concentrations (2, 4, 6, 8 µM) of compounds, made by serial dilution in culture medium, were incubated for 48 hr. A separate well containing 0.01% DMSO only (in absence of test compound) was run as DMSO-control, which was found to show no activity under applied conditions. The cell growth was
determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) reduction assay, which is based on the ability of viable cells to reduce a soluble yellow tetrazolium salt to blue formazan crystal\textsuperscript{26,27}. Briefly, after 48 hr of treatment, the 10 µL of MTT dye, prepared in phosphate buffered saline (PBS) were added to all wells. The plates were then incubated for 4 hr at 37°C. Supernatant from each well was carefully removed, formazan crystals were dissolved in 100 µL of DMSO and absorbance at 540 nm wavelength was recorded (Sharma \textit{et al.} 2010). Each concentration was tested in threefold.

Cell toxicity (% of control)=$\frac{(\text{OD}_i - \text{OD}_b)}{(\text{OD}_c - \text{OD}_d)} \times 100$

% Cell viability=100 – % Cell toxicity

where, OD\textsubscript{i} is mean optical density of treated wells, OD\textsubscript{b} is mean optical density of blank wells, OD\textsubscript{c} is mean optical density of control wells. The IC\textsubscript{50} values were determined as concentration of compounds that inhibited K562 cell growth by 50%.

Resazurin reduction assay (REMA)

The resazurin reduction assays using \textit{M. smegmatis} was used for screening antimycobacterial activity of compounds. The antimycobacterial drug susceptibility testing (DST) was performed using microtiter plate assay (Palomino \textit{et al.} 2002) with glycerol and acetate as two different carbon sources. The details are as provided: the \textit{M. smegmatis} log phase culture was diluted using MB7H9 medium to give an OD\textsubscript{600} of 0.05 and 100 µL of it was taken in micro titer plate. Also, 100 µL of MB7H9 medium with either glycerol or acetate as carbon source was used.

The drug concentration was initially adjusted to 50 µM. The sterility control, growth control and solvent controls were also included. All experiments were performed in duplicate. The plates were sealed properly and incubated for 48 hr at 37°C. At the end of incubation 30 µL of resazurin stock (0.02% w/v) was added, mixed thoroughly and incubated overnight at 37°C. The plates were visually scored after overnight incubation for colour change from blue to pink.

The visual observation method was used to determine growth inhibition of cells. Plates were read in duplicate on a micro plate fluorescence reader using 530/25 nm excitation filter and 590/35 nm emission filter. Data obtained was used to determine growth percentage.

Conclusion

A series of \textit{p}-substituted benzyl thiosemicarbazone was synthesized, among which 1a, 1b, 1e and 1f are newly prepared and their structure was confirmed by microanalysis, IR, \textsuperscript{1}H NMR, UV-Vis and mass spectral studies. Obtained results clearly indicates cytotoxic potential of these compounds in very low micro molar range of 3.01-5.67 µM. Antimycobacterial screening against \textit{M. smegmatis} indicates that these compounds have much inhibition potential with acetate as carbon source in comparison to glycerol. Among all compounds, 1f was found to have 99% growth inhibition activity at 50 µM of compound concentration.

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References