SYNTHESIS, ANTIMICROBIAL AND ANTITUMOR STUDY OF NEW PYRIDO[2,1-a]ISOQUINOLINES VIA ISOQUINOLINE-1-ACETONITRILE

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Abstract – Refluxing of enaminonitrile 3 with arylacetonitriles 2 in ethanol in the presence of piperidine afforded 4H-pyrido[2,1-a]isoquinoline-1-carbonitriles 6. Refluxing of 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile 1 with ethyl 3-aryl-2-cyanoacrylates 9 in acetonitrile in the presence of piperidine gave the corresponding 4H-pyrido[2,1-a]isoquinoline-1,3-dicarbonitriles 13. All the new synthesized compounds were identified by elemental analysis and spectral data. Cytotoxic assay was investigated for in vitro antitumor screening against MCF7, HepG2 and HCT-116 cell lines. Molecular docking using Mcule.com. software was carried out for the most potent compound 6b. The results are compared with doxorubicin standard anticancer drug. Antimicrobial activities were investigated, and all compounds revealed no antimicrobial activities against all tested strains except compounds 13b and 13e.

INTRODUCTION

Bridge headed nitrogen heterocyclic compounds showed a significant role as natural and synthetic products.1-3 Many compounds bearing such moiety exhibit beneficial biological activities, in addition to acting as agrochemical or pharmaceutical agents.4-11 For example, azaphenanthrene of type (I) and (II) (Figure 1) containing pyrido[2,1-a]isoquinoline moiety are useful for treatment and/or prophylaxis of diabetes associated with enzyme DPP IV (dipeptidyl peptidase IV), such as diabetes mellitus and firstly non-insuline dependent diabetes mellitus and disturbed tolerance of glucose.12-14 To our knowledge, only a few methods are available in literature for synthesis of pyrido[2,1-a]isoquinolines.15,16 Most of reported protocols for their synthesis need transition metals, multisynthetic steps and low yields.15,16 In
continuation to our work on utility of isoquinolines in the synthesis of such heterocyclic compounds,\textsuperscript{17-29} we reported herein efficient, metal free and one-pot synthesis of new 4\textit{H}-pyrido[2,1-\textit{a}]isoquinoline derivatives using 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile. Also, we studied the biological activities of the target compounds.

![Structure of azaphenanthrene](image)

**Figure 1. Structure of azaphenanthrene**

**RESULTS AND DISCUSSION**

Treatment of 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile 1 with \textit{N,N}-dimethylformamide dimethyl acetal (DMF-DMA) in refluxing dioxane afforded enaminonitrile 3.\textsuperscript{26} Refluxing of the latter enaminonitrile 3 with 2-arylacetonitriles 2 in ethanol in the presence of piperidine yielded 4\textit{H}-pyrido[2,1-\textit{a}]isoquinoline-1-carbonitrile derivatives 6a-d (Scheme 1). The latter products 6a-d were also prepared by treatment of enaminonitriles 4a-d\textsuperscript{30,31} [prepared by refluxing of 2-arylacetonitriles 2 with \textit{N,N}-dimethylformamide dimethyl acetal in dioxane] with 1 in refluxing ethanol in the presence of piperidine (Scheme 1).

The proposed mechanism was the reacting of 3, being the Michael acceptor, with 2-arylacetonitriles 2, being the Michael donor, to afford the corresponding Michael adduct which underwent \textit{in situ} cyclization to give intermediate 5 followed by hydrolysis during work up to yield the final product 6 (Scheme 2). The structures of products 6a-d were identified by their elemental analysis and spectral data. For example, the IR spectrum of compound 6d showed bands at 1620 and 2199 cm\textsuperscript{-1} assignable to CO and CN groups, respectively. Its \textit{1}H NMR spectrum revealed five singlet signals at \(\delta\) 3.82, 3.88, 7.10, 7.27 and 7.81 corresponding to two MeO groups, isoquinoline-H, the proton of the pyrido ring and another isoquinoline-H, respectively, a pair of doublet at \(\delta\) 7.73 and 8.28 corresponding to a \textit{para}-substituted benzene ring, in addition to two multiplet signals at \(\delta\) 2.94-2.96 (CH\textsubscript{2} group of isoquinoline) and 4.13-4.15 (CH\textsubscript{2} group of isoquinoline). Its \textit{13}C NMR revealed 20 signals for asymmetric carbon atoms. The mass spectrum showed a molecular ion beak at \textit{m}/\textit{z} = 403.
Scheme 1. Synthesis of 4\(H\)-pyrido[2,1-\(a\)]isoquinoline-1-carbonitrile derivatives 6a-d

