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Synthesis, molecular docking and antitumor activity of novel pyrrolizines with potential as EGFR-TK inhibitors

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ABSTRACT

A new series of pyrrolizine derivatives **4–8c** were synthesized, their structures were confirmed by spectral and elemental analyses. Cytotoxic activity of these compounds was evaluated against breast (MCF7), colon (HCT116) and liver (HEPG2) cancer cell lines using sulphorhodamine-B (SRB) assay method. All the tested compounds showed highly potent activity against MCF7 cell line with IC_{50} range equal 8–194 nM/ml and compound **8c** was the best active one (IC_{50} = 8.6 nM/ml). **8b** was the best active compound on both HCT116 and HEPG-2 cancer cell lines; its IC_{50} is 26.5 and 12.3 nM/ml respectively. Docking studies into ATP binding site of EGFR tyrosine kinase were performed to predict their scores and mode of binding to amino acids, moreover, inhibitory activity of these compounds against EGFR-TKs was evaluated; their inhibitory percent ranged from 40.4 to 97.6, compound **8c** and **8b** showed inhibitory activity at 97.6% and 88.4% respectively.

1. Introduction

Pyrrolizine is a bicyclic system consists of two fused five member rings with one nitrogen atom at the ring junction, 3*H*-pyrrolizine **I** (Fig. 1) is the parent compound of this class [1]. The saturated perhydro pyrrolizine derivatives (pyrrolizidines) constitute the main skeleton in several alkaloids [2], in addition, pyrrolizidine N-oxides proved to have antitumor activity [3–5].

Pyrrolizine analogues and their derivatives have attracted considerable interest in medicinal chemistry; this nucleus was adopted as a scaffold in many compounds with diverse biological activities as antitumor [6], anti-inflammatory [7], antiviral [8] and antimicrobial [9].

The antitumor antibiotic clazamycin A and B are natural pyrrolizine derivatives [10], moreover, phenyl pyrrolizinone derivative **II** (Fig. 2) showed a very fine cytotoxic activity against human KB cells, its IC_{50} value was 70 nM [11]. The licofelone derivative **III** showed two fold increase in activity over both 5-flurouracil and licofelone at MCF-7 and MDA-MB 231 carcinoma cells. Moreover, it was found to be as active as 5-FU and two fold more active than licofelone at colon carcinoma cells HT-29 [12]. The acylated pyrrolizine derivative **IV** (IPP) showed anticancer activity against different types of tumors [13]. Moreover, the pyrimidopyrrolizine derivative **V** (Fig. 2) was reported to inhibit the growth of Jensen's carcinoma 755 in rats by 30–40% [14]. Furthermore, our previous study for exploring the antitumor activity of pyrroloazepine system (7 and 5 fused rings) was promising [15] and this encouraged us to further explore the pyrrolizine system (5 and 5 rings) as well.

All these observations proved the importance of the pyrrolizine scaffold as a key nucleus for the synthesis of promising antitumor drugs, this paid our attention to synthesize a new pyrrolizines **4–8c** and evaluate their antitumor activity against liver (HEPG-2), breast (MCF-7) and colon cancer (HCT-116) cell lines.

The epidermal growth factor receptor (EGFR) is highly expressed in many types of cancers especially breast, colon and bladder cancers [16]. Targeting this receptor represents a good strategy for the design of new anticancer drugs [17]. Thus, molecular docking studies for all the synthesized compounds **4–8c** into ATP binding site of EGFR-TKs was performed to investigate the ability of these novel derivatives to inhibit these tumorogenic agents and explore their binding mode to EGFR-TKs. The synthesized compounds were also evaluated for their inhibitory activity against EGFR-TK.

2. Results and discussion

2.1. Chemistry

The present work includes preparation of new compounds **4–8c** based on pyrrolizine scaffold to evaluate their anticancer activity.

