Cytotoxicity of New 5-Phenyl-4,5-dihydro-1,3,4-thiadiazole Analogues

Mohammad Sayed Alam,1,a,b Lijun Liu,1,b and Dong Ung Lee∗,a

a Division of Bioscience, Dongguk University; Gyeongju 780–714, Republic of Korea; b College of Chemistry and Chemical Engineering, Ningxia University; Yinchuan 750021, China; and * Department of Chemistry, Jagannath University; Dhaka 1100, Bangladesh.

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A series of 5-phenyl-4,5-dihydro-1,3,4-thiadiazoles were synthesized and their cytotoxicity was examined against four human cancer cell lines, e.g. lung cancer (A549), ovarian cancer (SK-OV-3), skin cancer (SK-MEL-2), and colon cancer (HCT15). The title compounds were synthesized by condensation of thiosemicarbazide with substituted benzaldehydes, followed by cyclization with acetic anhydrides in good yields. Most of the compounds exhibited significant suppressive activity against the growth of all of the cancer cell lines. The 4-hydroxy analogue of 5-phenyl-4,5-dihydro-1,3,4-thiadiazole (2h) was most active in the inhibition of growth of the SK-MEL-2 cell line, with an IC 50 value of 4.27 µg/ml; followed by compound 2a (IC 50 5.16 µg/ml). The compounds 2j, 2h, and 2b, bearing 3-methoxy-4-hydroxy-, 4-hydroxy- and 4-methyl substituents in the C-5 phenyl ring respectively, exhibited the highest activity against the SK-OV-3 (IC 50 7.35 µg/ml), HCT15 (IC 50 8.25 µg/ml) and A549 (IC 50 9.40 µg/ml) cell lines, respectively. A structure–activity relationship study revealed that an optimal electron density on the C-5 phenyl ring of 1,3,4-thiadiazoles is crucial for their cytotoxic activity against the human cancer cell lines used in the present study.

Key words 5-phenyl-4,5-dihydro-1,3,4-thiadiazole; cytotoxicity; human cancer cell line

Phenyl-1,3,4-thiadiazoles are a class of small molecules that have received much interest in the fields of chemistry and biology due to their broad spectrum of activity. The 1,3,4-thiadiazole scaffold is an interesting building block that has been used to synthesize a variety of useful bioactive compounds. There is a progressive body of studies on the synthesis of 1,3,4-thiadiazole analogues and their wide range of pharmacological actions in the literature. Phenyl-1,3,4-thiadiazole derivatives have been reported to be antimicrobial,11 anticancer,12 anti-tubercular,13 anti-convulsant,14 anti-inflammatory,5 anti-anaesthetic,5 anti-anxiety, anti-depressant6 and anti-viral7 agents.

Cancer is one of the major causes of death globally. Many anticancer agents exert their effects through the destruction of rapidly dividing cells, and these cytotoxic agents remain the primary resource in cancer chemotherapy, despite advances in understanding of the cell cycle that may help develop more selective chemotherapeutic agents that target only cancer cells. During the last few decades, anticancer therapy has progressed significantly, but the management of malignancies in humans still constitutes a major concern for contemporary medicine. Phenyl-1,3,4-thiadiazole and its derivatives are well known, broad spectrum anticancer agents8–10 that have been proven in vivo conditions.11,12

As a part of our continuing search for novel biologically active molecules, we have designed and synthesized 5-phenyl-4,5-dihydro-1,3,4-thiadiazole analogues (2a–j) and evaluated their anticancer properties. The compounds 2a–j were synthesized, by modification of a known method,13 from their corresponding thiosemicarbazone analogues (1a–j). Furthermore, we investigated the in vitro cytotoxic activities of these new compounds against four culture cell lines: A549 (human lung cancer), SK-OV-3 (human ovarian cancer), SK-MEL-2 (human skin cancer), and HCT15 (human colon cancer).