Reaction of 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile 1 with ethyl 3-aryl-2-cyanoacrylates 9a-e\(^{32,33}\) in refluxing acetonitrile and in the presence of piperidine yielded the corresponding 4\(H\)-pyrido[2,1-\(a\)]isoquinoline-1,3-dicarbonitrile derivatives 13a-e (Scheme 3). The formation of 13 is supposed to be carried out by addition reaction of 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile 1 to ethyl 3-aryl-2-cyanoacrylates 9 to give the corresponding Michael adduct followed by cyclization via \textit{in situ} elimination of ethanol to afford intermediate 12. The latter underwent aromatization via \textit{in situ} oxidation to give 13. The structures of the isolated products 13 were identified by their elemental analysis and spectral data. For example, the IR spectrum of 13b revealed three characteristic bands at \(v\) 1651, 2188 and 2214 cm\(^{-1}\) assignable to CO group and two CN groups, respectively. Its \(^1\)H NMR showed a singlet signal at \(\delta\) 2.44 assignable to 4-MeC\(_6\)H\(_4\), a pair of doublet at \(\delta\) 7.35 and 7.45 corresponding to a \textit{para}-substituted benzene ring, in addition to the signals of isoquinoline moiety. Its \(^{13}\)C NMR showed 22 signals for asymmetric carbon atoms. The mass spectrum showed a molecular ion peak at \(m/z = 397\).

Antitumor Screening

The new synthesized compounds 6a-d were subjected to in vitro antitumor screening against different human cancer cell lines namely: HepG2, MCF7 and HCT-116. It is clear from IC$_{50}$ values in Table 1, according to Shier scale all the tested compounds showed moderate to weak antitumor activity against HepG2, MCF7 and HCT-116. Compound 6b revealed moderate activities against all tested cell lines followed by compound 6d compared to the positive doxorubicin drug. On the other hand, compounds 6a and 6c showed very weak activities against all tested cell lines compared to the positive doxorubicin drug.

Table 1. IC$_{50}$ of the tested compounds 6a-d for their in vitro antitumor activity against (HepG2), (MCF7) and (HCT-116).

<table>
<thead>
<tr>
<th>Compd.</th>
<th>HepG2 (µg)</th>
<th>MCF7 (µg)</th>
<th>HCT-116 (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>1.20</td>
<td>2.38</td>
<td>0.469</td>
</tr>
<tr>
<td>6a</td>
<td>43.4</td>
<td>35.0</td>
<td>34.7</td>
</tr>
<tr>
<td>6b</td>
<td>20.0</td>
<td>12.6</td>
<td>12.2</td>
</tr>
<tr>
<td>6c</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>45.3</td>
</tr>
<tr>
<td>6d</td>
<td>25.6</td>
<td>15.3</td>
<td>19.9</td>
</tr>
</tbody>
</table>
Modeling Studies
Docking studies visualization was performed using PLIP (Protein-Ligand Interaction Profiler). We performed these studies to anticipate the binding mode between the ligand and the binding site of target proteins which is EGFR (epidermal growth factor receptor), one of the human proteins which are highly expressed in most tumor cells to validate and specify the mechanism of action in the context of anticancer activity. Protein was selected and downloaded from the Protein Data Bank (PDB ID: 4i23). From the results, four binding sites have been detected, three hydrophobic interactions and one hydrogen bond for this complex. The hydrophobic interactions with Val229, Asp819 and Leu 1282 with bond distances 3.75, and 3.82 and 3.54 Å respectively. The fourth interaction is hydrogen bonding between Lys373 and oxygen atom with bond distance 2.95 Å and angle 136.65 (Figure 2). All these binding modes confirmed