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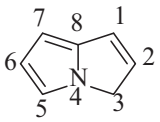
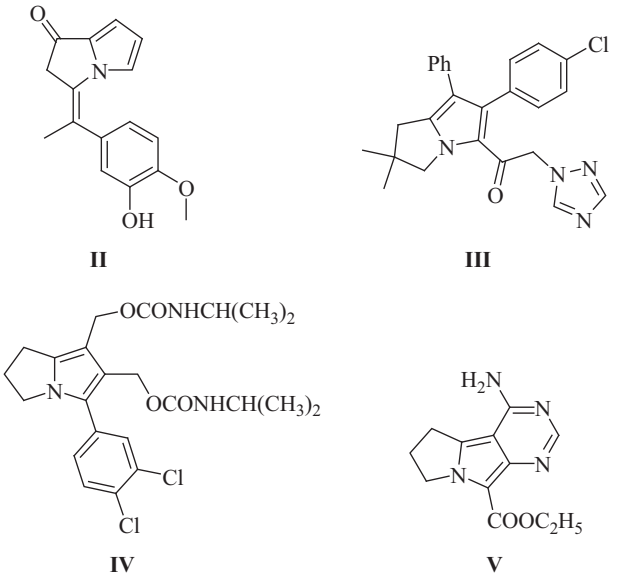


I

Fig. 1. The parent compound (3*H*-pyrrolizine).



II III IV V

Fig. 2. Antitumor active pyrrolizines and pyrimidopyrrolizines.

Compound **1** was obtained according to the previously reported method [18], heating compounds **1** and **2** under reflux for 20 h in dry acetone and in presence of dry potassium carbonate yielded a mixture of compounds **3** and **4** (^{1}H NMR spectrum of this mixture provided in the supplementary data), stirring of this mixture in 1% $NaOC_2H_5$ at r.t. for 24 h afforded compound **4** (Scheme 1). ^{1}H NMR spectrum of compound **4** (Supp. data) revealed characteristic signals for 3 CH_2 of pyrrolizine nucleus, aromatic protons signal at 7.31–7.50 ppm and a singlet at 5.39 corresponding to the NH_2 (exchanged with D_2O). Reaction of the obtained pyrrolizine derivative **4** (Scheme 2) with equimolar amount of acetyl chloride afforded the acylated pyrrolizine derivative **5**, which upon treatment with 1% sodium ethoxide at r.t. gave the pyrimidopyrrolizine derivative **6**, the ^{1}H NMR spectrum showed the appearance of additional signal characterizing CH_3 protons at 1.97 and 1.41 ppm respectively (Supp. data). Chloro-acetylamino derivative **7** was obtained by reacting compound **4** with chloroacetylchloride, ^{1}H NMR spectrum of compound **6** showed the appearance of a singlet signal at 5.39 ppm for CH_2Cl in addition to the other signals characterizing the structure (Supp. data). Compound **6** was refluxed in absolute ethanol with excess amount of different secondary amines to give compounds **8a–c** (Scheme 2), the ^{1}H NMR spectrum showed the appearance of additional signals characterizing the added secondary amine in addition to a singlet signal corresponding to CH_2-CO. Mass spectrum and ^{13}C NMR were used also in structure confirmation (Supp. data and experimental section).