Synthesis of 5-Phenyl-4,5-dihydro-1,3,4-thiadiazoles

Synthesis of the 5-phenyl-4,5-dihydro-1,3,4-thiadiazole analogues (2a–j) was achieved following a convenient two-step procedure; starting from commercially available starting materials as outlined in Chart 1. The intermediate arylidene-thiosemicarbazone analogues (1a–j) were synthesized by the condensation reaction of thiosemicarbazide and substitution of benzaldehyde in ethanol, with excellent yields (80–98%). The structures of compounds 1a–j were confirmed by IR and 1H-NMR spectral data. In the IR spectrum of the arylidenethiosemicarbazones (1a–j), the –NH– and –NH– protons showed an absorption band at around the 3490–3373 cm−1 and 3363–3239 cm−1 regions, respectively. In the 1H-NMR spectra of compounds 1a–k, two amino (–NH2) protons appeared at 7.67–8.26 and 7.81–8.38 ppm due to en-thiol tautomerism, while these protons disappeared in compounds 2a–j. The =N–NH– and –CH=NH– protons were observed as singlet at 11.17–11.71 and 7.93–8.41 ppm, respectively, equivalent to one proton each. The aromatic proton signals appeared at 6.67–8.63 ppm, either as multiplets or doublets, according to the substitution pattern on the phenyl ring.

The cyclization of arylidenethiosemicarbazones (1a–j), achieved by refluxing with acetic anhydrides in ethanol, yielded 5-phenyl-4,5-dihydro-1,3,4-thiadiazole (2a–j) analogues in good yields (67–80%). All the synthesized 5-phenyl-4,5-dihydro-1,3,4-thiadiazoles were characterized by IR, 1H-NMR, and electron ionization (EI)-MS spectral data together with elemental analysis. In the IR spectrum of compounds 2a–j, the characteristic N–H and >C=O stretching absorption bands appeared at the 3222–3203 cm−1 and 1713–1631 cm−1 regions, respectively. The 1H-NMR of compounds 2a–j showed a characteristic singlet for the C-5 proton at 6.75–6.89 ppm, with an upfield shift of 1.18–1.52 ppm from that of the imino proton (–CH=NH–) of the open-chain thiosemicarbazones (1a–j), which was in good agreement with other reported chemical shifts of 1,3,4-thiadiazole analogues.14,15 Two acetyl groups appeared as two singlets at 2.01–2.12 and 2.23–2.30 ppm. The amide pro-
ton (–NHCO–) of compounds 2a—j was observed as a singlet at 11.63—11.89 ppm. The phenyl protons were assigned in the usual way, according to their substitution pattern. EI-MS spectra of 2a—j showed a molecular ion peak with intensities from 85—100%.

Anticancer Activity

The anticancer activity of compounds 2a—j was evaluated by an in vitro assay performed on four human cancer cell lines: lung cancer (A549), ovarian cancer (SK-OV-3), skin cancer (SK-MEL-2), and colon cancer (HCT15). The activity was evaluated by measuring the inhibition of the net growth of cells, measured as a percent of the control samples, after incubation for 48 h with the test samples, using the SRB (sulforhodamine-B) method. All activities were compared with cisplatin as a positive control.

The activity results presented in Table 1 report the cytotoxic effects of 5-phenyl-4,5-dihydro-1,3,4-thiadiazole (2a—j) analogues against the various cancer cell lines. Among the tested compounds 2a, b, h and j showed significant cytotoxic effects against all the cancer cell lines. Compound 2h exhibited the highest activity (IC50 4.27 μg/ml) and selectivity, followed by 2a (IC50 5.16 μg/ml), against the skin cancer (SK-MEL-2) cell line. While compound 2j exhibited the highest cytotoxic activity (IC50 7.35 μg/ml) and selectivity against the ovarian cancer (SK-OV-3) cell line, followed by 2h (IC50 10.14 μg/ml). Compound 2b exhibited significant potency against the lung cancer (A549) cell line, with an IC50 value of 9.40 μg/ml; followed by compounds 2a (IC50 14.27 μg/ml) and 2h (IC50 15.58 μg/ml). Compound 2h exhibited significant potency against the colon cancer (HCT15) cell line, with an IC50 value of 8.25; followed by compounds 2a, j and b, at 10.25, 10.83 and 12.24 μg/ml, respectively.

The structure–activity relationship study revealed that the nature of the substituted group on the phenyl ring of 5-phenyl-4,5-dihydro-1,3,4-thiadiazole plays an important role in the cytotoxic activity of the compound. Compound 2a, which possessed an unsubstituted phenyl ring, was 2—3 times more cytotoxic against SK-MEL-2 cell lines (IC50 5.16 μg/ml) than against the A549, SK-OV-3 and HCT15 cell lines. Introduction of a methyl group, a weak electron-donating group, at the para-position on the phenyl ring resulted in compound 2b, which exhibited greater activity against A549 cell lines than against SK-MEL-2 and HCT15, but was simi-

