Pyrrolo[1,2]azepine is a heterocyclic system of two fused 7 and 5 membered rings with one nitrogen atom at the ring junction, it is considered as an analogue of 5,5- and 6,5-heterocyclic systems named pyrrolizine and indolizine, respectively. This nucleus represents a basic scaffold in a class of natural products known as stemona alkaloids, isolated from family Stemonaceae (1). Several pyrrolo[1,2-a]azepines exhibited remarkable anticancer activity (2-4) for example, cephalotaxine ester I that was reported to have an efficient anti-leukaemic activity (5, 6). Belal has reported novel, potent anticancer agents based on the pyrrolo[1,2-a]azepine scaffold e.g., compound II that showed to be more potent than doxorubicin against HEPG2 cancer cells (7), its IC50 value attained 1.6 nM/mL. The aforementioned facts proved the importance of this scaffold in designing new anticancer active agents and this was a motivation for us to continue our work in investigating its anticancer activity, hence we started to synthesize compound 3 as a mimic for compound II, replacing the amidic group at position 3 with ethyl ester group.

In addition, the 4-thiazolidinone nucleus was reported to possess anticancer properties and several publications have described the anticancer activity of this moiety (8-11). Compound II showed to have an efficient anti- proliferative activity (5, 6). Belal has reported novel, potent anticancer agents based on the pyrrolo[1,2-a]azepine scaffold e.g., compound II that showed to be more potent than doxorubicin against HEPG2 cancer cells (7), its IC50 value attained 1.6 nM/mL. The aforementioned facts proved the importance of this scaffold in designing new anticancer active agents and this was a motivation for us to continue our work in investigating its anticancer activity, hence we started to synthesize compound 3 as a mimic for compound II, replacing the amidic group at position 3 with ethyl ester group.

Moreover, it was previously reported (13), that substituting the thiazolidinone with p-chlorophenyl hydrazono group lead to obtaining active anticancer agent V, the same incorporation strategy was performed in compound 6 in a trial to get a potential anticancer active agent.
The present work is directed towards synthesis of pyrrolo[1,2-a]azepine system, its hybrids with thiazolidinone and aryl hydrazono derivatives using facile synthetic pathways and easily accessible chemicals, investigating their antitumor activity against a variety of human cancer cell lines (A549 “lung”, MCF7 “breast” and Hep3B “liver” cancers) in addition to normal fibroblast cells to access calculating the therapeutic index of these new derivatives.

RESULTS AND DISCUSSION

Chemistry

Compound 1 was prepared according to the previously reported methods (14), then refluxed with equimolar amount of ethyl chloroacetate in dry acetone and in the presence of potassium carbonate to afford the ethyl ester derivative of pyrrolo[1,2-a]azepine 2 (15). Chloroacylated derivative 3 was obtained by reacting compound 2 with chloroacetyl chloride in dioxane at room temperature (Scheme 1). 1H NMR spectrum revealed the presence of a singlet signal at 3.57 ppm indicating the COCH₂Cl in addition to the other signals characterizing azepane and ethyl ester protons. 4-Thiazolidinone nucleus was obtained through reaction of compound 3 with ammonium thiocyanate (Scheme 2) in absolute ethanol, structure of compound 4 was confirmed by 1H NMR and 13C NMR spectra. Coupling the active methylene of the thiazolidinone moiety with aldehydes afforded compounds 5a,b. 1H NMR spectra showed the appearance of signals characterizing N=CH and the aromatic protons. Hydrazono derivative 6 was obtained by coupling active CH₂ of thiazolidinone with diazonium salt and the structure was confirmed by the appearance of aromatic protons and additional signal for NH. Moreover, IR and C13 NMR were used in structure confirmation of the new compounds.