![Figure 2. 3D and 2D dimensional modeling representation of compound 6b with EGFR](image-url)
our suggestion that 9,10-dimethoxy-3-(4-methoxyphenyl)-4-oxo-6,7-dihydro-4H-pyrido[2,1-α]-isoquinoline-1-carbonitrile 6b may have weak anticancer activity and play a minor role in inhibiting the cancer progression.

**Antimicrobial Screening**

The antibacterial and antifungal activities of new synthesized compounds were studied by the disc diffusion method. The antibacterial activities were studied against the following pathogenic organisms; the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, the gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. Moreover, antifungal activities against *Candida albicans*

Table 2. *In vitro* antibacterial and antifungal activity of some new synthesized compounds (Inhibition zone in mm). (G− = Gram negative; G+ = gram positive; R. A. = relative activity)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Bacterial species (G⁺)</th>
<th>Bacterial species (G⁻)</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Bacillus subtilis</em></td>
<td><em>Staphylococcus aureus</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td></td>
<td>IZ</td>
<td>IZ</td>
<td>IZ</td>
</tr>
<tr>
<td><strong>Control: DMSO</strong></td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Ampicillin</strong></td>
<td>26</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td><strong>Amphotericin</strong></td>
<td>21</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>13b</strong></td>
<td>15</td>
<td>57.7</td>
<td>12</td>
</tr>
<tr>
<td><strong>13e</strong></td>
<td>10</td>
<td>38.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

were studied. The synthesized compounds were used at the concentration of 20 mg/mL using DMSO as a solvent. The Ampicillin and the Gentamicin 10 μL/disc were used as a standard antibacterial agents and the Nystatin 20 μg/mL as standard antifungal agent. All the tested compounds showed no antimicrobial activities except for compounds 13b and 13e.

**EXPERIMENTAL**

Melting points were measured with a Stuart melting point apparatus and are uncorrected. The IR spectra were recorded using a FTIR Bruker–vector 22 spectrophotometer as KBr pellets. The ¹H and ¹³C NMR spectra were recorded in CDCl₃ or DMSO–d₆ as solvent on Varian Gemini NMR spectrometer at 300 MHz and 75 MHz, respectively, using TMS as internal standard. Chemical shifts are reported as δ values in ppm. Mass spectra were recorded with a Shimadzu GCMS–QP–1000 EX mass spectrometer in EI (70
eV) model. The elemental analyses were performed at the Micro analytical center, Cairo University. The 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile 1, enaminonitrile 3, enaminonitrile 4a-d and ethyl 3-aryl-2-cyanoacrylate 9a-e were prepared using the reported procedures. In vitro antitumor activity was conducted at The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The in vitro antimicrobial testing was performed at Microanalytical Center, Cairo University. The agar disc diffusion method and a panel of standard strains (*S. aureus* ATCC 6588, *B. subtilis* CMGB 215, *E. coli* ATCC 11775, *P. aeruginosa* ATCC 15442, and *C. albicans* ATCC 7102) were employed.

**Synthesis of 3-aryl-9,10-dimethoxy-4-oxo-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1-carbonitrile (6a-d):**

**Method A:**

To the appropriate solution of 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)-3-(dimethylamino)-acrylonitrile 3 (0.57 g, 2.0 mmol) and 2-arylacetonitriles 2 (2.0 mmol) in EtOH (20 mL), piperidine (0.5 mL) was added and the reaction mixture was refluxed for 6 h. the solvent was evaporated under reduced pressure and the residue was treated with MeOH (50 mL) where it solidified. The crude product was collected and crystallized from suitable solvent to give 6 in yield (60-52%).