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**Table 1. In Vitro Cytotoxicity Data of the 5-Phenyl-4,5-dihydro-1,3,4-thiadiazole Analogues (2a—j) against Selected Human Cancer Cell Lines**

<table>
<thead>
<tr>
<th>Compd.</th>
<th>R1</th>
<th>R2</th>
<th>IC50 (μg/ml)</th>
<th>A549</th>
<th>SK-OV-3</th>
<th>SK-MEL-2</th>
<th>HCT15</th>
</tr>
</thead>
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<tr>
<td>2a</td>
<td>H</td>
<td>H</td>
<td>14.27</td>
<td>13.22</td>
<td>5.16</td>
<td>10.25</td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>Me</td>
<td>H</td>
<td>9.40</td>
<td>13.39</td>
<td>11.05</td>
<td>12.24</td>
<td></td>
</tr>
<tr>
<td>2c</td>
<td>NO2</td>
<td>H</td>
<td>102.11</td>
<td>108.67</td>
<td>105.25</td>
<td>98.71</td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td>H</td>
<td>NO2</td>
<td>135.25</td>
<td>175.58</td>
<td>139.21</td>
<td>140.65</td>
<td></td>
</tr>
<tr>
<td>2e</td>
<td>NMe2</td>
<td>H</td>
<td>144.85</td>
<td>187.62</td>
<td>179.56</td>
<td>135.15</td>
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</tr>
<tr>
<td>2f</td>
<td>Cl</td>
<td>H</td>
<td>72.61</td>
<td>78.52</td>
<td>53.33</td>
<td>60.27</td>
<td></td>
</tr>
<tr>
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<td>Br</td>
<td>H</td>
<td>60.29</td>
<td>21.76</td>
<td>28.73</td>
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<tr>
<td>2h</td>
<td>OH</td>
<td>H</td>
<td>15.58</td>
<td>10.14</td>
<td>4.27</td>
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<tr>
<td>2i</td>
<td>OMe</td>
<td>H</td>
<td>180.12</td>
<td>188.75</td>
<td>168.16</td>
<td>137.86</td>
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<tr>
<td>2j</td>
<td>OH</td>
<td>OMe</td>
<td>28.16</td>
<td>7.35</td>
<td>29.76</td>
<td>10.83</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td></td>
<td>1.4</td>
<td>0.9</td>
<td>0.8</td>
<td>2.2</td>
<td></td>
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</table>

a) IC50 values were obtained using a dose response curve by nonlinear regression using a curve fitting program, OriginPro 7.5.
lar to that against the ovarian cancer (SK-OV-3) cell lines. Introduction of a hydroxy group, which has more electron-donating capacity than a methyl group, yielded compound 2h, which had enhanced activity against all the cell lines, except A549, compared with that of 2a. Increasing the electron-donating capacity of the hydroxy group, by replacing it with methoxy group (compound 2i), led to a substantial loss in activity against all cell lines. N,N-dimethylamino (2c), reduced a potency and nitro group (2f) the SK-OV-3 and SK-MEL-2 cell lines compared to that of 70 eV). Elemental analyses (C, H, N) were performed by means of a Perkin-Elmer 2400 II CHN elemental analyzer.

Preparation of Thiourea Analogue

The synthesis of thioureas was carried out according to the previously described method. Briefly, an alcoholic solution of substituted benzaldehydes (1 mmol) was added slowly to a stirred solution of thiosemicarbazide (1 mmol) in an ethanol–water mixture, and refluxed for 10—20 min. After cooling the reaction mixture to an ambient temperature, the mixture was filtered to give a solid crude product, which was crystallized from ethanol to provide the pure compounds 1a—j with a yield of 80—98%.

2-Benzylidenehydracinecarbothioamide 1a: Yield 98%. mp 164—165 °C. 1H-NMR δ: 3.75—7.52 (m, 3H, Ar-H), 7.87—8.00 (m, 2H, Ar-H), 7.74 and 7.81 (2 × s, 2H, –NH2), 8.17 (s, 1H, CH), 11.40 (s, 1H, –NH–). IR (KBr) cm−1: 3422 (NH), 3251 (NH), 1590 (C=N), 1298 (C=S).

2-(4-Methylbenzylidenehydracinecarbothioamide 1b: Yield 80%. mp 175—176 °C. 1H-NMR δ: 2.42 (s, 3H, –CH3), 6.96 (d, 2H, J = 8.2 Hz, Ar-H), 7.76 (d, 2H, J = 8.2 Hz, Ar-H), 7.92 and 8.12 (2 × s, 2H, –NH2), 8.03 (s, 1H, CH), 11.33 (s, 1H, –NH–). IR (KBr) cm−1: 3390 (NH2), 3270 (NH), 1610 (C=N), 1260 (C=S).

