Synthesis of novel triazoloquinoxaline-pyrazole hybrids asantiproliferatives, EGFR inhibitors and apoptosis inducers

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ABSTRACT

Novel triazoloquinoxaline-pyrazole hybrids have been developedandsynthesized.All derivatives' anticancer activity has been evaluated using SRB assay for cancer cell lines MCF-7, HepG-2, and HCT-116. Compound **12b** was two-fold more cytotoxic than Doxorubicin, while **12a,c**demonstrated comparable cytotoxicity to the reference Doxorubicin. Further investigations on the most active derivatives **12a-c** were done to study their inhibitory activity on two EGFR subtypes wild EGFR and mutant EGFR (L858R) tyrosine kinases in MCF-7 cell lines. Compound **12b** exhibited potentinhibitory activity toward wild EGFR (IC_{50}: 0.98µM) when compared toGefitinib(IC_{50}:18.07µM).**12b** also possessed a marked inhibition againstmutant EGFR (L858R-TK) exhibiting (IC_{50}:27.45µM) in comparison to Lapatinib (IC_{50}: 61.06 µM).Compound **12b** improved the active Caspase-3 value and the BAX/Bcl-2 reference. Furthermore **12b** showed G2/M cell cycle arrest induced apoptosis in cell line MCF-7. In addition, the most active derivatives have been orally bioavailable as shown in the *in silico*determination of the ADME characters. The binding pattern of compound**12b** was also studied by molecular docking.

1. Introduction

Cancer is possessing high death rate cause in the world, and new treatments and solutions to this disease need a great deal of attention[1,2].Cells are regulated by proteolytic enzymes called caspases that induce cell death or apoptosis.The activation of apoptotic process is initiated by either extracellular or intracellular death signals, which cause activation of procaspases and caspases[3].In the last 10 years, the oncogenic mutations that change the apoptosis leading to tumor initiation , development, or metastasis have been thought to have triggered initiation of the cancer. Apoptosis offers a strong basis for understanding the relationship between cancer genetics and cancer therapy. For this reason, the analysis of the genes and pathways that make up the central machinery of the pathway of apoptosis offered new insights into tumor biology and enhanced new therapeutic benefits strategies in cancer treatment[4]. BAX and Bcl-2, which are the important members of the Bcl-2 family for their influential roles in the development of tumors and the pronostics of human malignancies, have also gained anenourmous deal of interest in recent years. In response to various cell pressures, BAX enhances cell death. In contrast, Bcl-2 abandons apoptosis by inhibiting the activity of BAX[5].Moreover, The transmembrane glycopritein with tyrosine-kinasa (EGFR) is the epidermal growth factor receptor which are overexpressed in many cancer cells such as (breast, ovarian and colon human cancers)[6].EGFR family overexpression leads to autophosphorylation of several tyrosine residues within the COOH-terminal tail of the receptors which initiates a certain cascade resulting in cell proliferation, differentiation and anti-apoptosis. So, inhibition of EGFR family identified to possess significant role in the development of targeted chemotherapeutic agents[6].

Literature survey clarified thattriazoloquinoxalineswere known asanticancer agents[7,8].In 2018,Mohamed Alswah*et al.*,reported that 1-(4-(1-ethyl-[1,2,4]triazolo[4,3-*a*]quinoxalin-4-ylamino)phenyl)-3-

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(substitutedphenyl)prop-2-en-1-one A had antiproliferative characteristics with EGFR kinase inhibition (IC_{50}=0.039µM)[2].Noteworthy,pyrazoles were proved to possess potent anticancer activity[9 ,10, 11].One of the reported anticancer pyrazoleswhich induced apoptosis via Caspase-3 activation dependent pathway wasthe reported phenyldiazenyl-1*H*pyrazoles-3,5-diamine derivative **B**[10].

Based on these findings we found that it is interesting to hybridize triazoloquinoxaline scaffold by pyrazole moieties (c.f.**figure 1)**hoping to establish more potent, and safer novel anticancer agent.

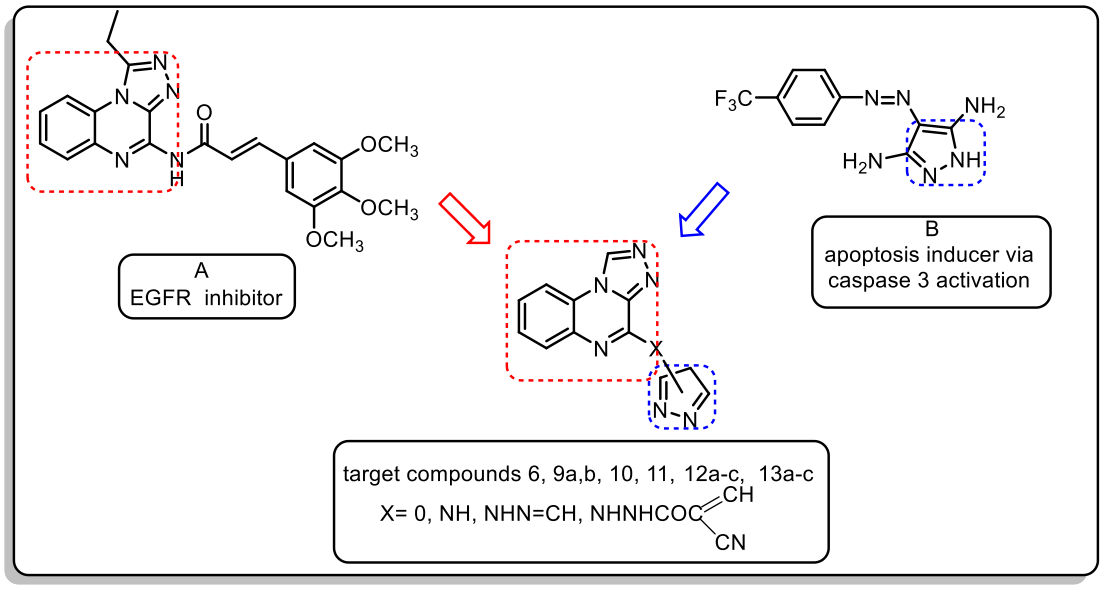


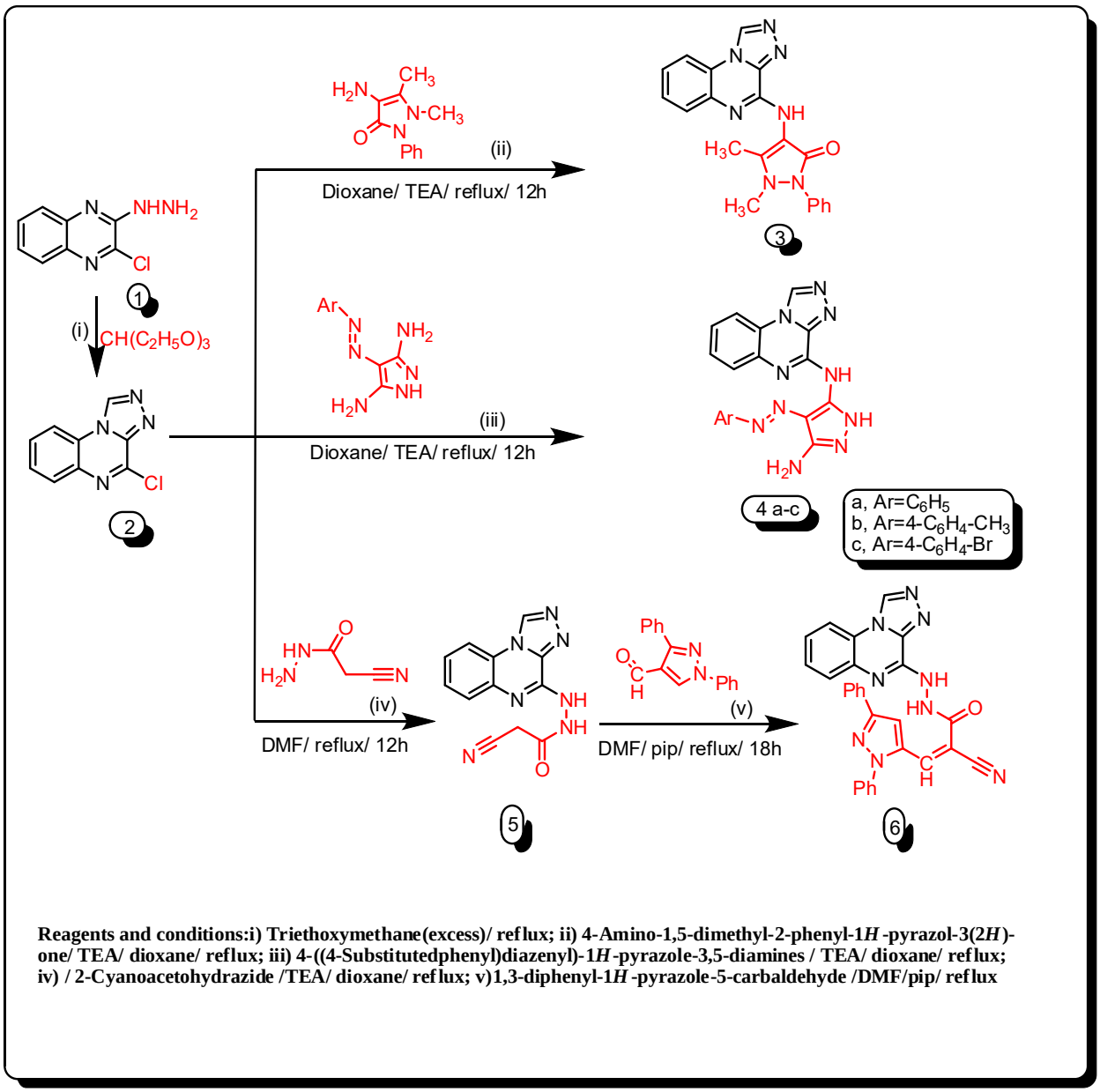
Figure1: General design of novel triazoloquinoxaline-pyrazoles hybrids