Pharmacological screening

In the present work, the newly synthesized compounds were tested on three cancer cell lines (lung A549, breast MCF7 and liver Hep3B) in addition to the normal fibroblast cells using sulforhodamine-B assay method (16). The surviving fraction of cancer cells was plotted against drug concentration to obtain the survival curve. IC₅₀ value (the concentration of the compound that causes 50% inhibition of viable cells) was calculated, data are represented in Table 1 (showing the IC₅₀ values for the tested compounds in µg/mL ± S.D.). Selectivity index also was calculated by dividing the IC₅₀ value of the compound against normal fibroblast cells on its IC₅₀ value against the cancer cell line (17). S.I. and IC₅₀ values expressed in µM/mL and nM/mL are represented in Table 2. The tested compounds exhibited remarkable antitumor activity, the obtained results can be summarized as follows: i) 3,
Table 1. IC₅₀ values of the tested compounds.

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>A549 (µg/mL ± S.D.)</th>
<th>MCF7 (µg/mL ± S.D.)</th>
<th>Hep3B (µg/mL ± S.D.)</th>
<th>Wish (µg/mL ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>23.777 ± 0.084</td>
<td>11.749 ± 0.016</td>
<td>8.0829 ± 0.009</td>
<td>9.696 ± 0.006</td>
</tr>
<tr>
<td>4</td>
<td>256.81 ± 0.068</td>
<td>4.231 ± 0.015</td>
<td>57.905 ± 0.021</td>
<td>6.0384 ± 0.006</td>
</tr>
<tr>
<td>5a</td>
<td>6.1218 ± 0.027</td>
<td>13.31 ± 0.007</td>
<td>33.329 ± 0.029</td>
<td>17.96 ± 0.054</td>
</tr>
<tr>
<td>5b</td>
<td>79.9855 ± 0.012</td>
<td>123.608 ± 0.007</td>
<td>7.2324 ± 0.015</td>
<td>78.48 ± 0.025</td>
</tr>
<tr>
<td>6</td>
<td>52.242 ± 0.01</td>
<td>117.7153 ± 0.005</td>
<td>4.2276 ± 0.009</td>
<td>1206.3 ± 0.014</td>
</tr>
</tbody>
</table>

Each value represents a mean of triplicate values.
Table 2. IC₅₀ values of the tested compounds expressed in µM/mL (nM/mL) and selectivity index against each type of cancer cell line.

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>A549 IC₅₀ (µM/mL) (nM/mL)</th>
<th>S. I.</th>
<th>MCF7 IC₅₀ (µM/mL) (nM/mL)</th>
<th>S. I.</th>
<th>Hep3B IC₅₀ (µM/mL) (nM/mL)</th>
<th>S. I.</th>
<th>Wish IC₅₀ (µM/mL) (nM/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.073438 (74)</td>
<td>0.407786</td>
<td>0.036288 (36)</td>
<td>0.825259</td>
<td>0.024965 (25)</td>
<td>1.199559</td>
<td>0.029947 (30)</td>
</tr>
<tr>
<td>4</td>
<td>0.741368 (741)</td>
<td>0.9023513</td>
<td>0.012214 (12)</td>
<td>0.1427215</td>
<td>0.167162 (167)</td>
<td>0.104282</td>
<td>0.017432 (17)</td>
</tr>
<tr>
<td>5a</td>
<td>0.013054 (13)</td>
<td>2.933813</td>
<td>0.028382 (28)</td>
<td>1.349376</td>
<td>0.07107 (71)</td>
<td>0.538877</td>
<td>0.038298 (38)</td>
</tr>
<tr>
<td>5b</td>
<td>0.166807 (167)</td>
<td>0.981176</td>
<td>0.25778 (258)</td>
<td>0.63491</td>
<td>0.015083 (15)</td>
<td>10.85109</td>
<td>0.163667 (164)</td>
</tr>
<tr>
<td>6</td>
<td>0.107724 (108)</td>
<td>23.09069</td>
<td>0.242732 (243)</td>
<td>10.24761</td>
<td>0.008717 (9)</td>
<td>285.353</td>
<td>2.487422 (2487)</td>
</tr>
</tbody>
</table>

S.I. = IC₅₀ on normal cells/IC₅₀ of cancer cells (17).