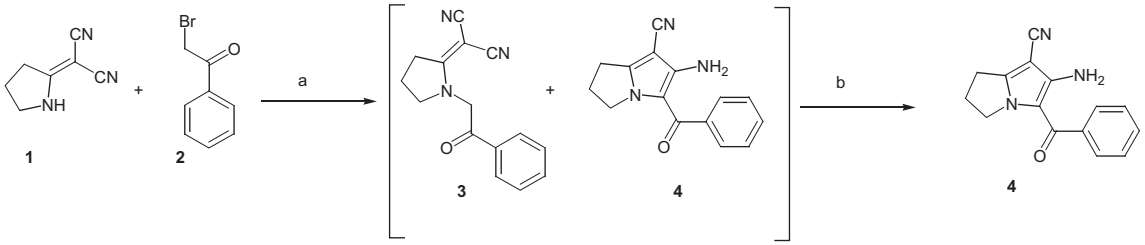
2.2. Pharmacological screening

In this work cytotoxic activity of the newly synthesized pyrrolizines **4–8c** were evaluated against breast (MCF7), colon (HCT116) and liver (HEPG2) cancer cell lines, using sulphorhodamine-B (SRB) assay method. IC_{50} was calculated and represented in μM/ml (Table 1), moreover, percent of viability at different concentrations of the tested compounds is presented in supplementary data (Tables 1–3). The tested compounds showed a marked anticancer activity against all the tested cell lines, as for their activity against MCF7 cell line; all compounds **4–8c** showed great activity against breast cancer cells with IC_{50} value in nanomole range from 8.6 to 194 nM/ml, compound **8c** was the best active one its IC_{50} = 8.6 nM/ml, followed by compound **5** and **6** with IC_{50} values of 20 and 24 nM/ml respectively, the rest of the tested compounds are arranged in the following order **7** > **8a** > **8b** > **4** according to their IC_{50} values (Table 1). Activity against colon cancer cell line (HCT116) revealed that; compound **8b** is the best active one with IC_{50} value equals 26.5 nM/ml followed by compound **6** and **7** with IC_{50} value of 40 and 106 nM/ml respectively, the other tested compounds showed IC_{50} values more than 200 nM/ml. All the tested compounds showed potent activity against HEPG2 cancer cells except compound **4** and **5** as their IC_{50} values exceeded 200 nM/ml, the active compounds against HEPG2 cancer cells are **8b** > **8c** > **7** > **6** > **8a**, their IC_{50} range is (12–112 nM/ml). Further exploration for the mode of action of these pyrrolizines was performed through docking the synthesized compounds into EGFR-TKs, moreover, the inhibitory properties against these receptors were also evaluated, compound **8c** was the most potent inhibitor (97.6%) followed by compound **8b** (88.4%) and the other compounds showed inhibitory range from 40.4% to 62.8% (Table 2).

2.3. Molecular docking

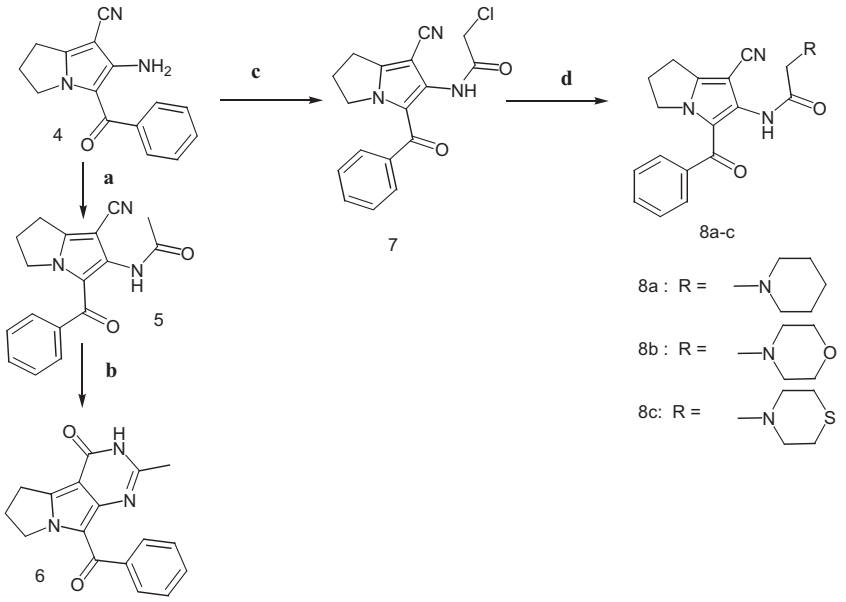
Epidermal growth factor receptor tyrosine kinases (EGFR-TKs) play a major role in cell survival and signal transduction pathways, over expression of these kinases can led to several types of human cancers, so, they are considered to be important targets in cancer treatment [19–23].

Pyrrolizines represent an important scaffold for anticancer drugs [24], however, its mechanistic studies are not sufficiently explored. In this work the synthesized compounds were docked into ATP binding site of EGFR-TK to explore the ability of the novel pyrrolizines to act as EGFR-TK inhibitors. AEE 788 was redocked into EGFR with a root mean standard deviation (RMDS) = 1.2357, AEE 788 revealed score energy (*S*) = −17.17 kcal/mol and hydrogen bonding with Thr 854 through the N atom of piperidine moiety



1 2 3 4 4

Scheme 1. Synthesis of compound **4**. Reagents and conditions: (a) Dry acetone, K_2CO_3, reflux 20 h. (b) 1% $NaOC_2H_5$, r.t. 24 h.



Scheme 2. Synthesis of compounds **5–8c**. Reagents and conditions: (a) Acetyl chloride, dioxan, r.t. 24 h. (b) 1% $NaOC_2H_5$, r.t. 24 h. (c) Chloro acetyl chloride, dioxan, r.t. 12 h. (d) Secondary amine, abs. ethanol, reflux 24 h.

Table 1
IC_{50} values for the newly synthesized pyrrolizine derivatives (μM/ml).

Comp. no.	MCF7	HCT116	HEPG2
4	0.19415	0.481379	0.89068
5	0.02087	0.58563	0.263767
6	0.02400	0.040561	0.096117
7	0.05892	0.106048	0.052709
8a	0.06298	0.20718	0.112743
8b	0.12501	0.026496	0.012286
8c	0.00862	0.31812	0.043859

Table 2
EGFR tyrosine kinase inhibitory activity of pyrrolizine derivatives at 10 μM.