2. Results and discussion:

2.1.Chemistry

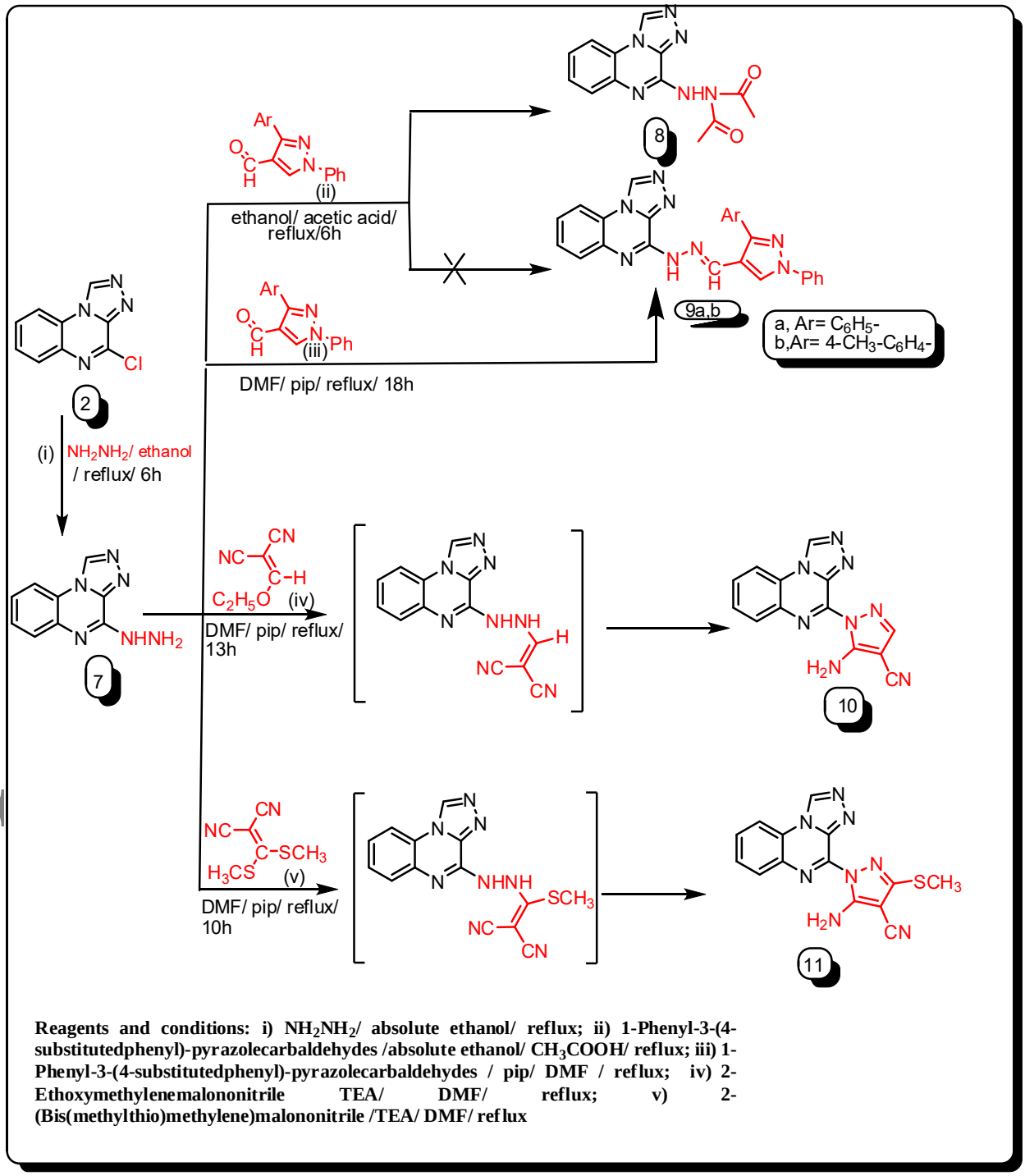
The key intermediates **2** and **7** were prepared according to reported procedure[2**]**. The nucleophilic displacement reaction of the 4-chlorotriazoloquinoxaline derivative **2** with 4-aminoantipyrine or with the aminopyrazole derivatives afforded compound **3** and diazinyl pyrazoles **4a-c**; respectively. In order to introduce pyrazole moiety at position 4 through longer linker, the intermediate cyanoacetohydrazide **5** was prepared from the reaction of 2-cyanoacetohydrazide with compound **2,** then the product **5** was further reacted with formylpyrazole derivative to yield the acrylohydrazide **6 (scheme 1)**. The ^{13}C NMR chart of **3**was characterized by a signal at 162.51 due to C=O carbon. In addition, the ^{1}H NMR spectrum of **3** showed two singlet signals at δ 2.25 and δ3.16 ppm corresponding to $2CH_3$ protons.The infrared spectrum of **4b** showed bands at a stretching frequencies (υ) 3375, 3290 cm^{-1} and 3205 cm^{-1} corresponding to the NH_2 and NH functions; respectively. Furthermore, the ^{1}H NMR spectrum of **4b** revealed a singlet signal at δ 2.32 due to CH_3 protons. The shifted value of carbonitrile stretching frequency (υ) from 2264 cm^{-1}in **5** into 2214 cm^{-1}proved the structure of **6**.When the hydrazine derivative **7** was condensed with pyrazole carbaldehyde derivatives in ethanol/acetic acid mixture, the expected Schiff's bases were not achieved, instead, the diacetylated hydrazide **8** was obtained. However, the target Schiff's bases **9a**,**b** were furnished when the same reaction was performed in DMF/ piperidine mixture. On the other hand, to synthesize triazoloquinoxaline hybrids with directly attached pyrazoles, the hydrazino derivative **7**was reacted with 2-(ethoxymethylene) malononitrile or 2-(*bis*(methylthio)-methylene)malononitrile, Michael addition of NH_2 on ylidenic bond resulting in an acyclic intermediates formation, which were cyclized by nucleophilic addition of NH on cyanocarbon to afford the [1,2,4]triazolo[4,3-*a*]quinoxaline-3-amino-pyrazole-4-

carbonitriles compounds **10** and **11**; respectively. The infrared spectrum of diacetyl hydrazide **8** represented a band at 1716 $cm^{-1}$for C=Oin addition to two singlet signals at δ1.91 and δ3.06 ppm in the ^{1}H NMR spectrum corresponding $2CH_3$ protons. While, The ^{1}H NMR of the Schiff's bases **9a**,**b** exhibited the singlet signal indicating the imine hydrogenN=CH at δ 9.67 ppm**.** In addition, the Infrared spectrum of **11** exhibited an absorption band at frequency (υ) 2210 cm^{-1}represented C≡N function group. Additionally, the ^{1}H NMR of compound**11**showed a signal which appeared as a singlet at δ2.64 ppm attributed to SCH_3 protons **(scheme 2)**. Moreover, cycloaddition of binucleophile hydrazine **7** on diazinyl malononitriles afforded the 1-(5*H*-[1,2,4]triazolo[4,3-*a*]quinoxalin-4-yl)-4-(aryl)-1*H*-pyrazole-3,5-diamines **12a-c**. Finally, the reaction of chalcone derivatives with hydrazine type **7**in basic medium assumed to be started with Michael addition followed by condensation to yield the diaryl pyrazoles **13a,b**.The ^{1}H NMR of the diaminopyrazole**12b** showed characteristic two singlets at δ 6.91and δ 9.30 ppm representative to the $2NH_2$ protons and another singlet signal at δ2.36ppm for CH_3 protons. Also**,** the IR of **13a**&**b** lacked any bands due to NH or NH_2.Besides,the ^{1}H NMR for derivative**13a**showed a singlet signal at δ3.86 ppm attributed to OCH_3 protons **(scheme 3)**. The ^{13}C NMR revealed signals of all the compounds in spectra are determined to be consistent with the expected signals for those structures. Additionally, mass spectrometry indicated molecular ion peaks at M^+.



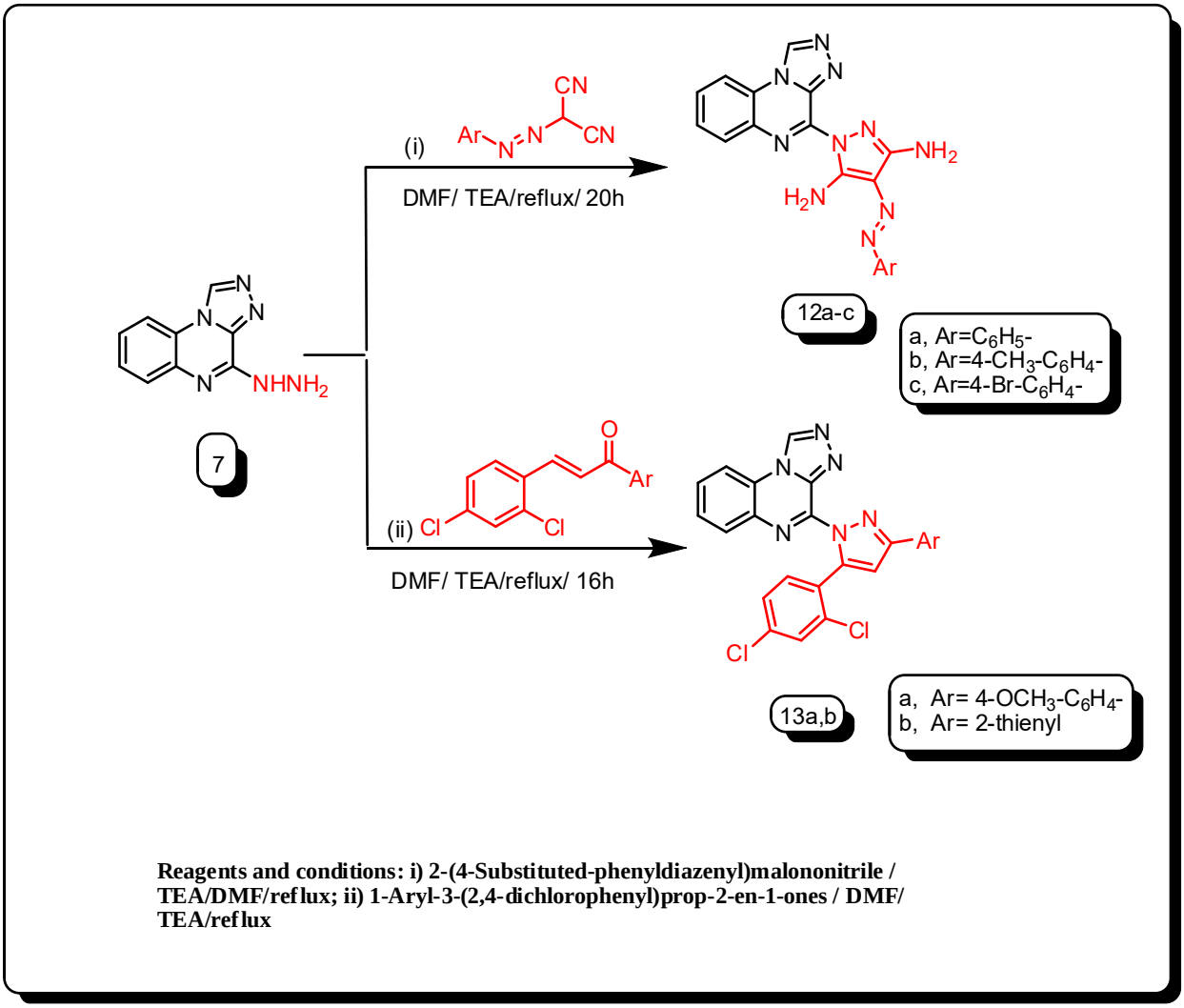
Reagents and conditions:i) Triethoxymethane(excess)/ reflux; ii) 4-Amino-1,5-dimethyl-2-phenyl-1*H* -pyrazol-3(2*H*)-one/ TEA/ dioxane/ reflux; iii) 4-((4-Substitutedphenyl)diazenyl)-1*H* -pyrazole-3,5-diamines / TEA/ dioxane/ reflux; iv) / 2-Cyanoacetohydrazide /TEA/ dioxane/ reflux; v)1,3-diphenyl-1*H* -pyrazole-5-carbaldehyde /DMF/pip/ reflux