Figure 1. Anticancer active compounds containing : pyrrolo[1,2-a]azepine I, II, 4-thiazolidinone III, IV or hydrazono group V.
5a and 6 are the most potent compounds on A549 cell line, with IC₅₀ range equal (13-108 nM/mL), selectivity index (S.I.) of compounds 5a and 6 on A549 was 3 and 23, respectively, however, that of compound 3 was less than 1. ii) Compounds 3, 4 and 5a are the best active on MCF7, their IC₅₀ range is (12-36 nM/mL), S.I. of compound 3 is less than 1, however, S.I. of 4 and 5a is 1.4 and 1.3, respectively. iii) Compounds 3, 5a, 5b and 6 are the best active on Hep3B cancer cell line, their IC₅₀ ranges from 9 to 71 nM/mL, S.I. of compounds 3, 5b and 6 are 1.1, 11 and 285, respectively, however, the S.I. of compound 5a is less than 1. iv) Compound 6 showed high potency and selectivity on Hep3B cancer cell line and compound 4 is the best active and selective one on MCF7 cell line. Compound 5a showed to be the best active on A549 with a good S.I. (3), however, compound 6 showed a greater selectivity on MCF7 than 5a but showed to be less potent. From these observed results we can conclude that: i) 2-Chloroacetylamino pyrrolo[1,2-a]azepine-3-ethyl ester 3 showed broad spectrum anticancer activity on the three tested cancer cell lines but with low selectivity towards A549 and MCF7 cancer cells. ii) Replacement of the chloroacetylamino group 3 with thiazolidin-4-one 4 moiety increased activity and selectivity towards MCF7 cancer cells and decreased the activity on both A549 and Hep3B cell lines. iii) Substituting the 5 position of the 4-thiazolidinone moiety with p-chlorobenzylidine lead to obtaining a highly potent compound 5a on the three cancer cell lines, but its selectivity towards Hep3B was low. iv) Substituting the 5 position of the 4-thiazolidinone moiety with p-nitrobenzylidine, in spite of the decrease in the activity and selectivity towards both A549 and MCF7, it afforded a highly

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>S (kcal/mol)</th>
<th>Interacting moieties</th>
<th>Amino acid residue</th>
<th>H bond length Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>-16.56</td>
<td>NH</td>
<td>Asp 86</td>
<td>2.97</td>
</tr>
<tr>
<td>4</td>
<td>-12.37</td>
<td>CO of thiazolidin</td>
<td>Asn 132</td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO of thiazolidin</td>
<td>Lys 129</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN</td>
<td>Lys 33</td>
<td>2.66</td>
</tr>
<tr>
<td>5a</td>
<td>-20.71</td>
<td>NH of thiazolidi</td>
<td>Asp 86</td>
<td>2.47</td>
</tr>
<tr>
<td>5b</td>
<td>-14.51</td>
<td>Phenyl moiety</td>
<td>Arene cation</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-19.73</td>
<td>CN</td>
<td>Leu 83</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenyl moiety</td>
<td>Arene cation</td>
<td></td>
</tr>
<tr>
<td>LS5</td>
<td>-18.95</td>
<td>NH of indole</td>
<td>Glu 81</td>
<td>2.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CO of indole</td>
<td>Leu 83</td>
<td>2.98</td>
</tr>
</tbody>
</table>
potent and selective compound on Hep3B cancer cells. v) Linking the pyrrolo[1,2-a]azepine ethyl ester scaffold with a hydrazino group through thiazolidinone ring lead to obtaining compound 6 with very high potency and selectivity towards Hep3B cancer cells, good activity and selectivity on A549 and decreased the activity on MCF7 cancer cells.

Molecular docking studies
Cyclin-dependent kinases (CDKs) are family of protein kinases, playing a role in cell cycle regulation. Interfering with CDKs action causes interruption in cell cycle regulation and lead to cancer cell death (18, 19). Molecular docking studies for the synthesized compounds were performed to investigate their mode of binding and possible interaction with these tumorogenic agents (CDK2).