Comp. no.	Percent of inhibition
4	45.2
5	58.24
6	54.27
7	40.4
8a	62.8
8b	88.43
8c	97.6
Erlotinib	100

(Fig. 3). All the docked compounds **4–8c** showed good docking scores and also showed hydrogen bonding with the receptor amino acids (Table 3), compounds **8b,c** showed better docking scores than the ligand itself, the best docking score was assigned for compound **8c** (−20.9 kcal/mol), it showed a hydrogen bonding with Asp 855 in addition to arene-cation interaction with Arg 841 amino acid (Fig. 5). Compound **8b** (Fig. 4) showed docking score (−17.8 kcal/-mol) better than **8a** (−15.6 kcal/mol) and both of them revealed a hydrogen bonding with Met 793 through the nitrogen atom of the cyano group. Compounds **5** and **6** also showed good docking scores and compound **6** revealed hydrogen bonding with LYS 745 and ASP 855 amino acids (Table 3), the least docking scores was assigned for compound **4** and **7**. The synthesized compounds **4–8c** were evaluated for their inhibitory activity against EGFR-TK and the obtained results (Table 2) proved molecular docking studies.

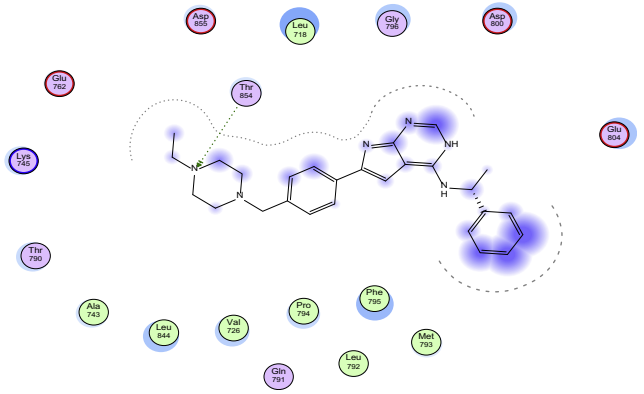


Fig. 3A. 2D interactions of AEE ligand with EGFR.

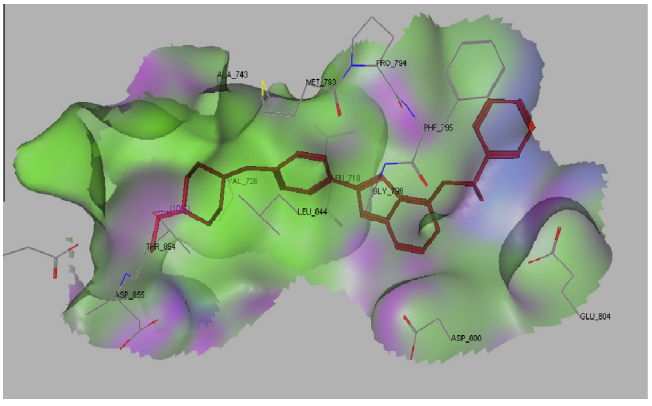


Fig. 3B. Docking predicted binding mode of AEE into the active site of EGFR.

3. Conclusion

From all the obtained data during this work we can conclude that (i) The synthesized pyrrolizine derivatives **4–8c** showed

Table 3
Docking scores, amino acid interactions and interacting groups of the docked compounds into the active site of EGFR-TK.

Comp. no.	*S* (kcal/mol)	Interacting moieties	Amino acid
4	−9.32	N of CN	Lys 745
5	−13.16		
6	−11.94	NH of pyrimidine	Asp 855
		CO in pyrimidine	Lys 745
7	−9.72		
8a	−15.59	N of CN	Met 793
8b	−17.77	N of CN	Met 793
8c	−20.92	N of thiomorpholine	Asp 855
		Phenyl moiety	Arg 841
AEE 788	−17.17	N-ethyl of piperidine	Thr 854

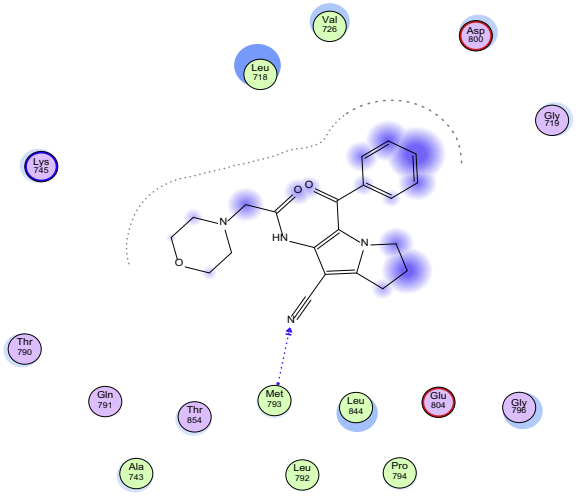


Fig. 4A. 2D interactions of compound **8b** with EGFR.