Scheme 1: synthesis oftriazolo-quinoxalin compounds **1-8**



Reagents and conditions: i) NH_2NH_2/ absolute ethanol/ reflux; ii) 1-Phenyl-3-(4-substitutedphenyl)-pyrazolecarbaldehydes /absolute ethanol/ CH_3COOH/ reflux; iii) 1-Phenyl-3-(4-substitutedphenyl)-pyrazolecarbaldehydes / pip/ DMF / reflux; iv) 2-Ethoxymethylenemalononitrile TEA/ DMF/ reflux; v) 2-(Bis(methylthio)methylene)malononitrile /TEA/ DMF/ reflux

Scheme 2: synthesis of triazolo-quinoxalin compounds **7-12**



Scheme 3: synthesis of triazolo-quinoxalin compounds **12**&**13**

2.2. Biology

2.2.1.Screening of anti-proliferative activity

The newly synthesized hybrids wereassessed*in-vitro* against MCF-7, HepG-2 and HCT-116 cell lines (Table 1). Four of the sixteen tested compounds **12a-c**&**4b** showed high cytotoxic activities (IC50s ranging from 2.79–10.77 µM). 1-([1,2,4]triazolo[4,3-*a*]quinoxalin-4-yl)-4-(substituted-phenyldiazenyl)-1*H*-pyrazole-3,5-diamine analogues**12b**showed about twice the Doxorubicin potency(IC_{50}= 2.79, 3.07 and 3.64 µM) and **12a** (IC_{50} = 6.25, 6.17 and 8.50µM) was more potent cytotoxic than the reference Doxorubicin (IC_{50}= 4.65,7.46 and8.29 µM) respectively, against the tested cell lines.

Comparing cytotoxic results of **4a-c**&**12a-c**, it was found that **12a-c**were potent anticancer agents while their analogues having NH linkers **4a-c** demonstrated good to poor activity. On the other hand, compounds **13a, b** did not show any cytotoxicity, which recommend the importance of aryldiazenyldiaminopyrazole for cytotoxic activity.

Up on the abovefindings, it appeared that, the hybrids of triazoloquinoxaline should be directly attached to pyrazole scaffold(linker X=0). Also,from our findings, the presence of aryldiazenylpyrazolediamine moiety is essential for cytotoxic activity. In addition,The cytotoxic activity evaluation of the most active compounds on the normal cells human diploid lung fibroblasts cell line WI-38 was done owing to study their toxicity and selectivity (Table 1). The

results showed that, these compounds are inactive against this normal cell exerting IC_{50}=83.21 , 78.34 & 80.75 µM for compounds **12a-c**; respectively. So, the most active compounds exhibited no cytotoxic activity against normal cells, a considerable safety margin and selectively toward the cancerous cell lines as idicated by their IC_{50} values.Due to this, compounds **12a-c** were selected for further biological investigation.

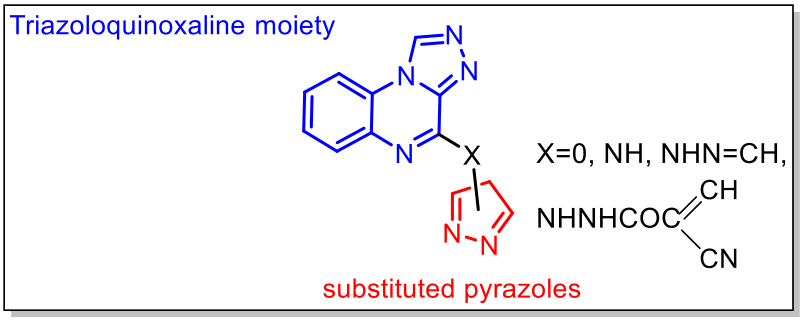


Table 1: Anti-proliferative screening of the 16 newly synthesized compoundsagainst MCF-7, HepG-2, HCT-116 and WI-38cell lines

Cpd. No.	**IC_{50} µM**			
	MCF-7	**HePG-2**	**HCT-116**	**WI-38**
3	35.11	47.23	45.57	-
4a	32.86	57.42	64.19	-
4b	10.75	11.66	10.89	-
4c	42.36	49.18	60.86	-
5	63.48	98.48	72.38	-
6	51.49	47.35	55.28	-
8	34.22	50.11	44.95	-
9a	85.37	79.43	82.38	-
9b	82.16	71.19	82.46	-
10	45.26	67.89	60.22	-
11	38.47	45.09	59.16	-
12a	**6.25**	**6.17**	**8.5**83.21	
12b	**2.79**	**3.07**	**3.64**78.34	
12c	**8.37**	**10.77**	**9.53**80.75	
13a	55.17	72.44	70.64	-
13b	65.38	84.37	92.14	-
Doxorubicin	**4.65**	**7.46**	**8.29**	-

2.2.2. EGFR inhibition assay

EGFR inhibitory assay for new representative active compounds **12a-c** was evaluated using MCF-7 cancer cells and the results obtained in table 2 showed that, compound**12b** exhibited reasonableinhibitoryagainstwt-EGFR expression showing IC_{50} value= 0.975 µMwhile the two other aryldiazenyl-pyrazole derivatives**12c** and **12a** exhibited less inhibitory activity towards the same enzymewithIC_{50} value= 1.35and 1.57 µM; respectively comparable to Gefitinib (IC_{50} value= 18.07µM)(Table2). In addition, the potent compounds **12a-c**representedgood activity on mutant type of EGFR (L858R-TK) with IC_{50} values (27.45-40.95µM)higher than Lapatinib(IC_{50} value=61.06µM)(Table3).

Table 2:IC_{50} of the hit anticancer compounds, **12a-c**on EGFR Wild type in MCF-7

Cpd. No.	IC_{50} EGFR Wild type (µM)
12a	1.547
12b	0.975
12c	1.35
Lapatinib	0.046
Gefitinib	18.07

Table 3:IC_{50} of the hit anticancer compounds**12a-c**on EGFR mutant (L858R-TK)in MCF-7

Cpd. No.	IC_{50} EGFR mutant (L858R-TK) (µM)
12a	32.72
12b	27.45
12c	40.95
Lapatinib	61.06

2.2.2.Apoptosis detection results

2.2.2.1. Effect on the active Caspase-3 level

The best understood mammalia Caspases is Caspase-3 in terms of specificity and the roles of apoptosis. The activation of Caspase-3 is a mark of appoptosis and its testing is used to study the most active hit **12b** was evaluated in the Caspase-3 cascade death pathway. Our findings of the investigation indicated the elevated level of active Caspase-3 across 9 folds than reference drug, which is considered an apoptotic marker(Table4).

2.2.2.2. Effect on mitochondrial apoptosis pathway proteins BAX and Bcl-2

BAX and Bcl-2 are the essential mitochondrial protein memberships of the Bcl-2 gene family. Apoptotic pathway regulates the balance between the two signature proteins. Hence, BAX is pro-apoptotic protein, which enhances the apoptosis.Several new BAX activators identified as targeted anticancer agents to hoping to overcome the resistance of other chemotherapeuticagents. The most promising compound **12b** was assessed for its effect on BAX, and Bcl-2, as apoptosis key markers. The high BAX/Bcl-2 ratio indicates the apoptosis susceptibility in the cell. Lower levels of that ratio can lead to resistance of apoptosis in

humancancer cell.Therefore, the findings clarified the higher BAX/Bcl-2 ratios that regulating apoptotic mechanism.(Table4).

Table 4: The effect of compound **12b** on assessment of some apoptotic markers

Cpd. No.	Caspase-3 pg/mL	BAX pg/mL	Bcl-2 pg/mL
12b	475.62	391.56	1.687
Cont.	51.27	46.85	6.165

2.2.5. Cell cycle analysis

The cell cycle analysis is used as a basis of the DNA content assessment for determining the cell proportion in each step of the cell cycle. Incubation with compound **12b** generated a shift in population from G0/G1 phase to G2/M phase and increase in apoptotic cells being represented by % of pre-G1population is seen.Cell cycle arrest on cancer cells with MCF-7, the obtained results are represented in (Figure 2&3),revealing that,apoptosis induced by compound **12b** at Pre-G1 phase, the percentage of death of the cellat Pre-G1 phase is (19.25 %). Also, MCF-7 cells accumulated at G2/M phase, the percent is (37.82 %) upon treating cells with compound**12b.** It is to be noted that this increment is accompaniedwitha decrease in the percentage of S phase (Table 5). The findings from Pre-G1 and G2/M phase indicate that compound **12b**is apoptoticinducer and can arrest the G2/M phase in the cell cycle.