Docking the synthesized derivatives into the binding site of CDK2 was performed using pdb file: 1KE9 (20) and molecular operating environment software (MOE). The obtained results (Table 3) revealed that all the docked compounds showed good docking score energy, moreover, compounds 5a and 6 showed a lower score energy than LS5 ligand itself. Figure 2 represents the binding mode of the cocrystallized ligand LS5 inside CDK2 active site. 2D and 3D interactions of the docked compounds are represented in Figures 3-7, compounds 3 and 5a showed arene-cation interaction with Lys 89 through the phenyl moiety. Compounds 3 and 5a showed a hydrogen bonding with Asp 86 through the NH group. Compound 4 revealed 2 hydrogen bonds with Asn 132 and Lys 129 through the CO of thiazolidinone nucleus and another hydrogen bond with Lys.
Figure 5. 2D and 3D interactions of compound 5a with CDK2

Figure 6. 2D and 3D interactions of compound 5b with CDK2

Figure 7. 2D and 3D interactions of compound 6 with CDK2
33 through the CN group. Compound 6 showed a hydrogen bond with Leu 83 through the CN group in addition to arene-cation interaction. All these results gave a promising venue for further exploration of these target compounds.

CONCLUSION

From the present investigation we can conclude the following: i) Pyrrolo[1,2-a]azepine-3-ethyl ester can be considered as a promising scaffold in designing new anticancer agents. ii) Substituting this scaffold with 2-chloroacetylamino group gave a broad spectrum anticancer activity on A549, MCF7 and Hep3B but with low selectivity. iii) Hybridization between the pyrroloazepine system and thiazolidin-4-one lead to obtaining a highly potent and selective compound towards MCF7 cancer cells. iv) Further substitution in position 5 of thiazolidin-4-one with p-chlorobenzylidine gave a highly potent and selective compound towards both A549 and MCF7 cancer cells. However, the 5-p-nitro derivative showed a decreased activity and selectivity on both cell lines and superior activity and selectivity on Hep3B cancer cells. v) Molecular docking studies gave an evidence for the ability of the target compounds to quick fit the active site of CDK2 and act as modulators for these receptors.

EXPERIMENTAL

Chemistry

Compounds 1 and 2 were prepared according to the previously reported methods (14, 15). Melting points were uncorrected and were performed using Electrothermal Stuart SMP3 digital melting point apparatus. Reactions were monitored by TLC, Kieselgel 0.25 mm, 60 G F 254, Merck silica gel plates were used and visualization was performed by UV lamp short and long wavelength. Chloroform/ethanol (9 : 1, v/v) was the running solvent system. IR (KBr discs) and elemental microanalyses were performed at the microanalytical center, Faculty of Science, Cairo University, the 1H-NMR spectra were recorded in DMSO-d6 on a Varian Mercury spectrometer (400 MHz) and 13C-NMR (DMSO-d6) spectra were recorded at 100.62 MHz at the magnetic resonance unit Beni-Suef University. Chemical shifts are expressed in ppm values and tetramethylsilane (TMS) was the internal standard.

4.1.1. 2-(2-Chloroacetylamino)-1-cyano-6,7,8,9-tetrahydro-5H-pyrrolo[1,2-a]azepine-3-carboxylic acid ethyl ester 3

A mixture of compound 2 (0.25 g, 1 mmol) and chloroacetyl chloride (1 mmol) in dioxane (20 mL) was stirred for 24 h at room temp. The solvent was evaporated, the obtained residue was washed with water, filtered and recrystallized from ethanol to give white solid, 0.2 g, 62% yield, m.p. 168-170°C. IR (KBr, cm⁻¹): 3266 (NH), 2928, 2856 (CH aliph.), 2210 (CN), 1732 (C=O, ester), 1693 (C=O of amide). 1H NMR (400 MHz, DMSO-d6, δ, ppm): 1.51-1.55 (m, 2H, H2), 1.59 (t, J = 7.5 Hz , 3H, CH3), 1.60-1.61 (m, 2H, CH3), 1.65-1.68 (m, 2H, CH3), 2.50-2.51 (m, 2H, CH3), 2.65-2.68 (m, 2H, CH3), 3.41 (q, J = 7.5 Hz , 2H, CH3), 3.57 (s, 2H, CH3). 8.98 (s, H, NH); 13C NMR (DMSO-d6, δ, ppm): 24.53, 28.17, 29.56, 31.21, 39.35, 40.60, 45.11, 47.19, 49.45, 51.11, 116. 25, 117.80, 118.70, 120.90, 128.55, 162.33, 177.47. Microanalyses: for C15H18ClN3O3 (323.77) calcd.: C, 55.64; H, 5.60; N, 12.98%; found: 55.48; H, 5.55; N, 13.02%.