**Method B:**

To the appropriate solution of 2-aryl-3-(dimethylamino)acrylonitriles 4a-d (2.0 mmol) and 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile 1 (0.46 g, 2.0 mmol) in EtOH (20 mL), piperidine (0.5 mL) was added and the reaction mixture was refluxed for 6 h. The solvent was evaporated under reduced pressure and the residue was treated with MeOH (50 mL) where it solidified. The crude product was collected and crystallized from suitable solvent to give 6. The compounds prepared with their physical data are listed below in yield (81-79%).

**9,10-Dimethoxy-4-oxo-3-phenyl-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1-carbonitrile (6a).** Yellow crystals; mp 200-202 °C (MeCN); yield (79%); IR (νmax, cm⁻¹) ν 1631 (CO), 2204 (CN); ¹H NMR (300 MHz, DMSO-d6) δ 2.94-2.99 (m, 2H, CH₂), 3.81 (s, 3H, CH₃O), 3.87 (s, 3H, CH₃O), 4.15-4.20 (m, 2H, CH₂), 7.10 (s, 1H, isoquinoline-H), 7.12 (s, 1H, Ar-H), 7.30-7.41 (m, 5H, Ar-H), 7.79 (s, 1H, isoquinoline-H); ¹³C NMR (75 MHz, DMSO-d6) δ 26.49, 40.18, 55.35, 55.63, 87.03, 110.82, 113.75, 119.43, 127.55, 128.36, 128.47, 128.95, 128.98, 131.84, 132.72, 137.22, 146.96, 148.63, 151.75, 159.34; MS (EI, 70 eV) m/z (%): 358 (M⁺, 34.41), 356 (100), 178 (13.88), 102 (22.83), 51 (66.64). Anal. Calcd. for C₂₂H₂₈N₂O₃ (358.40): C, 73.73; H, 5.06; N, 7.82. Found: C, 73.83; H, 4.93; N, 7.71.

**9,10-Dimethoxy-3-(4-methoxyphenyl)-4-oxo-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1-carbonitrile (6b).** Yellow crystals; mp 244-246 °C (MeCN); yield (81%); IR (νmax, cm⁻¹) ν 1643 (CO), 2206 (CN); ¹H
NMR (300 MHz, DMSO-d_6) δ 2.92-2.96 (m, 2H, CH_2), 3.78 (s, 3H, CH_3O), 3.82 (s, 3H, CH_3O), 3.86 (s, 3H, CH_3O), 4.13-4.16 (m, 2H, CH_2), 6.93 (d, J = 9 Hz, 2H, Ar-H), 7.08 (s, 1H, isoquinoline-H), 7.68 (d, J = 9 Hz, 2H, Ar-H), 7.88 (s, 1H, Ar-H), 7.92 (s, 1H, isoquinoline-H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 26.81, 40.13, 55.20, 55.69, 55.92, 86.39, 110.93, 113.53, 118.46, 119.55, 124.33, 127.50, 129.77, 131.24, 132.45, 137.09, 147.06, 148.41, 151.90, 159.11, 159.60; MS (EI, 70 eV) m/z (%): 388 (M^+, 100), 373 (44.03), 194 (14.91), 75 (9.48), 51 (9.020). Anal. Calcd. for C_{23}H_{20}N_2O_4 (388.42): C, 71.12; H, 5.19; N, 7.21. Found: C, 71.05; H, 5.31; N, 7.35.