Table 5: Effect of **12b** on the cell cycle of MCF-7

Cpd. No.	%G0-G1	%S	%G2/M	%Pre-G1	Comment
12b/MCF-7	36.51	25.67	37.82	19.25	PreG1apoptosis & Cell growth arrest atG2/M
Cont. MCF-7	53.71	37.67	8.62	2.15	

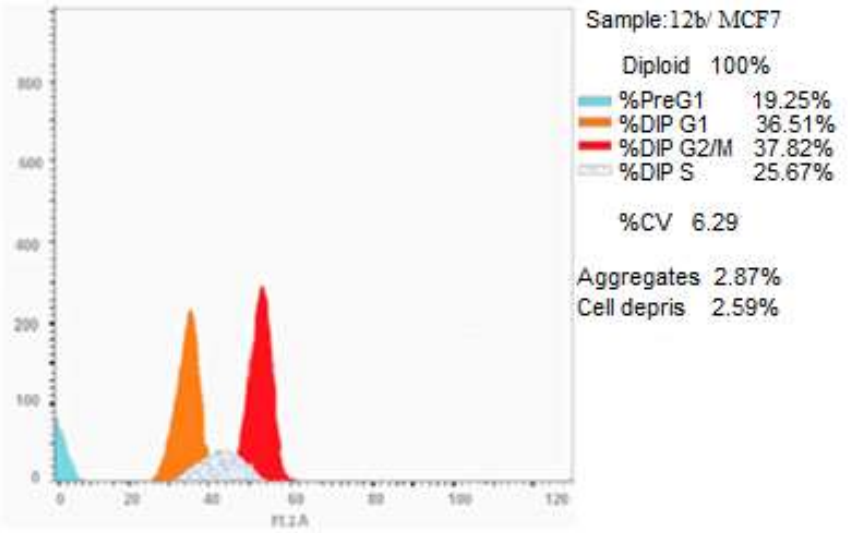


Figure 2: Cell cycle analysis of MCF-7 treated with **12b**

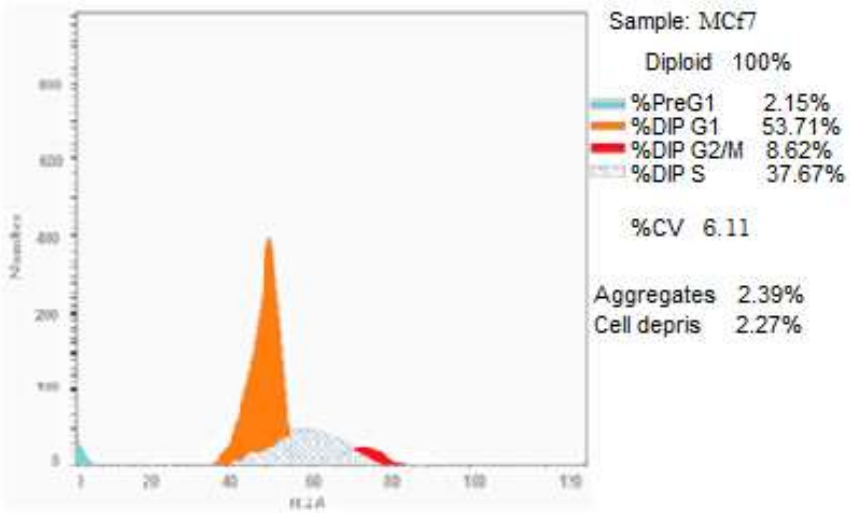


Figure 3: Cell cycle analysis of MCF-7 treated with DMSO (negative control)

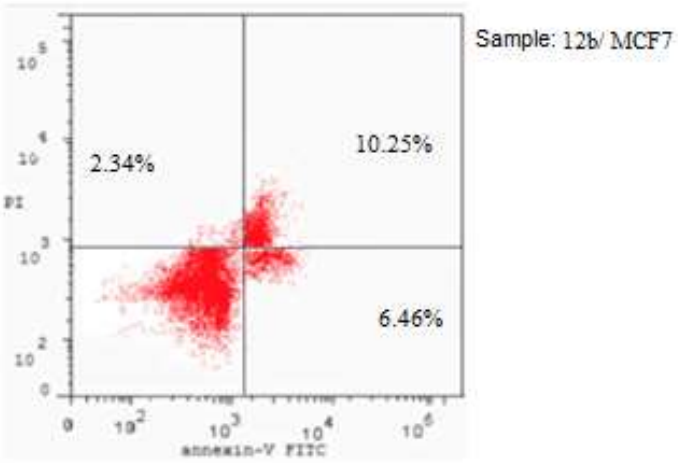
2.2.5. Annexin V-FITC apoptosis determination

Annexin V assay labeled fluorescent used to detect phosphatidylserine (PS) [12] that was transfered during early apoptosis from the inner to outer side of the cell membrane [13]. Furthermore, the florescent propidium iodide (PI) material was needed to detect the percentage of cells that died from necrosis. For this reason, Annex V / propidium iodide (PI) double staining assay was used to differentiate between apoptosis and necrosis. The early apoptosis ratio (lower right cytogram quadrant) increases from 0.36 to 10.25 per cent when MCF7-cells treated with the tested compound **12b** for 24 hours; it causes increment in the latter apoptosis ratio (higher right cytogram quadrants)) from 1.37% to 6.46%. This means that compound **12b** was induced in comparison with control at approximately 19.25 (total) in both early and late apoptosis (Table 6)(Figure 4). Compound **12b** induced apoptosis rather than necrotic pathway by the programmed cell death pathways.

Table 6: Effect of **12b** and DMSO(control) on the percentage of annexin V-FITC-positive staining in MCF-7 cells

Cpd. No.	Apoptosis			Necrosis
	Total	Early	Late	
12b/MCF-7	19.25	10.25	6.46	2.34
Cont. MCF-7	2.15	0.36	1.37	0.42

(A)



(B)

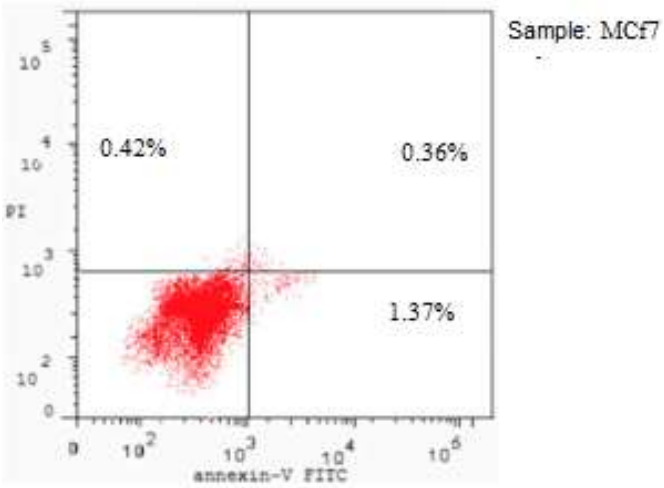


Figure 4: Effect of **12b**(A) and DMSO(control) (B) on the percentage of annexin V-FITC-positive staining in MCF-7

2.3. In silico evaluation of physicochemical and ADME

Lipinski's rule were previously reported in literature to give an idea about the expected physicochemical properties of the promising target compounds[14].

Computational study was performed for the derivatives which possess promising anticancer activity, **4b** and **12a-c** to assesstheir physicochemical and ADME properties using SwissADME[15]. With respects to physicochemical properties (Table 7**)**, the most active synthesized compounds havenoviolation for Lipinski's rule for oral drugs (M log P less than 2.40) so it is orally bioavailable compounds. All the topological polar surface areas (TPSA) are ranging from (134.53- 137.66) Å 2. Also, absorption (% ABS) was determined by applying the equation % ABS = 109 – (0.345 × TPSA)[16], found that the calculated % ABS of the four compounds ranged between 61.51% and 62.59.the four compounds do not pass blood brain barrier.

Table 7: Physicochemical properties of the hit compounds **4b** and**12a-c**based on Lipinski's rules, TPSA, % ABS and number of rotatable bonds

	Lipinski's parameters							
Cpd. No.	**M.Wt**	**H-bond donors**	**H-bond acceptors**	**MLOGP**	**Lipinski violation**	**TPSA**	**% ABS**	**No of rotatable bonds**
4b	370.37	3	6	2.11	0	134.53	62.59	4
12a	370.37	2	6	1.71	0	137.66	61.51	3
12b	384.40	3	6	1.94	0	134.53	62.59	4
12c	449.27	2	6	2.32	0	137.66	61.51	3

2.3.3. Docking study on EGFR

The minor binding energy comparable to the reference, Erlotinib (score-6.28 and-5.35 Kcal / mol) was shown on the docking of **12b** on an active site of EGFR. Owing to its two bulky aryl rings, the orientation of **12b** pyrazolo triazoloquinoxaline was parallel to the hinge area. The hydrogen bond accepter (distance: 3,23Å) with the backbone of NH Met769 in the hinge region was nitrogen from position 2 of triazoloquinoxaline of **12b**. (Figure 5).