1-Cyano-2-(4-oxo-thiazolidin-ylideneamino]-6,7,8,9-tetrahydro-5H-pyrrolo[1,2-a]azepine-3-carboxylic acid ethyl ester 4

A mixture of compound 3 (0.32 g, 1 mmol) and ammonium thiocyanate (1 mmol) in absolute ethanol (20 mL) was refluxed for 5 h. The solvent was removed under vacuum, the obtained residue was washed with water, filtered and recrystallized from acetone. Yellowish white solid, 0.24 g, 70% yield, m.p. 173-175°C. IR (KBr, cm⁻¹): 3265 (NH), 2969, 2928 (CH aliph.), 2194 (C≡N), 1730 (C=O, ester), 1692 (C=O of amide); 1H NMR (400 MHz, DMSO-d6, δ, ppm): 1.29 (t, J = 7.8 Hz, 3H, CH3), 1.50-1.51 (m , 2H, CH2), 1.67-1.68 (m, 2H, CH3), 2.50-2.51 (m, 2H, CH3), 2.65-2.68 (m, 2H, CH3), 23.37 (m, 2H, CH2), 3.40 (q, J = 7.5 Hz , 2H, CH3), 3.42 (s, 2H, CH3). 8.98 (s, H, NH). 13C NMR (DMSO-d6, δ, ppm): 24.53, 28.17, 29.57, 31.33, 39.33, 40.59, 45.11, 47.19, 116. 25, 117.80, 118.70, 120.90, 128.55, 162.33, 177.47. Microanalyses: for C16H18N4O3S (346.40) calcd.: C, 55.48; H, 5.24; N, 16.17%; found: 55.35; H, 5.50; N, 16.02%.

General procedure for the preparation of 5a,b.

Compound 4 (0.35 g, 1 mmol) was dissolved in absolute ethanol (20 mL) and the appropriate aldehyde (1 mmol) in absolute ethanol (20 mL) was refluxed for 5 h. The solvent was removed under vacuum, the obtained residue was washed with water, filtered and recrystallized from acetone. Yellowish white solid, 0.24 g, 70% yield, m.p. 173-175°C. IR (KBr, cm⁻¹): 3265 (NH), 2969, 2928 (CH aliph.), 2194 (C≡N), 1730 (C=O, ester), 1692 (C=O of amide); 1H NMR (400 MHz, DMSO-d6, δ, ppm): 24.53, 28.17, 29.57, 31.33, 39.33, 40.59, 45.11, 47.19, 116. 25, 117.80, 118.70, 120.90, 128.55, 162.33, 177.47. Microanalyses: for C16H18N4O3S (346.40) calcd.: C, 55.48; H, 5.24; N, 16.17%; found: 55.35; H, 5.50; N, 16.02%.

The mixture was stirred under reflux for 10 h, solvent was evaporated under vacuum. The obtained product was crystallized from methanol.
2-[(4-Chloro-benzylidene)-4-oxo-thiazolidin-2-ylidenamino]-1-cyano-6,7,8,9-tetrahydro-5H-pyrrolo[1,2-a]azepine-3-carboxylic acid ethyl ester 5a

Yellowish white solid, 0.23 g, 50% yield, m.p. 183-185°C. IR (KBr; cm⁻¹): 3266 (NH), 1697 (C=O), 2210 (CN) at room temperature and the separated residue was filtered, washed with water and crystallized from acetone/ethanol mixture.