3-(4-Chlorophenyl)-9,10-dimethoxy-4-oxo-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1-carbonitride (6c). Yellow crystals; mp 249-251 °C (MeCN); yield (80%); IR (νmax, cm\(^{-1}\)) ν 1630 (CO), 2201 (CN); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.94-2.96 (m, 2H, CH_2), 3.82 (s, 3H, CH_3O), 3.88 (s, 3H, CH_3O), 4.15-4.17 (m, 2H, CH_2), 7.10-7.56 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 26.63, 40.23, 55.20, 55.67, 86.53, 110.40, 113.93, 119.69, 127.67, 129.00, 129.53, 129.89, 130.12, 132.52, 134.40, 137.10, 147.15, 148.43, 151.82, 159.01; MS (EI, 70 eV) m/z (%): 392 (M^+, 74.85), 391 (100), 178 (28.73), 140 (22.55), 75 (22.36). Anal. Calcd. for C_{22}H_{17}ClN_3O_3 (392.84): C, 67.26; H, 4.36; Cl, 9.02; N, 7.13. Found: C, 67.39; H, 4.24; Cl, 9.11; N, 7.23.

9,10-Dimethoxy-3-(4-nitrophenyl)-4-oxo-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1-carbonitride (6d). Red crystals; mp 260-262 °C (DMF); yield (81%); IR (νmax, cm\(^{-1}\)) ν 1620 (CO), 2201 (CN); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.94-2.96 (m, 2H, CH_2), 3.82 (s, 3H, CH_3O), 3.88 (s, 3H, CH_3O), 4.13-4.15 (m, 2H, CH_2), 7.10 (s, 1H, isoquinoline-H), 7.27 (s, 1H, Ar-H), 7.73 (d, 2H, Ar-H), 7.81 (s, 1H, isoquinoline-H), 8.28 (d, 2H, Ar-H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 26.38, 40.09, 55.46, 55.83, 86.67, 110.54, 113.57, 119.67, 122.73, 127.72, 129.81, 130.19, 132.58, 137.05, 139.30, 146.99, 148.22, 148.53, 152.16, 159.27; MS (EI, 70 eV) m/z (%): 403 (M^+, 46.21), 238 (26.89), 156 (42.33), 91 (76.94), 51 (100). Anal. Calcd. for C_{22}H_{17}N_3O_5 (403.39): C, 65.50; H, 4.25; N, 10.42. Found: C, 65.58; H, 4.32; N, 10.30.

**Synthesis of 2-aryl-9,10-dimethoxy-4-oxo-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1,3-dicarbonitride (13a-e):**

To a solution of the appropriate ethyl 3-aryl-2-cyanoacrylates 9a-e (10 mmol) and 2-(6,7-dimethoxy-3,4-dihydroisoquinolin-1-yl)acetonitrile 1 (2.3 g, 10 mmol) in MeCN (10 mL) was added piperidine (0.08 mL, 10 mmol) at room temperature. The reaction mixture was refluxed for 6 h, the solvent was evaporated under reduced pressure and the residue was treated with MeOH (5 mL) where it solidified. The crude products was collected and crystallized from suitable solvent to give 13.

9,10-Dimethoxy-4-oxo-2-phenyl-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1,3-dicarbonitride (13a). Yellow crystals; mp 226-228 °C (MeCN); yield (81%); IR (νmax, cm\(^{-1}\)) ν 1649 (CO), 2187 (CN), 2213 (CN); ^{1}H NMR (300 MHz, CDCl_3) δ 2.97-3.01 (m, 2H, CH_2), 3.95 (s, 3H, CH_3O), 3.99 (s, 3H, CH_3O),
4.28-4.32 (m, 2H, CH₂), 6.84 (s, 1H, isoquinoline-H), 7.51-7.55 (m, 5H, Ar-H), 7.96 (s, 1H, isoquinoline-H); ¹³C NMR (75 MHz, CDCl₃) δ 26.73, 40.98, 56.36, 56.52, 89.12, 100.83, 109.86, 110.89, 113.64, 117.26, 117.52, 127.69, 128.73, 128.99, 133.29, 133.57, 148.02, 153.35, 153.52, 158.63, 160.86; MS (EI, 70 eV) m/z (%): 383 (M⁺, 100), 382 (61.48), 368 (35.20), 352 (7.77), 296 (5.74). Anal. Calcd. for C₂₉H₁₇N₃O₃ (383.41): C, 72.05; H, 4.47; N, 10.96. Found: C, 72.11; H, 4.59; N, 10.89.