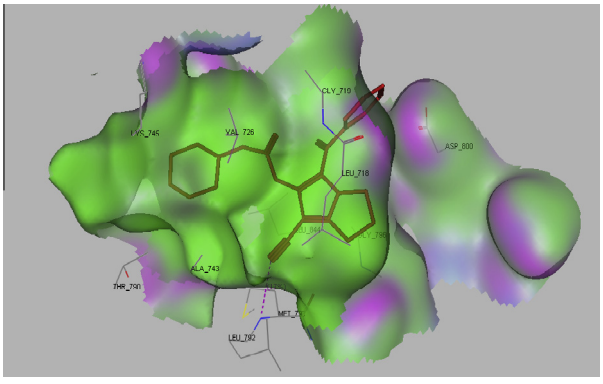


Fig. 4B. Docking predicted binding mode of compound **8b** with the active site of EGFR.

cytotoxic activity against breast cancer cells MCF7 (all are active) greater than their cytotoxity against both liver cancer HEPG2 (five compounds are active **6–8c**) and colon cancer HCT116 (three compounds are active **6**, **7**, **8b**). (ii) Compound **8c** (thiomorpholin-4-yl-acetamide derivative) is highly potent against MCF7 (IC_{50} = 8.6 nM/ml), in addition to its great activity against HEPG2 (IC_{50} = 44 nM/ml). (iii) Replacing thiomorpholine with morpholine revealed compound **8b** which is the most potent compound against HEPG2 and HCT116 cancer cell lines with IC_{50} values equal 12.3 and 26.5 nM/ml respectively, in addition to its activity against MCF7 (IC_{50} = 125 nM/ml), it can be considered as a broad spectrum

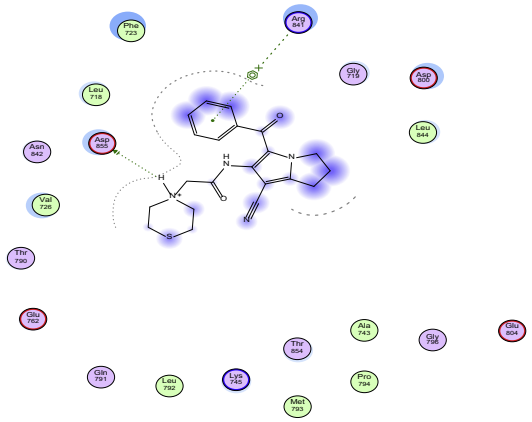


Fig. 5A. 2D interactions of compound **8c** with EGFR.

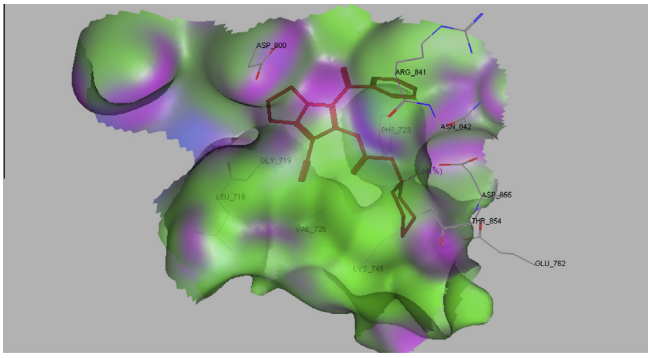


Fig. 5B. Docking predicted binding mode of compound **8c** with the active site of EGFR.

cytotoxic compound against the three tested cell lines. (iv) Docking studies proved the ability of the synthesized pyrrolizines to quick fit into the active site of EGFR-TK and can act as promising inhibitors for these receptors, compounds **8a–c** revealed the best score energy. (v) Compound **8c** was the most potent inhibitor for EGFR-TK (97.6%) followed by compound **8b** (88.4%) and **8a** (62.8%) these results confirmed the molecular docking studies.

