SYNTHESIS OF N-\{5-(2,4-DICHLOROPHENYL)-1, 3, 4-OXADIAZOL-2-YL\} M ETHYL}AMINE DERIVATIVES AS ANTICANCER PRECURSORS

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ABSTRACT
In the present study N-[5-(2,4-dichlorophenyl)-1,3,4-oxadiazol-2-yl]methyl}amine derivatives5(a-h) were synthesized and tested for invitro anticancer activity. Cyclisation of 2, 4-dichlorobenzohydrazide in chloroacetic acid and phosphorous oxy chloride gives 2-(chloromethyl)-5-(2,4-dichlorophenyl)-1,3,4-oxadiazole. This on reaction with various primary and secondary aliphatic or aromatic amines gives the title compounds. The anticancer activity of some of the prepared compounds was evaluated using three human tumour cell lines, representing cervic, liver and breast. The compounds tested were, in most of cases, selective towards liver cancer, where the most potent compound showed IC₅₀ = 2.46 μg/mL. The synthesized compounds were purified by column-chromatography and characterized by LCMS, TLC, IR, and ¹HNMR spectral data. Three different cell lines are used for the present study namely (Hela, Hep-G2 and MCF7).

Keywords: Hela, Phosphorus oxy chloride, Hep-G, Anticancer, Triethylamine, MCF7.

INTRODUCTION
In recent years 1,3,4-oxadiazoles and its derivatives have received considerable attention owing to their synthetic and effective biological importance [1]. The heterocycles bearing a symmetrical oxadiazole moiety were reported to show a broad spectrum of pharmacological properties viz., anticonvulsant, insecticidal [2], fungicidal [3], antiviral, anti-cancer [4] activities etc. Cancer is a known medically as a malignant neoplasm is a broad group of diseases involving unregulated cell growth that affecting millions of people worldwide. The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. Cancer is usually treated with chemotherapy, radiation therapy and surgery. In the present study N-[5-(2,4-dichlorophenyl)-1,3,4-oxadiazol-2-] methyl}amine derivatives have been found to be more potent molecules in inhibiting cancer cell lines. 1, 3, 4 - oxadiazole ring are known to have potent anticancer properties. This importance of oxadiazole nucleus and continuing demand for new anticancer agents, prompted us to synthesis about eight 1,3,4-oxadiazole derivatives.

EXPERIMENTAL
The chemicals required for the study were obtained from spectrochem and s-d fine chemicals. The melting points of these synthesized compounds were determined in open capillary tube. The IR spectra were recorded by preparing KBr pellets containing 1% compounds using FTIR-8400 spectrometer. Liquid Crystal Mass spectra(LCMS) of the samples were recorded using Agilent and the ¹HNMR spectra was recorded using Varian (400MHz) NMR spectrometer.

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EXPERIMENTAL PROCEDURE

Step 1: Synthesis of ethyl 2,4-dichlorobenzoate

2,4-Dichlorobenzoic acid (0.0523mol, 10g) was refluxed with concentrated H\textsubscript{2}SO\textsubscript{4} (2.5ml) in absolute ethanol (100cm\textsuperscript{3}) for 4 hours. The formation of ester was monitored by TLC and the solvent was removed under reduced pressure. Ice cold water was added and later the aqueous was neutralised with saturated solution of NaHCO\textsubscript{3}. The product is extracted with ethyl acetate (25ml x 3) and organic layer was washed with brine (10ml), dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated under reduced pressure. The product is taken as such for next step. Yield (%) 88, \textsuperscript{1}HNMR-1.15(t,3H), 3.85(q,2H), 7.5(m,2H), 8.5 (s,1H) TLC ethyl acetate: hexane (2:8).

Step 2: Synthesis of 2,4-dichlorobenzohydrazide

The ethyl 2, 4-dichlorobenzoate (8.8g) was refluxed with excess of hydrazine hydrate in ethanol for 8 hrs. The reaction was monitored by TLC. After the completion of reaction solvent was removed under reduced pressure. The liquid residue was added with few ice pieces and the solid thus obtained was filtered, washed with cold water, and dried under vacuum. The crude solid was recrystallized with ethanol and used for the next step (3). Yield (%) 68, \textsuperscript{1}HNMR-4.3(bs,2H), 7.5(m,2H), 7.8(d,1H), 9.4 (s,1H). IR 1350cm\textsuperscript{-1}(C-N stretching), 3458 cm\textsuperscript{-1} (NH- stretching), 1570 cm\textsuperscript{-1} (NH bend), TLC Ethyl acetate: hexane (5:5).