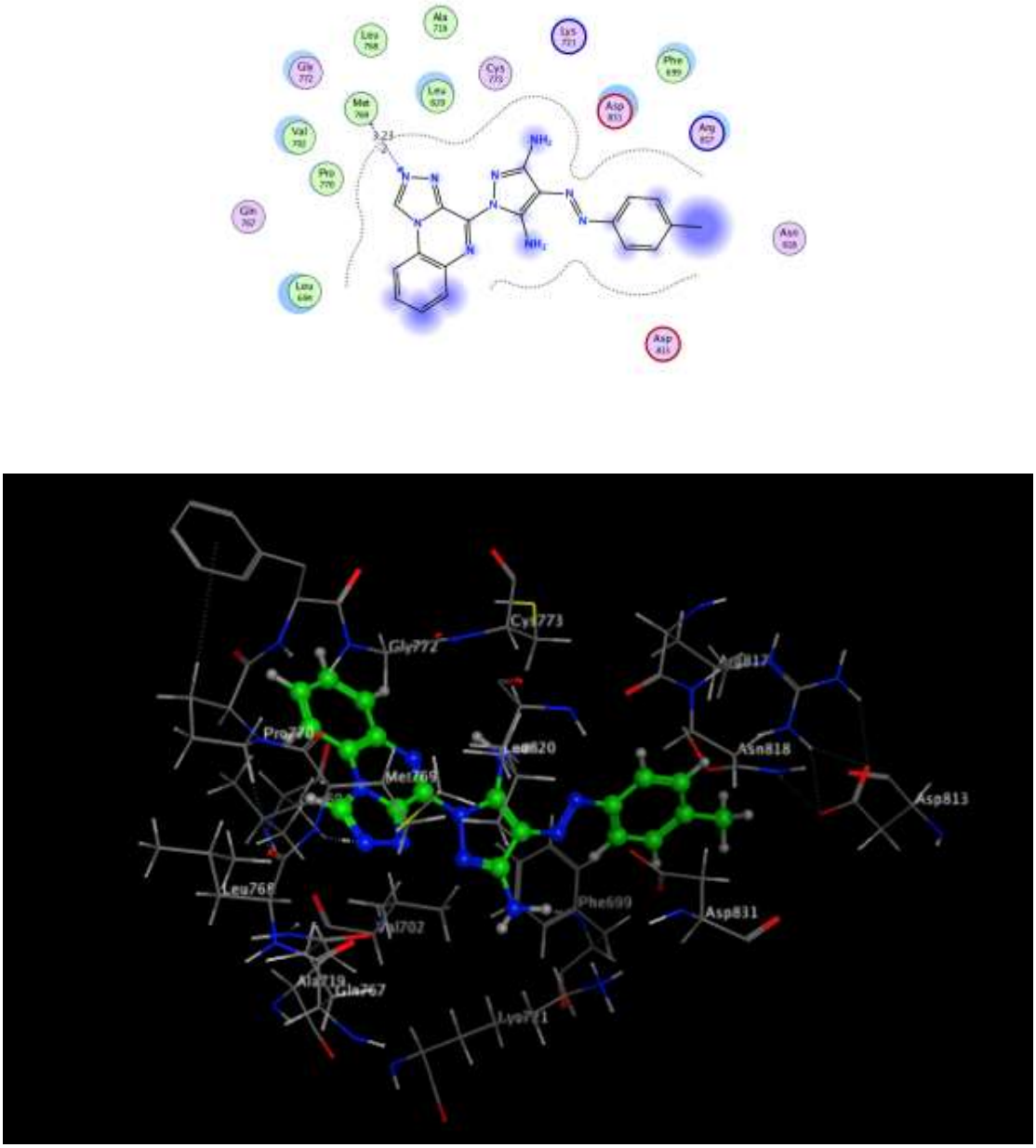


Figure 5: The 12b expected binding mode docked in the active site of EGFR. A and B exertingthe 2D and 3D ligand-receptor interactions (C atoms are colored green, N blue)

3. Conclusion

In this analysis, new hybrids of triazoloquinoxaline pyrazoles was produced and synthesized. Tests have shown that four hits have triggered cytotoxic activity of HCT-116, HepG-2, and MCF-7 cell lines.However, The most potent compounds **12a-c** showed no cytotoxic effect toward the normal cellline WI-38.Two forms of the inhibitory activity of EGFR kinase (normal and mutant) were tested for **12a-c**, where **12b** were found to be strong inhibitory against the EGFR wild. In order to assess the pro-apoptotic ability of our compounds, apoptosis studies for the **12b** most active compound have been performed. The results showed that **12b** increased active Caspase-3 value and the BAX / Bcl-2 ratiowhen compared to control level. In addition, **12b** has been identified as arresting MCF7 cells at G2 / M and increasing the pre-G1 cell percentage to 8.95 times.

4. Materials and Methods

4.1 Chemistry

Solvents and reagents were bought from specific commercial suppliers and used without further purification. The yields reported are for the purified products. All reactions were regularly tested using a Thin Layer Chromatography (TLC) on Merck Silica Gel 60 F254 (0.25 mm thick) and a UV lamp simulation took place. Melting points measurement was done in the capillary tubes using Stuart SMP3 device. A Simadzu FT / IR 1650 (Perkin Elmer) spectrometer was used for the test of IR spectra (KBr). The Bruker Advance-400 (400 MHz for 1H and 100 MHz for 13C) was captured with ^{1}H and ^{13}C spectra in DMSO-*d6*. Chemical shifts (δ) are seen in ppm relative to the TMS as a standard, or to the solvent used in the spectrum. A Shimadzu 2010 GS / MS-QP plus spectrometer with 70 eV was used to perform the mass spectrum.Anticancer research was conducted on local strain found in the Regional Center for Genetic Engineering, Faculty of Science (Boys), University of Al-Azhar and the findings were within ± 0.4 percent of the measured outcome.

Compounds4-(phenyldiazenyl)-1*H*-pyrazole-3,5-diamine derivatives[17], 2-cyanoacetohydrazide[18], 1,3-diphenyl-1*H*-pyrazole-5-carbaldehydes[19], ethoxymethylenemalononitrile[20,21],*bis*(methylthio)-methylenemalononitrile[22,21], 2-(phenyldiazenyl)malononitrileanalogues[23, 24] and 3-(2,4-dichlorophenyl)-1-(aryl)prop-2-en-1-one derivatives[25, 26] were prepared according to reported procedures.

4-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-ylamino)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one(3)

Triazoloquinoxaline**2** (2.05g, 0.01mol.) and 4-aminoantipyrine (2.03g, 0.2mL, 0.01 mol.) washeated under reflux for 12 h in dioxane(20 mL)containing 3drops of TEA. The solid product was filtered, dried and crystallized from ethanol to afford **3**.

White powder, yield: (46.3%), m.p. 223^{0}C;IR (KBr, cm^{-1}): 3379 (NH); 3082 (C-H aromatic); 2985(C-H aliphatic); 1660 (C=O); 1589 (C=N); 1539 (C=C);^{1}H NMR (DMSO-d_6, δ ppm): 2.25 (s, 3H, pyrazole-C_5-CH_3); 3.16(s, 3H, pyrazole-N_1-CH_3); 7.36(t, 1H, *J*=8Hz,N-C_6H_5-C_4-H); 7.38-7.7.50 (m, 4H, N-C_6H_5-$C_{2,3,5,6}$-H); 7.51-7.60 (m, 3H, triazoloquinoxaline-$C_{7,8,9}$-H); 8.23 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_6-H); 9.29 (s, 1H, NH, D_2O exchangeable); 10.04 (s, 1H, quinoxaline-C_1-H);^{13}CNMR (DMSO-*d6*): 11.06 (CH_3-C), 36.51(N-CH_3-C), 108.32, 116.63, 122.79, 123.90, 124.56, 126.63, 127.03, 128.03, 129.59, 135.64, 136.79, 138.47, 138.72(Ar-C), 145.41, 153.98(C=N), 162.51(C=O);MS *m/z* (relative intensity %): 371 ($M^{\cdot+}$, 26.71); 292(65.97); 143(42.21); 75(62.15); 56.30 (100); Anal. form: $C_{20}H_{17}N_7O$. Calcd.(%) C, 64.68; H, 4.61; N, 26.40; O, 4.31. Found (%): C,64.91; H, 4.85; N, 26.64.

General procedure for preparation of:

***N*5-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-4-(aryldiazenyl)-1*H*-pyrazole-3,5-diamine(4a-c)**

Chlorotriazoloquinoxaline**2** (2.05 g, 0.01 mol.) and 4-(aryldiazenyl)-1H-pyrazole-3,5-diamines were heated under refluxin dioxane (15mL) containing 3 drops of TEA for 12 h.The solid product was filtered, dried and crystallized from ethanol to afford the different 1*H*-pyrazole-3,5-diamine derivatives **4a-c**.

***N*5-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-4-(phenyldiazenyl)-1*H*-pyrazole-3,5-diamine(4a)**

Reddish brown powder, yield: (56%), m.p. $>300^{0}$C.IR (KBr, cm^{-1}): 3375, 3294 (NH_2&NH); 3070 (C-H aromatic); 2904 (C-H aliphatic); 1624 (C=N); 1508 (C=C); 1462 (N=N);^{1}H NMR (DMSO-$d_{6,}\delta$ ppm):4.10(br.s, 2H, NH_2, D_2O exchangeable); 7.34 (t, 1H, *J*=7.3Hz, C_6H_5-C_4-H) 7.47 (t, 2H, *J*=7.3Hz,C_6H_5-$C_{3,5}$-H); 7.60-7.78 (m, 2H, C_6H_5-$C_{2,6}$-H); 7.88 (d, 2H, *J*=7.7Hz, triazolquinoxaline- C_9-H); 8.15-8.23 (m,

2H, triazoloquinoxaline- $C_{7,8}$-H); 8.42-8.55 (m, 1H, triazoloquinoxaline-C_6-H); 8.86 (s, 2H, two NH, D_2O exchangeable); 10.38 (s, 1H, triazoloquinoxaline-C_1-H); ^{13}CNMR (DMSO-d_6, δ ppm): 113.29, 116.82, 117.26, 120.00, 121.58, 123.67, 123.89, 128.57, 128.76, 128.85,129.17, 129.49, 133.82, 137.79, 138.52, 139.23(Ar-C), 141.69, 153.15(C=N); MS *m/z* (relative intensity %): 370 (M, 36.72); 367 (M-3, 34.10); 343 (100). Anal. form: $C_{18}H_{14}N_{10}$. Calcd.(%) C, 58.37; H, 3.81; N, 37.82. Found (%): C,58.48; H, 4.39; N, 26.28.

5-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-4-(*p*-tolyldiazenyl)-1*H*-pyrazole-3,5-diamine(4b)

Reddish brown powder, yield: (52%), m.p.>300^{0}C.IR (KBr, cm^{-1}): 3375, 3290 (NH&NH_2); 3074 (C-H aromatic); 2978 (C-H aliphatic); 1620 (C=N); 1566 (C=C); 1462 (N=N);^{1}H NMR (DMSO-d_6,δ ppm): 2.37 (s, 3H, CH_3); 6.31(s, 2H, NH_2, D_2O exchangeable); 7.28 (d, 2H,*J*=8.2Hz, 4-CH_3-C_6H_4-$C_{3,5}$-H); 7.66-7.71 (m, 2H, triazolquinoxaline-$C_{7,8}$-H); 7.76 (d, 2H, *J*=8.2Hz, 4-CH_3- C_6H_4-$C_{2,6}$-H); 8.10-8.18 (m, 1H, triazoloquinoxaline-C_9-H); 8.37-8.46 (m, 1H, triazoloquinoxaline-C_6-H); 8.59 (s, 2H,two NH, D_2O exchangeable); 10.21 (s, 1H, triazoloquinoxaline-C_1-H); ^{13}CNMR (DMSO-d_6): 21.23 (CH_3-C), 68.23, 73.15, 121.36, 123.08, 128.34, 128.73, 130.07, 138.32(Ar-C), 143.13, 151.32, 165.12(C=N); MS*m/z* (relative intensity %): 384 (M, 12.45); (356, 56.69); (330, 75.06); (254, 85.83);(240, 76.85); (110, 85.02); 111 (100). Anal. form: $C_{19}H_{16}N_{10}$. Calcd.(%) C, 59.37; H, 4.20; N, 36.44. Found (%): C,59.53; H, 4.31; N, 36.28.