2-(Nitrobenzylidenehydracinecarbothioamide 1c: Yield 98%. mp 250—231 °C. 1H-NMR δ: 8.07 (d, 2H, J = 8.2 Hz, Ar-H), 8.22 (d, 2H, J = 8.2 Hz, Ar-H), 8.26 and 8.38 (2 × s, 2H, –NH2), 8.41 (s, 1H, CH), 11.71 (s, 1H, –NH–). IR (KBr) cm−1: 3490 (NH2), 3363 (NH), 1589 (C=N), 1247 (C=S).

2-(3-Nitrobenzylidenehydracinecarbothioamide 1d: Yield 98%. mp 215—216 °C. 1H-NMR δ: 7.64—7.81 (m, 2H, Ar-H), 8.42—8.63 (m, 2H, Ar-H), 7.67 and 7.81 (2 × s, 2H, –NH2), 8.19 (s, 1H, CH), 11.60 (s, 1H, –NH–). IR (KBr) cm−1: 3393 (NH2), 3239 (NH), 1603 (C=N), 1299 (C=S).

2-(4-Methylamino)benzylidenehydracinecarbothioamide 1e: Yield 86%. mp 193—194 °C. 1H-NMR δ: 2.94 (s, 6H, CH3), 6.67 (d, 2H, J = 8.2 Hz, Ar-H), 7.55 (d, 2H, J = 8.2 Hz, Ar-H), 7.75 and 7.91 (2 × s, 2H, –NH2), 7.99 (s, 1H, CH), 11.17 (s, 1H, NH). IR (KBr) cm−1: 3373 (NH), 3330 (NH), 1600 (C=N), 1269 (C=S).

2-(4-Chlorobenzylidenehydracinecarbothioamide 1f: Yield 98%. mp 209—211 °C. 1H-NMR δ: 7.43 (d, 2H, J = 8.2 Hz, Ar-H), 7.84 (d, 2H, J = 8.2 Hz, Ar-H), 8.00 and 8.02 (2 × s, 2H, –NH2), 8.23 (s, 1H, CH), 11.47 (s, 1H, –NH–). IR (KBr) cm−1: 3436 (NH2), 3278 (NH), 3105 (C=S), 1600 (C=N), 1282 (C=S).

2-(4-Chloro-3-methylbenzylidenehydracinecarbothioamide 1g: Yield 91%. mp 219—220 °C. 1H-NMR δ: 7.43 (d, 2H, J = 8.1 Hz, Ar-H), 7.84 (d, 2H, J = 8.1 Hz, Ar-H), 8.01 and 8.07 (2 × s, 2H, –NH2), 8.23 (s, 1H, CH), 11.47 (s, 1H, –NH–). IR (KBr) cm−1: 3431 (NH2), 3278 (NH), 1609 (C=N), 1279 (C=S).

2-(4-4-Hydroxybenzylidenehydracinecarbothioamide 1h: Yield 92%. mp 217—218 °C. 1H-NMR δ: 6.73 (d, 2H, J = 8.2 Hz, Ar-H), 7.60 (d, 2H, J = 8.2 Hz, Ar-H), 7.71 and 7.91 (2 × s, 2H, –NH2), 8.04 (s, 1H, CH), 9.84 (s, 1H, OH), 11.22 (s, 1H, –NH–). IR (KBr) cm−1: 3476 (NH2), 3359 (NH), 1608 (C=N), 1232 (C=S).

2-(4-Methoxybenzylidenehydracinecarbothioamide 1i: Yield 88%. mp 172—173 °C. 1H-NMR δ: 3.80 (s, 3H, CH3), 6.96 (d, 2H, J = 8.3 Hz, Ar-H), 7.76 (d, 2H, J = 8.3 Hz, Ar-H), 7.92 and 8.12 (2 × s, 2H, –NH2), 8.03 (s, 1H, CH), 11.32 (s, 1H, –NH–). IR (KBr) cm−1: 3390 (NH2), 3270 (NH), 1610 (C=N), 1260 (C=S).