Step 3: Synthesis of 2-(chloromethyl)-5-(2,4-dichlorophenyl)-1,3,4-oxadiazole

The 2,4-dichlorobenzohydrazide (5.9 g, 0.042mol) was added with phosphorous oxychloride (10ml) and chloroacetic acid (8.116g, 3eq, 0.0863mol). The reaction mixture was irradiated using microwave for about 5 minutes (30sec/interval). The reaction mixture was neutralised with saturated solution of NaHCO\textsubscript{3}. The solid thus obtained was filtered, washed with water and dried under vacuum. Yield (%) 45, NMR-7.97(d,1H), 7.60(d,1H), 7.42(dd,1H), 4.56(s,2H), IR 1400cm\textsuperscript{-1}(C-N stretching), 3490cm\textsuperscript{-1} (NH- stretching), 1580 cm\textsuperscript{-1} (NH bend), TLC Ethyl acetate: hexane (2:8).

Scheme 2: General procedure for the synthesis of N-[(5-(2,4-dichlorophenyl)-1, 3, 4-oxadiazol-2-yl)methyl]alkyl or arylamine derivatives

The 2-(chloromethyl)-5-(2,4-dichlorophenyl)-1,3,4-oxadiazole (250mg, 1eq.) was dissolved in 1,4-dioxan (10ml) containing triethylamine (100µl). To this reaction mixture various substituted aliphatic or aromatic amines (1.2eq) was added while stirring. The reaction mixture was heated to 80°C for 2-8hrs. Reaction was monitored by TLC. After the completion of the reaction, reaction mixture was concentrated under reduced pressure, ice cold water was added and the product was extracted using ethyl acetate (10ml x 2). The organic layer was washed with brine (10ml), dried over sodium sulphate and evaporated to dryness. (Synthetic scheme-2)

PURIFICATION

All the final compounds were purified by column chromatography using silica gel 100-200 mesh. 100% n-hexane has been used as eluent for column and later using ethyl acetate it was decreased to 75%. Yield (%) 68. TLC ethyl acetate: hexane (5:5). Table -02 summarizes the physical and analytical data of compounds.

ANTICANCER ACTIVITY

Eight compounds (5a, 5b, 5c, 5d, 5e, 5f, 5g and 5h) have been selected for the screening: Four different concentrations 1, 2.5, 5 and 10 mg/mL of each compound were employed. Three human cell lines were used in this experiment namely: a) human cervic carcinoma cell lines b) human liver carcinoma cell line (HepG2) and c) human breast carcinoma cell line (MCF7). Stock cultures were grown in T-75 flasks containing 50 mL of RPMI-1640 medium with glutamine bicarbonate and 5% fet al., calf serum. Medium was changed at 48 hr intervals. Cells were dissociated with 0.25% trypsin. Experimental cultures were plated in micro-titre plates, containing 0.2 mL of growth medium per well at a densities of 1,000-200,000 cells per well.

Cell fixation

Cells attached to the plastic substratum were fixed by gently layering 50 mL of cold 50% TCA (4°C) on the top of the growth medium in each well to produce a final TCA concentration of 10%. The cultures were incubated at 4°C for one hour and then washed five times with tap water to remove TCA, growth medium and low-molecular weight metabolites, and serum proteins. Plates were air dried and then stored until its further use. Background optical densities were measured in wells incubated with growth medium without cells. The anionic dye sulforhodamine B (SRB, Sigma Chemical Co.) was dissolved in 1% acetic acid for cell staining and extracted from cells with 10 mM buffered trisbase [tris (hydroxymethyl) amino methane].