9,10-Dimethoxy-4-oxo-2-(p-tolyl)-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1,3-dicarbonitrile (13b). Yellow crystals; mp 236-238 °C (MeCN); yield (80%); IR (v max, cm⁻¹) ν 1651 (CO), 2188 (CN), 2214 (CN); ¹H NMR (300 MHz, CDCl₃) δ 2.44 (s, 3H, 4-CH₃C₆H₄), 2.96-3.01 (m, 2H, CH₂), 3.95 (s, 3H, CH₃O), 3.99 (s, 3H, CH₃O), 4.28-4.32 (m, 2H, CH₂), 6.84 (s, 1H, isoquinoline-H), 7.35 (d, 2H, Ar-H), 7.45 (d, 2H, Ar-H), 7.96 (s, 1H, isoquinoline-H); ¹³C NMR (75 MHz, CDCl₃) δ 21.48, 27.36, 40.58, 56.30, 56.62, 89.43, 101.08, 110.21, 111.91, 114.54, 117.37, 117.83, 128.22, 129.54, 130.51, 133.15, 141.46, 147.85, 153.43, 153.68, 158.59, 161.14; MS (EI, 70 eV) m/z (%): 397 (M⁺, 100), 396 (32.06), 383 (18.36), 382 (64.47), 366 (9.45). Anal. Calcd. for C₂₉H₁₉N₃O₃ (397.43): C, 72.53; H, 4.82; N, 10.57. Found: C, 72.64; H, 4.68; N, 10.64.

9,10-Dimethoxy-2-(4-methoxyphenyl)-4-oxo-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1,3-dicarbonitrile (13c). Yellow crystals; mp 272-274 °C (DMF); yield (85%); IR (v max, cm⁻¹) ν 1652 (CO), 2191 (CN), 2214 (CN); ¹H NMR (300 MHz, CDCl₃) δ 2.96-2.99 (m, 2H, CH₂), 3.92 (s, 3H, CH₃O), 3.95 (s, 3H, CH₃O), 4.00 (s, 3H, CH₃O), 4.26-4.30 (m, 2H, CH₂), 6.83 (s, 1H, isoquinoline-H), 7.39 (d, J = 9 Hz, 2H, Ar-H), 7.51 (d, J = 9 Hz, 2H, Ar-H), 7.98 (s, 1H, isoquinoline-H); ¹³C NMR (75 MHz, CDCl₃) δ 27.26, 41.35, 56.11, 56.26, 56.45, 89.32, 101.22, 109.54, 111.67, 114.35, 116.19, 117.38, 117.61, 124.83, 129.19, 133.33, 147.98, 153.44, 153.82, 158.61, 160.40, 160.93; MS (EI, 70 eV) m/z (%): 413 (M⁺, 100), 412 (27.11), 399 (11.70), 398 (41.08), 382 (18.27). Anal. Calcd. for C₂₉H₁₉N₂O₄ (413.43): C, 69.72; H, 4.63; N, 10.16. Found: C, 69.81; H, 4.72; N, 10.27.