5-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-4-((4-bromophenyl)diazenyl)-1*H*-pyrazole-3,5-diamine(4c)

Reddish brown powder, yield: (45%), m.p.>300^{0}C.IR (KBr, cm^{-1}): 3390, 3290 (NH &NH_2); 3070 (C-H aromatic); 2970 (C-H aliphatic); 1624 (C=N); 1566 (C=C); 1462(N=N); 1064 (*p*-substituted-phenyl);^{1}H NMR (DMSO-d_6,δ ppm): 7.66 (d, 2H, *J*=8Hz, 4-Br- C_6H_4-$C_{2,6}$-H); 7.68-7.76 (m, 2H, triazoloquinoxaline-$C_{7,8}$-H); 7.84 (d, 1H, *J*=8Hz, 4-Br- C_6H_4-$C_{3,5}$-H); 8.11 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_9-H); 8.44 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_6-H); 8.85 (br.s, 2H, twoNH, D_2O exchangeable); 10.28 (s, 1H, triazoloquinoxaline-C_1-H);^{13}CNMR (DMSO-d_6): 74.51, 123.54, 128.60, 129.62, 132.36(Ar-C), 141.11, 151.91, 162.25(C=N); MS *m/z* (relative intensity %): 449 (M, 33.45);275.76(66.13); 224.12 (88.25); 205 (100); (114.02, 78.81). Anal. form: $C_{18}H_{13}BrN_{10}$. Calcd.(%) C, 48.12; H, 2.92; Br, 17.79; N, 31.18. Found (%): C,48.31; H, 3.16; N; 31.40.

N'-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-2-cyanoacetohydrazide(5)

Equivalnts of triazoloquinoxaline **2** (2.05 g, 0.01mol.)and 2-cyanoacetohydrazide (0.99gm, 0.01 mol) was heated under reflux in DMF (10 mL) for 12 h. The reaction mixture was trated with ice cold water (20 mL), and the solid mass was filtered, dried and crystallized from dioxane to afford the target compound**5**.

Light brown powder, yield(84%), m.p. >300^{0}C.IR (KBr, cm^{-1}): 3323, 3213 (NH_2); 3105 (C-H aromatic); 2908, 2839(C-H aliphatic); 2264 (C$\equiv$N) 1740 (C=O); 1620 (C=N); 1550 (C=C);^{1}H NMR (DMSO-d_6,δ ppm): 3.91 (s, 2H, CH_2CN); 7.32-7.64(m, 2H, triazoloquinoxaline-C_7-H& NH, D_2O exchangeable); 7.68-7.73 (m, 1H,triazolo quinoxaline-C_8-H); 8.31-8.42 (m, 1H, triazoloquinoxaline-C_9-H); 8.41-8.45 (m, 1H, triazoloquinoxaline-C_6-H),10.02 (s, 1H, triazoloquinoxaline-C_1-H); 10.20 (s, 1H, NH, D_2O exchangeable); ^{13}CNMR (DMSO-d_6):24.64(CH_2-C),116.23(C$\equiv$N), 116.80, 118.33, 128.24, 128.42, 138.50(Ar-C), 138.88, 139.53(C=N), 153.12(C=O); MS *m/z* (relative intensity %): 267 (M, 38); 264 (74.14); 215 (86.23); 121 (100). Anal. form: $C_{12}H_9N_7O$.Calcd(%):C, 53.93; H, 3.39; N, 36.69; O, 5.99. Found (%): C,54.14; H, 3.54; N, 36.51.

N'-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-5-yl)acrylohydrazide(6)

Cyanoacetohydrazide analogue **5**(2.67g, 0.01 mol.) and1,3-diphenyl-1*H*-pyrazole-5-carbaldehyde (2.48g, 0.01mol.) was heated under reflux in DMF (10mL) as solvent and piperidine (3drops) as basic catalyst for 18 hours. The reaction mixture was concentrated and treated with ice cold water and then the obtained precipitate was filtered, dried, and crystallized from DMF to afford the acrylohydrazide **6**.

Yellow powder, yield(57%) ,m.p.(above 300^0C).IR (KBr, cm^{-1}): 3394 (NH) ; 3062 (C-H aromatic); 2947 (C-H aliphatic); 2214 (C≡N); 1700 (C=O); 1631 (C=N); 1597 (C=C); ^{1}H NMR (DMSO-d_6, δ ppm): 7.16-8.18 (m, 12H, C=C<u>H</u>, pyrazole- C_4-H, pyrazole-*N*1-C_6H_5-$C_{2,3,4,5,6}$-H, pyrazole-C_3-C_6H_5-$C_{3,4,5}$–H& triazoloquinoxaline-$C_{7,8,9}$-H,); 8.38-8.53 (m, 3H, triazoloquinoxaline-C_6-H, pyrazoleC_3-C_6H_5-$C_{2,6}$ -H); 9.61 (s, 1H, NH, D_2O exchangeable); 10.09 (s, 1H, triazoloquinoxaline-C_1-H);. 10.11(s, 1H, NH, D_2O exchangeable). MS *m/z* (relative intensity %): 498(M+1, 23.83); 497 (M, 26.83); 62 (100). Anal. form: $C_{28}H_{19}N_9O$. Calcd(%) C, 67.60; H, 3.85; N, 25.34; O, 3.22.Found (%): C,67.49; H, 4.02; N, 25.57.

***N*'-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-*N*-acetylacetohydrazide(8)**

An equimolar mixture of 4-Hydrazino triazoloquinoxaline **7** (2.05 g, 0.01mol.) and1-phenyl-3-aryl-1H-pyrazole-4-carbaldehydeanalogueswere heated under reflux in ethanol/ acetic acid (15mL/10 drops) for 6hours. The solid product was filtered, dried and crystallized from ethanol to afford *N*-acetylacetohydrazide**8.**

Yellow powder, yield (55%), m.p.>300^0C.IR (KBr, cm^{-1}):3286 (NH); 3059 (C-H aromatic); 2947 (C-H aliphatic); 1716 (C=O); 1643 (C=N); 1581 (C=C). ^{1}H NMR (DMSO-d_6,δ ppm): 1.91 (s,3H,CO<u>CH$_3$</u>); 3.06 (s, 3H, CO<u>CH$_3$</u>); 7.71(t, 2H, triazoloquinoxaline-$C_{7,8}$-H,) ; 8.31 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_9-H); 8.46 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_6-H);10.01 (s, 1H, triazoloquinoxaline-C_1-H); 11.99 (s, 1H, NH, D_2O exchangeable). ^{13}CNMR (DMSO-d_6):15.27, 21.53, 118.41, 118.45, 123.05, 124.32, 128.05, 128.43, 138.67, 149.23, 172.58.MS *m/z* (relative intensity %): 284 (M, 25.91); 283 (M-1, 44.58); 229(54); 169 (100); 69(98). Anal. form: $C_{13}H_{12}N_6O_2$. Calcd. (%) C, 54.93; H, 4.25; N, 29.56; O, 11.26.Found (%): C,55.19; H, 4.38; N, 29.78.

General procedures for synthesis of (9a,b):

Compound**7** (2.05g, .0.01 mol.) and 1,3-diaryl-1*H*-pyrazole-4-carbaldehyde derivativeswasheated under reflux for 18 hours in dioxane / DMF (10mL/5mL) containing catalytic amount of piperidinefor 12 h. The obtained solid was filtered, washed with ethanol, dried and crystallized from dioxane to yield the triazoloquinoxalines **9a, b.**

4-(2-((1,3-Diphenyl-1*H*-pyrazol-4-yl)methylene)hydrazinyl)-[1,2,4]triazolo-[4,3-*a*]quinoxaline (9a)

Yellow powder, yield: (35%), m.p.>300^0C).IR (KBr, cm^{-1}): 3425, (NH); 3055 (C-H aromatic); 2947 (C-H aliphatic); 1631 (C=N); 1543 (C=C). ^{1}H NMR (DMSO-d_6,δ ppm): 7.22-7.37 (m, 2H, pyrazole-C_3-phenyl-C_4-H & pyrazole-*N*-C_6H_5-C_4-H); 7.39-7.46 (m, 4H, pyrazole- C_3-C_6H_5- $C_{3,5}$-H & pyrazole-*N*-C_6H_5-$C_{3,5}$-H); 7.50-7.69 (m, 2H pyrazole-*N*-C_6H_5-$C_{2,6}$-H); 7.70-8.25 (m, 3H, triazoloquinoxaline-$C_{7,8,9}$-H); 8.67-8.69 (m, 2H, pyrazole- C_3-C_6H_5-$C_{2,6}$-H); 8.86 (d, 1H, *J*=12Hz, triazoloquinoxaline-C_6-H); 9.69 (s, 1H, N=<u>CH</u>); 10.08 (s, 1H, triazoloquinoxaline-C_1-H); 10.62 (s,1H, NH, D_2O exchangeable). ^{13}CNMR (DMSO-d_6): 22.39, 117.04, 118.36, 119.47, 122.95, 127.77, 128.46, 128.93, 129.82, 129.95, 130.14, 130.37, 138.89, 139.57, 146.43, 156.23. MS *m/z* (relative intensity %): 432 (M+2, 22.48); 430 (M, 34.48), 389 (61.42); 227(100); 189(76). Anal. form: $C_{25}H_{18}N_8$. Calcd. (%) C, 69.75; H, 4.21; N, 26.03Found (%): C,69.61; H, 4.39; N, 26.28.