SRB Assay

TCA-fixed cells were stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and cultures were quickly rinsed for four times with 1% acetic acid to remove unbound dye. The acetic acid was poured directly into the culture wells from a beaker. This procedure permitted rinsing to be performed so quickly such that desorption of protein-bound dye does not occur. Residual solution was removed by sharply flicking plates over a sink, which ensured the complete removal of rinsing solution. Because of the strong capillary action in 96-well plates, draining by gravity alone often failed to remove the
rinsing solution by simple inverting technique. After being rinsed, the cultures were air dried until no standing moisture was visible. Bound dye was solubilized with 10 mM unbuffered tris base (pH 10.5) for 5 min on gyratory shaker. OD (optical density) was read on a UVmax microtitre plate reader at 564 nm for maximum sensitivity.

RESULTS AND DISCUSSION

Starting from 2,4-dichloro ethyl benzoate [5], which are prepared according to the method of kaushik and darpan [6], reaction with hydrazine hydrate with ethyl 2,4-dichloro benzoate yields the appropriate 2,4-dichlorobenzohydrazide [7]. Cyclisation of the hydrazine in the presence of excess chloroacetic acid and phosphorous oxychloride gave the corresponding 2-(chloromethyl)-5-(2,4-dichlorophenyl)-1,3,4-oxadiazole-2-yl methyl amine 5(a) respectively. The presence of methyl amine side chain might overcome the water insolubility problem of 1,3,4-oxadiazole compounds, thus increasing their Bio availability (Scheme 2). On the other hand, since many 2,4-disubstituted phenyl 1,3,4-oxadiazole derivatives possess anticancer activity [13] it prompted us synthesize some novel 2,4-disubstituted phenyl derivatives.

ANTICANCER SCREENING

Above eight compounds were screened for anticancer activity at Centre for Cellular and Molecular Platforms (C-CAMP),Tata institute of Fundamental Research (TIFR), Bangalore, India. Three cell lines were used for the evaluation (Human cervic carcinoma cell line, human liver carcinoma cell line and human breast carcinoma cell line).The results are expressed in the form of the concentration of compound that causes 50% inhibition of cells growth. The invivo evaluation revealed that the activity of the tested compounds was higher towards both the liver cancer and cervic cancer than the breast cancer. Two compounds (5c, 5d,) were showed the activity towards all the 3-cell lines while compounds 5b, 5c, 5d, 5g, 5e and 5f were selective towards the liver cancer. Compound 5h was the only compound not selective towards any cell line. The cytotoxicity of the compound 5h is very high towards all the 3 cell lines. The results of the anticancer screening of the tested compounds are illustrated in Table no 3.