4. Experimental

4.1. Chemistry

Compound **1** was prepared according to the previously reported procedure [18]. Melting points were uncorrected; they were detected using Electrothermal Stuart $5MP_3$ digital melting point apparatus. Thin layer chromatography (TLC) was performed using Kiesel gel 0.25 mm, 60 G F 254, Merck Silica gel plates and the running solvent system was chloroform/methanol (9.5:0.5), ultraviolet light was used to detect the spots. Elemental microanalyses were performed at the micro analytical center, Faculty of Science, Cairo University, Mass spectra were detected on Fennigan MAT, SSQ 7000, Mass spectrometer, (70 eV (EI)) at the micro analytical Center, Faculty of Science, Cairo University. The ^1H NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 on a Varian Mercury spectrometer (400 MHz) and ^{13}C NMR ($CDCl_3$) spectra were recorded at 100.62 MHz at the magnetic resonance unit at Beni-Suef University. Chemical shifts are expressed in values (ppm) and tetramethylsilane (TMS) is the internal standard, addition of D_2O was used to confirm the exchangeable protons.

*4.1.1. 2-Amino-3-benzoyl-6,7-dihydro-5H-pyrrolizine-1-carbonitrile **4***

To a mixture of **1** (0.13 g, 1 mmol) and **2** (0.2 g, 1 mmol) in dry acetone (20 ml), dry potassium carbonate (0.5 g) was added and the reaction mixture was refluxed for 20 h. The solvent was removed under vacuum, the obtained residue was washed with water, dried then dissolved in 20 ml (1% $NaOC_2H_5$) at R.T. for 24 h, filtered and recrystallized from ethanol to give white solid, 0.2 g, 80% yield, mp 125–127 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.36 (m, 2H, CH_2-6), 2.92 (t, 2H, CH_2-7), 3.63 (t, 2H, CH_2-5), 5.39 (s, 2H, NH_2 exchangeable with D_2O), 7.31–7.50 (m, 5H, aromatic protons). ^{13}C NMR ($CDCl_3$) :25.2, 50.8, 52.1, 114, 114.6, 127.2, 128.3, 132.9, 140.1, 149.7, 150.9, 184.2. MS (EI): *m*/*z* (%): 250 (100), 251 (69). Anal. Calcd for $C_{15}H_{13}N_3O$ (251.28): C, 71.70; H, 5.21; N, 16.72. Found: C, 71.81; H, 5.10; N, 16.43.

*4.1.2. N-(3-Benzoyl-1-cyano-6,7-dihydro-5H-pyrrolizin-2-yl)-acetamide **5***

Compound **4** (0.25 g, 1 mmol) was dissolved in dioxan (20 ml) and acetyl chloride (2 mmol) was added, the reaction mixture was stirred at room temperature for 24 h, solvent was evaporated under vacuum. The obtained product was crystallized from acetone. White solid, 0.2 g, 69% yield. mp 130–132 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.97 (s, 3H, CH_3), 2.48 (m, 2H, CH_2-6), 3.05 (t, 2H, CH_2-7), 3.92 (t, 2H, CH_2-5), 7.46-7.67 (m, 5H, aromatic protons), 8.67 (s, 1H, NH exchangeable with D_2O). ^{13}C NMR($CDCl_3$): 23.4, 25.2, 25.5, 50.4, 114.2, 119, 127.9, 128.1, 128.6, 132, 134.7, 138.7, 149.6, 168.5, 186.1. MS (EI): *m*/*z* (%): 293 (11), 250 (100). Anal. Calcd for $C_{17}H_{15}N_3O_2$ (293.32): C, 69.61; H, 5.15; N, 14.33. Found: C, 69.89; H, 5.04; N, 14.44.

*4.1.3. 8-Benzoyl-6-methyl-2,3-dihydro-1H,5H-5,7,8a-triaza-cyclopenta[a]inden-4-one **6***

Compound **6** was prepared from compound **5** by dissolving (0.29 g, 1 mmol) in 20 ml (1%$NaOC_2H_5$) at R.T. for 24 h, white crystals were obtained, filtered and dried, 0.15 g, 50% yield, mp 148–150 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.41 (s, 3H, CH_3), 2.37 (m, 2H, CH_2-6), 2.93 (t, 2H, CH_2-7), 3.75 (t, 2H, CH_2-5), 5.38 (s, 1H, NH exchangeable with D_2O), 7.48-7.71 (m, 5H, aromatic protons). Anal. Calcd for $C_{17}H_{15}N_3O_2$ (293.32): C, 69.61; H, 5.15; N, 14.33. Found: C, 69.47; H, 5.13; N, 14.56.