4-(2-((1-Phenyl-3-(*p*-tolyl)-1*H*-pyrazol-4-yl)methylene)hydrazinyl)-[1,2,4]triazolo[4,3-*a*]quinoxaline (9b)

Orange yellow powder, yield: (55%), m.p.>300^{0}C.IR (KBr, cm^{-1}): 3410, (NH); 3082 (C-H aromatic); 2943 (C-H aliphatic); 1631 (C=N); 1543 (C=C);^{1}H NMR (DMSO-d_6,δ ppm): 2.42 (s, 3H, $\underline{CH_3}$); 7.24 (t, 1H,*J*=8Hz ,*N*-C_6H_5-C_4-H); 7.35-7.43 (m, 4H, 4-CH_3-C_6H_4-$C_{3,5}$-H&*N*-C_6H_5-$C_{3,5}$-H); 7.54-7.68 (m, 2H, triazoloquinoxaline-$C_{7,8}$-H); 7.71-7.75 (m, 1H,*N*-C_6H_5-$C_{2,6}$-H); 7.86 (d, 1H, *J*=8Hz, 4-CH_3-C_6H_4-$C_{2,6}$-H) 7.94 (s, 1H, pyrazole-C_5-H); 8.11 (d, 1H, *J*=8Hz triazoloquinoxaline-C_9-H); 8.43-8.47 (m, 1H, triazoloquinoxaline-C_6-H); 8.42-8.48 (m, 1H, triazoloquinoxaline-C_6-H); 9.67 (s, 1H, N=$\underline{CH}$); 10.05 (s, 1H, triazoloquinoxaline-C_1-H); 11.38 (s,1H, NH, D_2O exchangeable); ^{13}CNMR (DMSO-d_6): 66.81(CH_3), 116.92, 119.29, 119.54, 119.77, 127.76, 128.42, 128.77, 128.89, 129.12, 129.23, 129.40, 129.53, 130.16, 130.31, 134.25, 138.72, 139.12, 139.45, 139.54(Ar-C), 142.06, 149.26, 152.69(C=N).MS *m/z* (relative intensity %): 445 (M, 53.94); 444 (M-1, 73.82); 257(77); 209(93); 96 (100). Anal. form: $C_{26}H_{20}N_8$. Calcd. (%) C, 70.26; H, 4.54; N, 25.21. Found (%): C,69.98; H, 4.67; N, 25.47.

1-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-3-amino-1*H*-pyrazole-4-carbonitrile (10)

Hydrazine analogue**7** (2.05g, 0.01 mol) and 2-(ethoxymethylene)malononitrile (1.22g, 0.01mol.) was heated under reflux in dioxane/ DMF mixture (10/ 5mL), containing few drops of TEAfor 13 h. The reaction mixture was filtered, dried and crystallized from ethanol to afford the carbonitrile **10.**

Brown powder, yield (43%), m.p.> 300^{0}CIR (KBr, cm^{-1}): 3305, 3170 (NH_2); 3080 (C-H aromatic); 2214 (C≡N); 1635 (C=N); 1539 (C=C). ^{1}H NMR (DMSO-d_6,δ ppm): 7.06 (s, 2H, NH_2, D_2O exchangeable); 7.19 (s, 1H, pyrazole-C_5-H); 7.69-7.74 (m, 2H, triazoloquinoxaline-$C_{7,8}$-H); 8.41-8.48(m, 1H, triazoloquinoxaline-$C_{6,9}$-H); 10.04 (s, 1H, triazoloquinoxaline-C_1-H).^{13}C-NMR (DMSO-d6): 82.01, 118.40(C≡N), 128.52, 138.90(Ar-C), 147.31, 157.14(C=N).MS *m/z* (relative intensity %): 276 (M, 52.01); 275 (M-1, 52.27); 200(44.50); 102 (100). Anal. form: $C_{13}H_8N_8$. Calcd.(%) C, 56.52; H, 2.92; N, 40.56. Found (%): C,56.76; H, 3.18; N, 40.34.

1-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-3-amino-5-(methylthio)-4,5-dihydro-1*H*-pyrazole-4-carbonitrile(11)

An equimolar mixture of **7** (2.05 g, 0.01 mol) and 2-(bis(methylthio)methylene) malononitrile **(**1.70g, 0.01mol.) was heated under reflux in dioxane/ DMF mixture (10/ 5mL) containing few drops of TEAfor 10 h. The reaction mixture was filtered, dried and crystallized from ethanol to yield the title compound**11.** Orange powder, yield (60.6%), m.p.> 300^{0}C.IR (KBr, cm^{-1}): 3394, 3294 (NH_2); 3116 (C-H aromatic); 2924(C-H aliphatic); 2210 (C≡N); 1631 (C=N); 1566 (C=C);^{1}H NMR (DMSO-d_6,δ ppm): 2.64 (s, 3H, pyrazole-C_5-S-$\underline{CH_3}$); 7.73 (t, 1H,*J*=8Hz, triazoloquinoxaline-C_7-H); 7.81 (t, 1H, *J*=8Hz, triazoloquinoxaline -C_8-H); 8.19 (s, 2H, NH_2, D_2O exchangeable); 8.25 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_9-H); 8.46 (d, 1H, *J*=8Hz, triazoloquinoxaline -C_6-H),10.25 (s, 1H, triazoloquinoxaline-C_1-H); ^{13}CNMR (DMSO-d_6): 13.46(S-$\underline{CH_3}$), 72.88(pyrazole-C_4), 114.04(C≡N), 116.81, 124.87, 128.47, 129.57, 129.87, 133.50, 138.58, 139.01(Ar-C), 141.87, 151.89, 155.90(C=N).MS *m/z* (relative intensity %): 323 (M+1, 17.48); 322 (M, 60.01); 313.25 (100); (254, 77); (212, 78); (66, 69).Anal. form: $C_{14}H_{12}N_8S$. Calcd. (%):C, 51.84; H, 3.73; N, 34.55; S, 9.89. Found (%): C,52.34; H, 3.29; N, 34.53.

General procedure for synthesis of (12a-c):

Hydrazino derivative**7** (2.05g, .0.01mol.) and different malononitrile derivatives (0.01mol) was heated under reflux in DMF (10 mL) containing catalytic amount of TEA for 20 hours. The reaction mixture was then treated with ice cold water; the precipitated solid was filtered, dried, and crystallized from ethanol to afford 4-(4-substituted-phenyldiazenyl)-1*H*-pyrazole-3,5-diamine**12a-c.**

1-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-4-(4-phenyldiazenyl)-1*H*-pyrazole-3,5-diamine (12a)

Reddish brown powder, yield: (47%), m.p.>300℃. IR (KBr, cm^{-1}): 3394, 3275 (NH_2); 3097, 3062 (C-H aromatic); 1616 (C=N); 1504 (C=C); 1477(|N=N);^{1}H NMR (DMSO-d_6,δ ppm): 6.96 (s, 2H, NH_2, D_2O exchangeable); 7.31 (t, 1H, *J*=7.2Hz, C_6H_5-C_4-H); 7.37 (t, 2H, C_6H_5-C_3-H); 7.43-7.53 (m, 2H, , C_6H_5-$C_{2,6}$-H); 7.67-7.75 (m, 2H, triazoloquinoxaline-$C_{7,8}$ -H); 8.01 (d, 1H, *J*=7.6Hz, triazoloquinoxaline-C_9-H); 8.42-8.47 (m, 1H, triazoloquinoxaline-C_6-H); 9.40 (s, 2H, NH_2, D_2O exchangeable); 10.03 (s, 1H, triazoloquinoxaline-C_1-H); ^{13}CNMR (DMSO- d_6): 108.10, 118.31, 121.26, 122.16, 122.76, 128.45, 128.65, 129.60, 129.65, 138.86, 139.46 (Ar-C), 152.64, 152.71, 153.56(C=N). MS *m/z* (relative intensity %): 372 (M+2, 17.78); 370 (M, 17.00) ; (335, 100); (314, 78); (304, 84); (104, 47); Anal. form: $C_{18}H_{14}N_{10}$. Calcd. (%) C, 58.37; H, 3.81; N, 37.82. Found (%): C,58.59; H, 3.94; N, 37.59.

1-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-4-(*p*-tolyldiazenyl)-1*H*-pyrazole-3,5-diamine(12b)

Reddish brown powder, yield: (44%), m.p.>300℃.IR (KBr, cm^{-1}): 3390, 3271 (NH_2); 3101 (C-H aromatic); 2912, 2839 (C-H aliphatic); 1604(C=N); 1504 (C=C);1473 (N=N). ^{1}H NMR (DMSO- d_6,δ ppm): 2.36 (s, 3H, $\underline{CH_3}$); 6.91 (s, 2H, NH_2, D_2O exchangeable); 7.29 (d, 2H, *J*=8.4Hz, 4-CH_3- C_6H_5-$C_{3,5}$-H); 7.59 (d, 1H, *J*=8Hz, 4-CH_3- C_6H_5-C_2-H); 7.70-7.72 (m, 3H, triazoloquinoxaline-$C_{7,8,9}$-H); 7.91 (d, 1H, *J*=8Hz, 4-CH_3-C_6H_5-C_6-H); 7.86 (d, 1H, *J*=8Hz, 4- CH_3- C_6H_5-C_5-H); 8.44 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_6-H); 9.30 (s, 2H, NH_2, D_2O exchangeable); 10.03 (s, 1H, triazoloquinoxaline-C_1-H);^{13}CNMR (DMSO- d_6): 21.32 (CH_3-C), 118.29, 121.16, 122.08, 122.76, 128.62, 130.09, 130.14, 138.85, 139.30(Ar-C), 139.45, 150.69(C=N).MS *m/z* (relative intensity %): 384 (M ,76.50); 382 (M-2, 62.13) ; 284(66); 224(71); 174 (78.50). Anal. form: $C_{18}H_{14}N_{10}$. Calcd.(%) C, 58.37; H, 3.81; N, 37.82. Found (%):C, 59.54; H, 4.36; N, 36.21.