<table>
<thead>
<tr>
<th>Compounds Name/No</th>
<th>R²</th>
<th>m/z</th>
<th>Yield(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>(a)</td>
<td>364</td>
<td>85</td>
</tr>
<tr>
<td>5b</td>
<td></td>
<td>338</td>
<td>76</td>
</tr>
<tr>
<td>5c</td>
<td></td>
<td>378</td>
<td>65</td>
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Table 1. Percentage yield and m/z of the various 1, 3, 4-oxadiazole derivatives.
Table 2. Physicochemical characteristics of compounds 5(a-h)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular formula</th>
<th>IR cm⁻¹</th>
<th>LCMS (m+1)</th>
<th>M.P (°C)</th>
<th>¹H NMR (dms-o-d₆)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>C₁₆H₁₁Cl₂N₃O₃</td>
<td>3300(NH-stretch), 1650 (C=O), 3186 (Ar-H)</td>
<td>366</td>
<td>161</td>
<td>3.7(s,2H),7.1(dd,1H),7.6 (m,2H),8.6(m,3H),8.9 (dd,2H)</td>
</tr>
<tr>
<td>5b</td>
<td>C₁₅H₁₀Cl₂F₃N₂O</td>
<td>3322(NH-stretch),3146 (Ar-H)</td>
<td>340</td>
<td>143</td>
<td>5.1 (bs,2H),6.85 dd,2H),7.15 (dd,2H)</td>
</tr>
<tr>
<td>5c</td>
<td>C₁₇H₁₅Cl₂N₃O₃</td>
<td>3414 (NH-stretch), 1690(C=O), 3032 (Ar-H)</td>
<td>380</td>
<td>148</td>
<td>1.5(s,1H),3.7(s,2H),7.5 (m,3H),7.7(m,3H),11.5 (bs,1H)</td>
</tr>
<tr>
<td>5d</td>
<td>C₁₃H₁₇Cl₂N₅O₃</td>
<td>3373(NH-stretch), 3142 (Ar-H)</td>
<td>392</td>
<td>155</td>
<td>2.5 (q,2H),2.7(q,2H),3.3 (s,2H),6.9(dd,2H),7.10 (dd,2H),7.7 (m,2H),7.85(m,1H)</td>
</tr>
<tr>
<td>5e</td>
<td>C₁₃H₁₅Cl₂N₅O₃</td>
<td>3350(NH-stretch), 3122 (Ar-H)</td>
<td>301</td>
<td>208</td>
<td>0.6 (t,6H),2.3(q,4H),3.6 (s,2H),7.6(dd,1H),7.45 (m,2H)</td>
</tr>
<tr>
<td>5f</td>
<td>C₁₁H₁₁Cl₂N₂O₂</td>
<td>3120(Ar-H),1280(C-N), 1560 (NH-bend), 3340(NH-stretch), 1529(C-O) bend</td>
<td>289</td>
<td>211</td>
<td>2.6(m,4H),3.6 (s,2H),7.45 (m,2H),7.6(dd,1H),12.35(bs,1H)</td>
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<tr>
<td>5g</td>
<td>C₁₃H₁₁Cl₂N₃O₃</td>
<td>3152 (Ar-H),1300 (C-N), 1580(NH-bend), 3360(NH-stretch),</td>
<td>321</td>
<td>189</td>
<td>5.1(s,1H),3.7 (s,2H),6.9 m,2H),7.7(dd,2H)</td>
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<tr>
<td>5h</td>
<td>C₁₄H₁₀Cl₂N₄O₂</td>
<td>3120(Ar-H), 1200(C-N), 1560(NH-bend),3340(NH-stretch),</td>
<td>328</td>
<td>176</td>
<td>1.85(s,3H),2.6(q,4H),3.7(s,2H),7.6 (dd,,2H),7.45(m,2H)</td>
</tr>
</tbody>
</table>

Table 3. Effect of the synthesised compounds on Hela, Liver carcinoma and Breast carcinoma cell lines

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC 50(µg/ml)</th>
<th>Hela</th>
<th>Hep-G2</th>
<th>MCF7</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>6.08</td>
<td>-</td>
<td>-</td>
<td>7.89</td>
</tr>
<tr>
<td>5b</td>
<td>4.56</td>
<td>6.78</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5c</td>
<td>6.78</td>
<td>4.34</td>
<td>5.45</td>
<td></td>
</tr>
<tr>
<td>5d</td>
<td>3.54</td>
<td>2.46</td>
<td>8.98</td>
<td></td>
</tr>
<tr>
<td>5e</td>
<td>-</td>
<td>3.40</td>
<td>4.56</td>
<td></td>
</tr>
<tr>
<td>5f</td>
<td>-</td>
<td>4.10</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5g</td>
<td>9.04</td>
<td>5.62</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5h</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Reaction Scheme 1. Synthesis of 2-(chloromethyl)-5-(2,4-dichlorophenyl)-1,3, 4-oxadiazole

\[
\begin{align*}
\text{C1} & \quad \text{C1} \\
\text{OH} & \quad \text{H}_2\text{SO}_4 \\
\text{EtOH/Reflux} & \quad \text{EtOH/Hydrazine Hydrate} \\
\text{C1} & \quad \text{C1} \\
\end{align*}
\]

N-Z=205

m/z=263.5

Reaction Scheme 2. Synthesis of N-[[5-(2, 4-dichlorophenyl)-1, 3, 4-oxadiazol-2-yl]methyl] alkyl or aryl amine.

\[
\begin{align*}
\text{C1} & \quad \text{C1} \\
\text{N} & \quad \text{Dioxan/TEA} \\
\text{N} & \quad \text{R}^2 \\
\end{align*}
\]

m/z=263.5

N-[[5-(2,4-dichlorophenyl)-1,3,4-oxadiazol-2-yl]methyl]alkyl or aryl amine

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