Yield 72% (0.35 g), m.p. 205-207°C. IR (KBr, cm⁻¹): 3266, 3197 (2NH), 3006 (CH arom.), 2944 (CH aliph.), 2195 (CN), 1710 (C=O, ester), 1685 (C=O of amide); ¹H NMR (400 MHz, DMSO-d₆, δ ppm): 1.40 (t, J = 7.2 Hz, 3H, CH₃), 1.49-1.50 (m, 2H, CH₂), 1.57-1.61 (m, 2H, CH₂); 2.23-2.43 (m, 2H, CH₂); 2.65-2.67 (m, 2H, CH₂); 3.38 (q, J = 8.1 Hz, 2H, CH₂), 7.73 (s, H, CH=); 7.69 (dd, J = 5.1 and 4.8 Hz, 2H, CH arom.) 7.94 (dd, J = 5.1 and 4.8 Hz 2H, CH arom.), 8.41 (dd, J = 7.2 Hz, 2H, CH₂), 8.15 (s, H, CH=), 8.17 (dd, J = 7.5 and 4.8 Hz, 2H, CH₂), 8.41 (dd, J = 7.5 and 4.8 Hz, 2H, CH₂ arom.), 10.17 (s, H, NH).

¹³C NMR (DMSO-d₆, δ ppm): 24.53, 28.17, 31.14, 31.21, 39.75, 45.11, 47.19, 116.25, 117.79, 120.55, 122.45, 127.45, 128.25, 131.11, 132.75, 133.65, 135.14, 151.09, 177.47, 192.80. Microanalysis: for C₂₃H₂₁ClN₆O₃S (484.96) calcd.: C, 54.70; H, 4.40; N, 17.33%; found: 54.70; H, 4.40; N, 17.10%.

Pharmacological studies

cytotoxicity of the tested compounds was evaluated using the method reported by Skehan et al. (16). The tested cancer cell lines were obtained from the American Type Culture Collection (ATCC, Minnesota, U.S.A.) through the Tissue Culture Unit, The Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt). Anticancer activity evaluation was performed at the Center for Genetic Engineering, Al-Azhar University, Cairo, Egypt. Reagents and chemicals were purchased from Sigma Aldrich Chemical Company (St. Louis, U.S.A.). Data are representative of the individual experiment, performed in three replicates for each individual dose of the tested compounds. Plates were plated in 96-multiwell plate for 24 h before treatment with the prepared compounds. The tested compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted 1000-fold in the assay. Concentrations 0, 6.25, 12.5, 25, 50 and 100 µg/mL of the tested compounds were added to the cell monolayer. The monolayer cells were incubated with the compounds for 48 h at 37°C, in atmosphere of 5% CO₂. After 48 h, the cells were fixed, washed and stained with sulforhodamine-B stain (SRB). Excess stain was washed with acetic acid. The attached stain was recovered with Tris EDTA buffer. ELISA reader was used to detect cell survival by measuring color intensity. The percent of surviving cells was plotted against different concentrations of the tested compounds to obtain the survival curve. The IC₅₀ values were calculated using sigmoidal concentration-response curve fitting models (SigmaPlot software) and standard deviation were determined by SPSS 11 software.
Molecular docking

Molecular docking studies were carried out on an Intel Core i5, processor 2.5 GHz, memory (RAM) 4 GB, windows XP operating system and Molecular Operating Environment (MOE, 10.2008) software. The X-ray crystallographic structure of CDK2 with its co-crystallized ligand (LS5) in the PDB file (ID: 1KE9) was obtained from the protein data bank. The obtained pdb file was refined by MOE program, water chains were removed, hydrogen atoms were added, the active site was detected and saved as moe file. Verification process was performed by redocking the ligand into the active site of CDK2 (Fig. 2). 2D structures of the docked compounds were drawn using Chemdraw, their 3D structures were generated using MOE, energy minimizations were performed also with MOE until a RMSD gradient of 0.05 kcal/mol and with MMFF94X forcefield and the partial charges were automatically calculated, then saved in one mdb file to be used in docking process to predict the ligand enzyme interactions at the active site.

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Conflict of interests

No conflict of interests.

REFERENCES


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