*4.1.4. N-(3-Benzoyl-1-cyano-6,7-dihydro-5H-pyrrolizin-2-yl)-2-chloro-acetamide **7***

To a solution of compound **4** (0.5 g, 2 mmol) in dioxan (20 ml) chloro acetyl chloride (0.22 g, 2 mmol) was added drop wise, the reaction mixture was stirred at room temperature for 12 h, then evaporated under vacuum. The obtained white precipitate was recrystallized from ethanol, 0.5 g, 77% yield, mp 132–134 °C. ^{1}H NMR (DMSO-d_6): δ 2.05 (m, 2H, CH_2-6), 3.03 (t, 2H, CH_2-7), 3.75 (t, 2H, CH_2-5), 5.39 (s, 2H, CH_2-Cl), 7.44-8.14 (m, 5H, aromatic protons), 10 (s, 1H, NH exchangeable with D_2O). MS (EI): *m*/*z* (%): 327 (19.6), 105 (100). Anal. Calcd for $C_{17}H_{14}ClN_3O_2$ (327.76): C, 62.30; H, 4.31; N, 12.82. Found: C, 61.98; H, 4.63; N, 12.32.

*4.1.5. General procedure for the preparation of **8a–c***

Compound **7** (0.33 g, 1 mmol) was refluxed with the appropriate secondary amine (5 mmol) in absolute ethanol for 24 h, the reaction mixture was cooled to room temperature, the obtained white precipitate was filtered and recrystallized from ethanol.

*4.1.5.1. N-(3-Benzoyl-1-cyano-6,7-dihydro-5H-pyrrolizin-2-yl)-2-piperidin-1-yl-acetamide **8a**.* Compound **8a** was prepared from compound **7** and piperidine. White solid, 0.19 g, 50% yield. mp 135–138 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.36 (m, 2H, CH_2-piperidine), 1.49 (m, 4H, $2CH_2$-piperidine), 2.45 (m, 4H, $2CH_2$-piperidine), 2.52 (m, 2H, CH_2-6), 2.78 (s, 2H, CH_2-Cl), 3.11 (t, 2H, CH_2-7), 4.08 (t, 2H, CH_2-5), 7.41–7.65 (m, 5H, aromatic protons), 9.53 (s, 1H, NH exchangeable with D_2O). MS (EI): *m*/*z* (%): 376 (5.19), 98 (100). Anal. Calcd for $C_{22}H_{24}N_4O_2$ (376.45): C, 70.19; H, 6.43; N, 14.88. Found: C, 70.49; H, 6.04; N, 14.44.

*4.1.5.2. N-(3-Benzoyl-1-cyano-6,7-dihydro-5H-pyrrolizin-2-yl)-2-morpholin-4-yl-acetamide **8b**.* Compound **8b** was prepared from compound **7** and morpholine. White crystals, 0.18 g, 50% yield, mp 138–140 °C. ^{1}H NMR (DMSO-d_6): δ 2.15 (m, 2H, CH_2-6), 2.47 (m, 4H, $2CH_2$-morpholine), 3.07 (m, 4H, $2CH_2$-morpholine), 3.74 (m, 4H, CH_2-7, CH_2-Cl), 3.77 (t, 2H, CH_2-5), 7.45–7.60 (m, 5H, aromatic protons), 9.69 (s, 1H, NH exchangeable with D_2O). MS (EI): *m*/*z* (%): 378 (2.2), 100 (100). Anal. Calcd for $C_{21}H_{22}N_4O_3$ (378.42): C, 66.65; H, 5.86; N, 14.81. Found: C, 66.47; H, 5.63; N, 14.56.

*4.1.5.3. N-(3-Benzoyl-1-cyano-6,7-dihydro-5H-pyrrolizin-2-yl)-2-thiomorpholin-4-yl-acetamide **8c**.* Compound **8c** was prepared from compound **7** and thiomorpholine. Yellowish crystals, 0.2 g, 50% yield, mp 140–142 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.48 (m, 2H, CH_2-6), 2.66 (m, 4H, $2CH_2$-thiomorpholine), 2.92 (m, 4H, $2CH_2$-thiomorpholine), 3 (m, 4H, CH_2-7, CH_2-Cl), 4 (t, 2H, CH_2-5), 7.53–7.66 (m, 5H, aromatic protons), 9.69 (s, 1H, NH exchangeable with D_2O). ^{13}C NMR($CDCl_3$): 25.1, 25.2, 25.6, 26.3, 50, 50.4, 114.2, 127.6, 127.9, 128.5, 128.7, 132, 132.07, 138.8, 138.9, 148.7, 185.5. MS (EI): *m*/*z* (%): 394 (2.6), 116 (100). Anal. Calcd for $C_{21}H_{22}N_4O_2S$ (394.49): C, 63.94; H, 5.62; N, 14.20. Found: C, 63.97; H, 5.63; N, 14.56.