9,10-Dimethoxy-4-oxo-2-(3,4,5-trimethoxyphenyl)-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1,3-dicarbonitrile (13d). Yellow crystals; mp 252-254 °C (MeCN); yield (82%); IR (v max, cm⁻¹) ν 1653 (CO), 2188 (CN), 2214 (CN); ¹H NMR (300 MHz, CDCl₃) δ 2.96-3.00 (m, 2H, CH₂), 3.92 (s, 3H, CH₃O), 3.93 (s, 6H, 2CH₃O), 3.96 (s, 3H, CH₃O), 4.00 (s, 3H, CH₃O), 4.28-4.32 (m, 2H, CH₂), 6.78 (s, 2H, Ar-H), 6.82 (s, 1H, isoquinoline-H), 7.98 (s, 1H, isoquinoline-H); ¹³C NMR (75 MHz, CDCl₃) δ 27.39, 40.66, 56.30, 56.33, 56.51, 60.92, 89.35, 101.09, 105.99, 110.20, 112.02, 114.49, 117.21, 117.78, 128.26, 133.19, 140.37, 147.88, 153.25, 153.43, 153.75, 158.53, 160.69; MS (EI, 70 eV) m/z (%): 473 (M⁺, 100), 458 (22.72), 430 (17.84), 398 (11.32), 344 (10.66). Anal. Calcd. for C₂₉H₂₃N₃O₆ (473.49): C, 65.95; H, 4.90; N, 8.87. Found: C, 65.84; H, 4.84; N, 8.98.

9,10-Dimethoxy-4-oxo-2-(thiophen-2-yl)-6,7-dihydro-4H-pyrido[2,1-a]isoquinoline-1,3-dicarbonitrile (13e). Red crystals; mp 238-240 °C (MeCN); yield (81%); IR (v max, cm⁻¹) ν 1652 (CO), 2188 (CN), 2214
H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 2.98-3.02 (m, 2H, CH\(_2\)), 3.83 (s, 3H, CH$_3$O), 3.91 (s, 3H, CH$_3$O), 4.13-4.17 (m, 2H, CH$_2$), 7.16-8.02 (m, 5H, Ar-H); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 26.12, 40.40, 55.80, 56.08, 89.17, 99.80, 110.91, 112.48, 115.19, 117.46, 117.57, 127.91, 131.29, 131.82, 132.73, 134.43, 146.88, 152.98, 153.48, 153.94, 158.27; MS (EI, 70 eV) \(m/z\) (%): 389 (M$^+$, 100), 388 (55.03), 375 (10.37), 374 (37.74), 358 (9.49). Anal. Calcd. for C$_{21}$H$_{15}$N$_3$O$_3$S (389.43): C, 64.77; H, 3.88; N, 10.79; S, 8.23. Found: C, 64.87; H, 3.82; N, 10.87; S, 8.36.

Cytotoxicity Assay

The cells were propagated in DMEM supplemented with 10% heat-inactivated FBS, 1% L-glutamine, HEPES buffer and 50 µg/mL gentamycin. All cells were maintained at 37 °C in humidified atmosphere with 5% CO$_2$ and were subcultured two times a week. Cell toxicity was monitored by determining the effect of the test samples on cell morphology and cell viability. The cells were seeded in 96-well plate at a cell concentration of 1×10$^4$ cells per well in 100 µL of growth medium. Fresh medium containing different concentration of the test sample was added after 24 h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO$_2$ for a period of 48 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells for 24 h at 37 °C, various concentrations of sample (50, 25, 12.5, 6.25, 3.125 and 1.56 µg) were added, and the incubation was continued for 48 h and viable cells yield was determined by a colorimetric method.

Molecular Modeling

Modelling studies were done for the most active compound 6b, to predict the protein-ligand interactions at the active site. Protein structure of EGFR (epidermal growth factor receptor) were downloaded from the Protein Data Bank (PDB ID: 4i23). Proteins were prepared for docking, and other instructions were described according to the previous literatures for the modeling investigation using Mcule.com. By the end, the final data was visualized using PLIP (Protein-Ligand Interaction Profiler).

Antimicrobial Activity Evaluation

Antimicrobial activity of the tested compounds was determined using a modified Kirby-Bauer disc diffusion method. Briefly, 100 µL of the test bacteria/fungi were grown in 10 mL of fresh media until they reached a count of approximately 10$^8$ cells/mL for bacteria or 10$^5$ cells/mL for fungi 100 µL of
microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Of the many media available, NCCLS recommends Mueller-Hinton agar due to: it results in good batch-to-batch reproducibility. Disc diffusion method for yeasts developed by using approved standard method (M44-P) by the (NCCLS, 2009).40

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