2-(4-Hydroxy-3-methoxybenzylidenehydracinecarbothioamide 1j: Yield 85%. mp 198—199 °C. 1H-NMR δ: 3.81 (s, 3H, CH3), 7.45—7.73 (m, 3H, Ar-H), 7.90 and 8.09 (2 × s, 2H, –NH2), 7.93 (s, 1H, CH), 9.43 (s, 1H, OH), 11.24 (s, 1H, –NH–). IR (KBr) cm−1: 3434 (NH2), 3276 (NH), 1587 (C=N), 1276 (C=S).

Preparation of 5-Phenyl-4,5-dihydro-1,3,4-thiadiazol Analogue 2a—j A mixture of thiosemicarbazide (5 mmol) and acetic anhydride (7 ml) in ethanol was refluxed with constant stirring for 30 min and then cooled to ambient temperature. The mixture was poured into ice cold water and neutralized with dilute sodium hydroxide solution. A white precipitate was produced, the mixture was filtered and washed with water to give a crude product. Then it was purified by recrystallization with N,N-dimethylformamide–EtOH, affording pure thiadiazole derivatives 2a—j.

2-(4-Acetyl-5-phenyl-4,5-dihydro-1,3,4-thiadiazol-2-yl)acetamide 2a: Yield 78%. mp 201—202 °C (liq.) 222—223 °C. 1H-NMR δ: 2.01 (3H, CH3), 7.45—7.73 (m, 3H, Ar-H), 7.90 and 8.09 (2 × s, 2H, –NH2), 7.93 (s, 1H, CH), 9.43 (s, 1H, OH), 11.24 (s, 1H, –NH–). IR (KBr) cm−1: 3434 (NH2), 3276 (NH), 1587 (C=N), 1276 (C=S).

Preparation of 5-Phenyl-4,5-dihydro-1,3,4-thiadiazol Analogue 1a—j The synthesis of thiosemicarbazones was carried out according to the previously described method. Briefly, an alcoholic solution of substituted benzaldehydes (1 mmol) was added slowly to a stirred solution of thiosemicarbazide (1 mmol) in an ethanol–water mixture, and refluxed for 10—20 min. After cooling the reaction mixture to an ambient temperature, the mixture was filtered to give a solid crude product, which was crystallized from ethanol to provide the pure compounds 1a—j with a yield of 80—98%.
C_{12}H_{12}N_{4}O_{4}S: C, 46.75; H, 3.92; N, 18.17. Found: C, 46.79; H, 3.89; N, 18.14.

N-(4-Acetyl-5-(4-nitrophenyl)-4,5-dihydro-1,3,4-thiadiazol-2-yl)acetamide 2c: Yield 70%. mp 218—219 °C (lit. 15) mp 210—211 °C. 1H-NMR δ: 2.07 (3H, –CH₃), 6.87 (1H, H-5), 7.08—7.16 (m, 4H, Ar-H), 11.72 (1H, –NH–). IR (KBr) cm⁻¹: 3207, 1695, 1633, 1608, 1519, 1489. EI-MS m/z (%): 309 (M⁺, 100), 265 (30). Anal. Caled for C_{12}H_{12}N_{4}O_{4}S: C, 46.75; H, 3.92; N, 18.17. Found: C, 46.79; H, 3.89; N, 18.14.

N-(4-Acetyl-5-(4-nitrophenyl)-4,5-dihydro-1,3,4-thiadiazol-2-yl)acetamide 2d: Yield 67%. mp 233—234 °C (lit. 15) mp 222 °C. 1H-NMR δ: 2.07 (3H, –CH₃), 2.19 (3H, –COCH₃), 6.86—7.00 (m, 2H, Ar-H), 6.94 (1H, –NH–). IR (KBr) cm⁻¹: 3215, 1695, 1633, 1608, 1519, 1485. EI-MS m/z (%): 307 (M⁺, 100), 265 (30). Anal. Caled for C_{12}H_{12}N_{4}O_{4}S: C, 46.75; H, 3.92; N, 18.17. Found: C, 46.79; H, 3.89; N, 18.14.

C_{13}H_{15}N_{3}O_{3}S: C, 53.23; H, 5.45; N, 15.08.

**Cytotoxicity Assay**
Cytotoxicity after treatment of the tumor cells with the test materials was determined using the SRB (sulforhabdoid-B) method, currently adopted in the NCI’s in vitro anti-cancer drug screening, i.e., the inhibition rate of cell proliferation was estimated after continuous exposure to the test materials for 48 h. All samples were tested in triplicate and the mean IC₅₀ values (µg/ml) (the concentration of the compound that resulted in a 50% inhibition of cell proliferation) and the S.E.M. were calculated, respectively.

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