4.2. Pharmacological studies

4.2.1. In vitro cytotoxic activity evaluation by SRB assay

Cytotoxic activity of the newly synthesized compounds was evaluated against MCF7, HEPG2, HCT116 cancer cell lines using Sulphorhodamine-B (SRB) assay method as previously reported by Skehan et al. [25]. Antitumor 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, Mo, U.S.A.).The tested cell lines were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.) through the Tissue Culture Unit, The Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt).

Cells were seeded for 24 h in a 96 well microtiter plates at a concentration of 1000–2000 cells/well, 100 μl/well, then cells were incubated for 48 h with various concentrations (0, 6.25, 12.5, 25, 50, 100 μg/ml) of the tested compounds, 3 wells were used for each concentration, after incubation for 48 h the cells were fixed with 10% trichloroacetic acid 150 μl/well for 1 h at 4 °C, washed by distilled water for 3 times. Wells were stained for 10–30 min at r.t. with 0.4% SRB, dissolved in 1% acetic acid 70 μl/well. Washed with acetic acid 1% to remove unbound dye till colorless drainage obtained. The plates were subjected to air drying, 24 h not exposed to UV. The dye was solubilized with 150 μl/well of 10 mMTrise-EDTA (PH 7.4) for 5 min on a shaker at 1600 rpm. The optical density (OD) of each well was measured spectrophotometrically at 545 nm with an ELISA microplate reader. Survival curve was obtained by plotting the percent of surviving cells against different concentrations of the tested compounds. The IC_{50} values were calculated using sigmoidal concentration–response curve fitting models (Sigmaplot software).

4.2.2. EGFR tyrosine kinase inhibitory activity

Kinase activity was determined using Kinase-Glo Plus luminescence kinase assay kit, by quantitating the amount of ATP remaining in the solution of kinase reaction [26]. The luminescent signal is correlated with the amount present and it was inversely related

with kinase activity. The tested compounds were diluted to 100 mM in 10% DMSO, then 5 mL of the dilution was added to a 50 mL reaction. All of the enzymatic reactions were performed at 30 °C for 40 min, 50 mL of reaction mixture contains 10 mM $MgCl_2$, 40 mM Tris, pH 7.4, 0.1 mg/mL BSA, 0.2 mg/mL Poly (Glu, Tyr) substrate, 10 mM ATP and EGFR. Incubate the plate for 5 min at r.t. then add 50 mL of Kinase-GloPlus Luminescence kinase assay to each reaction. ADP-Glo assay kit is the protein kinase assays used to determine IC_{50} values in which ADP generation was measured as it leads to an increase in luminescence signal. The reaction mixture was incubated in a 96-well plate at 30 °C for 30 min, after the incubation period add 25 mL of ADP-Glo reagent to terminate the assay. Shake the 96-well plate for 30 min at ambient temperature then incubate it, then add 50 mL of kinase detection reagent. Read the 96-well plate using the ADP-Glo Luminescence reader. All the assay components were added to the blank control except the substrate. By removing the blank control value you can obtain the corrected activity for each protein kinase target.

4.3. Molecular docking

Docking studies were performed by downloading the pdb file: 2J6M [27], refined by removing water molecules then protonated and the pocket was detected using Molecular Operating Environment software 10.2008 (MOE) provided with chemical computing group, Canada. 2J6M represent the EGFR-TK cocrystallized with AEE 788, verification process was performed by redocking of the co crystallized ligand into the active site using the default settings (Fig. 3). The synthesized pyrrolizines 2D was converted to 3D by chemdraw, then protonated, subjected to energy minimization by Merck Molecular force field (MMff 94x) and saved in an mdb data base file to be docked into the active site of the receptor. Docking scores, hydrogen bonds and interacting groups are represented in Table 3.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2015.02.006. These data include MOL files and InChiKeys of the most important compounds described in this article.

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