1-([1,2,4]Triazolo[4,3-*a*]quinoxalin-4-yl)-4-((4-bromophenyl)diazenyl)-1*H*-pyrazole-3,5-diamine(12c)

Reddish brown powder, yield: (44%), m.p.>300℃. IR (KBr, cm^{-1}): 3379, 3298 (NH_2); 3070 (C-H aromatic); 1620 (C=N); 1566 (C=C); 1473(N=N). ^{1}H NMR (DMSO-d_6, δ ppm): 7.00 (s,2H, NH_2, D_2O exchangeable); 7.53 (d, 2H, *J*=8Hz, 4-Br-C_6H_4-$C_{2,6}$-H); 7.66-7.70 (m, 2H, triazoloquinoxaline-$C_{7,8}$-H); 7.76 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_9-H); 7.82 (d, 1H, *J*=8Hz, 4-Br-C_6H_4-C_3-H); 7.85 (s,2H, NH_2, D_2O exchangeable); 7.86 (d, 1H, *J*=8Hz, 4-Br-C_6H_4-C_5-H); 8.40 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_6-H); 10.21 (s, 1H, triazoloquinoxaline -C_1-H).^{13}C-NMR (DMSO- d_6): 116.70, 118.05, 120.83, 123.38, 124.12, 124.30, 128.37, 132.33, 132.70(Ar-C), 133.87, 142.91, 152.69(C=N).MS m/z (relative intensity %): 449 (M,34.50); 421(38.59), 315(68); 81(73); 175 (100). Anal. form: $C_{18}H_{13}BrN_{10}$. Calcd.(%) C, 48.12; H, 2.92; Br, 17.79; N, 31.18. Found (%): C,48.31; H, 3.20; N, 31.37.

General procedure for synthesis of (13a,b)

Equimolar amounts of **7**(2.05g, .0.01 mol.) andproper chalcone derivative (0.01 mol) were dissolved in dioxane/ DMF mixture (10mL/5mL) containing 3drops of TEA. The reaction mixture was heated at reflux for 16 h, the solid product was then filtered, dried and crystallized from dioxane to afford derivatives **13a,b.**

4-(5-(2,4-Dichlorophenyl)-3-(4-methoxyphenyl)-1*H*-pyrazol-1-yl)-[1,2,4]triazolo[4,3-*a*]quinoxaline(13a)

Orange powder. Yield: (35%), m.p.>300℃.IR (KBr, cm^{-1}): 3086, 3062 (C-H aromatic); 2931, 2897 (C-H aliphatic); 1620 (C=N); 1504 (C=C); 1033(*p*-substituted phenyl);^{1}H NMR (DMSO-d_6,δ ppm): 3.86 (s, 3H, O$\underline{CH_3}$); 7.09 (d, 2H, *J*=8Hz, 4-OCH_3-C_6H_4-$C_{3,5}$-H); 7.17 (s, 1H, pyrazole-C_4-H); 7.36-7.41 (m, 1H, triazoloquinoxaline-C_7-H); 7.54, 7.56 (dd, 1H, *J*=8, 4Hz, triazoloquinoxaline-C_8-H); 7.66-7.72 (m, 1H, triazoloquinoxaline-C_9-H); 7.73 (d, 2H, *J*=8Hz, 2,4-$(Cl)_2$-C_6H_3-C_5-H); 7.76 (d, 2H,*J*=8Hz, 4-OCH_3- C_6H_4-$C_{2,6}$-H); 7.82 (d, 2H, *J*=8Hz, 4-OCH_3- C_6H_4-C_5-H); 7.93 (d, 1H, *J*=8Hz, 2,4-$(Cl)_2$- C_6H_3-C_6-H); 8.00 (s, 1H,

2,4-$(Cl)_2$-C_6H_3-C_3-H); 8.08 (d, 1H, *J*=8Hz, triazoloquinoxaline-C_6-H); 8.46; 9.38 (s, 1H, triazoloquinoxaline -C_1-H); ^{13}CNMR (DMSO-d_6): 55.78(OCH_3-C), 114.18, 118.41, 118.46, 123.07, 128.04, 128.40, 128.60, 129.12, 129.75, 131.46, 134.13(Ar-C), 138.68, 140.81, 160.95(C=N);MS *m/z* (relative intensity %): 487 (M, 18.80); (416, 81.17); (128, 80.09); 118 (100). Anal. form: $C_{25}H_{16}Cl_2N_6O$. Calcd.(%) C, 61.61; H, 3.31; Cl, 14.55; N, 17.24; O, 3.28. Found (%): C,61.50; H, 3.48; N, 17.51.

4-(5-(2,4-Dichlorophenyl)-3-(thiophen-2-yl)-1*H*-pyrazol-1-yl)-[1,2,4]triazolo[4,3-*a*]quinoxaline(13b)

Brick red powder, yield: (44%), m.p>300^{0}C. IR (KBr, cm^{-1}): 3105 (C-H aromatic); 1647 (C=N); 1535 (C=C); 1327, 1049(C-S-C);^{1}H NMR (DMSO-d_6,δ ppm): 7.12-7.22 (m, 1H, thiophene- C_4-H); 7.30-7.40 (m, 1H, thiophene-C_3-H); 7.42-7.50(m, 1H, thiophene-C_5-H);7,56 (d, 1H, *J*=7.9Hz, 2,4-$(Cl)_2$-C_6H_3-C_5-H) ; 7.62-7.75 (m, 3H, triazoloquinoxaline-$C_{7,8}$-H& pyrazole-C_4-H); 7.91 (s, 1H, 2,4-$(Cl)_2$-C_6H_3-C_3-H); 8.10 (d, *J*=7.9Hz, 1H, 2,4-$(Cl)_2$-C_6H_3-C_6-H); 8.30-8.41(m, 2H, triazoloquinoxaline-$C_{6,9}$-H); 9.92 (s, 1H, triazoloquinoxaline-C_1-H); ^{13}CNMR (DMSO-*d6*): 116.62, 118.35, 122.54, 122.93, 123.66, 126.07, 127.98, 128.47, 138.38(Ar-C), 138.65, 138.89, 139.55, 147.65 (C=N);MS *m/z* (relative intensity %): 463 (M, 48.69); (415, 64.86); (294, 51.46); 92 (100). Anal. form: $C_{22}H_{12}Cl_2N_6S$. Calcd.(%) C, 57.03; H, 2.61; Cl, 15.30; N, 18.14; S, 6.92. Found (%): C,57.31; H, 2.87; N, 18.28.

4.2. Biological evaluation

4.2.1. Antiproliferative screening

The newly synthesized compounds underwent cytotoxicity in this study. All the chemicals used in this analysis are highly analytical in nature. The American Type Culture Collection (ATCC) is the source of the selected cancer cell lines; hepatocellular carcinoma cell lines (HepG-2), human breast adenocarcinoma cell lines (MCF-7), human colon carcinoma cell lines (HCT-116) and human diploid lung fibroblasts WI-38; which were obtained frozen in liquid nitrogen (-180 ° C) and maintained at the National Type Culture Collection (NTCC). Doxorubicin has been used as a standard reference drug, for comparison.

The cytotoxicity on human cancer cell line (HepG-2), MCF-7, HCT-116 and the normal cell lineWI-38 was assessed *in vitro* using Sulforhodamine-B stains (SRB) assay following the Skehan process. [27] Cells were cultured for 24 hours before treatment with the compounds in 96 multi well plates allowing the cells to fasten the plate wall. To the cell monolayer is applied the following concentrations of the compound under examination (0, 6.25, 12.5, 25, 50 and 100 µg / mL)

Triplicate wells have been prepared for any single dose. Monolayer cells were cultured at 37^{o}C for 48 hours with the compounds and 5 percent CO_2 in the atmosphere. After couple of days the cell was fixed, rinsed and stained with Sulforhodamine B stain. To get rid of the extra stain, it was washed with acetic acid, and stain attached with Tris EDTA buffer was retrieved. In an ELISA reader, color intensity was assayed. The relationship between surviving part of cells and concentration of drugs was plotted and Sigmaplot software calculated IC_{50} (the concentration needed for 50 per cent cell viability inhibition) for every compound.

4.2.2.EGFRwt-TK assay

Using the EnzyChromTM Kinase Assay Kit (EKIN-400) Kinase Reaction [28], the inhibiton of compounds **12a-c** on EGFRwt-TK activities was determined. Users can offer their own enzyme, ultra-pure ATP (e.g. Sigma # A7699), and substrates. Place a 20 µL reaction mixture containing the kinase, ATP, and substrate in the assay buffer given (pH 7.0) or any appropriate kinase buffer. Perform a Blank Control containing ATP and substrate, but no enzyme. Incubate for the targeted time period e.g. 30 min at desired temperature. Principles: Prepare 900 µL 10 µM ADP Premix by making a mixture of the standard 3 µL 3 mM and the

distilled water 897 µL. Dilute so as to be below normal. Transfere 20 µL standards to separate ADP Detection Plate wells. Prepare adequateworking reagent by combining 25 µL Reagent A and 25 µL Reagent B for every well. Add 40 µL Working Reagent well to every assay. Tap mixing pad. Incubate these pad for 10 min at room temperature. the 530 nm platewas readed, and the activity of kinasewas measured. Dilute standard according to the following procedure. Add 20 µL standards inside separate wells of the plate.*ADP Determination*: Prepare adequateworking reagent for every well by blending 25 µL Reagent A and 25 µL Reagent B. Add 40 µL Working Reagent to each assay well. Tap a plate to insure mixing. Incubate at room temperature for 10 min. *Read plat* at 530nm and the kinase activity was calculated.

4.2.3.EGFRL858R-TK assay

The assay EGFRL858R-TK was done in the kinase reaction buffer (50 mM HEPES, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, and 0.01 percent Tween 20). EGFRL858R-TK (the final concentration is 0.09 ng / mL) and various concentrations of **12a-c** or reference drug compounds (final concentration which is 2000e0.488 nM) were placedin each well and incubated for two hours, then pre-mixed ATP (final concentration: 5 mM) and ULight-poly GT (final concentration was 0.1 mM) were placed inside each well. After 120 minutes of incubation at room temperature (r.t.), EDTA was supplied and incubated for another five minutes with a final concentration of 10 mM.

4.2.4. Cell cycle analysis

MCF-7 cells were seeded at a concentration of$1x10^5$ cells per well in a 6-well plate, after which they incubated for one day. The cells were subjected toa vehicle (0.1 percent DMSO) or 2.04 µM of **12b** compound for 24 h. After this, cells were collected and fixed for 12 hours using ice-cold 70 percent ethanol at 4°C. Ethanol was eleminated and the cells were rinsed with cold PBS followed by incubation in 0.5 mL of PBS which contain 1 mg / mL Rnase for 30 min at 37°C auxiliary cells. The cells were subjected to stain in the dark using propidium iodide for 30 min. Thereafter,Flow cytometer was used to measure the contents of DNA[29].

4.2.5. Apoptosis detection studies

4.2.5.1. Annexin V-FITC assay

MCF-7 cells were cultured in a 6-well plate ($1 X10^5$ cell / well), incubated for one day (24 hours), then it was subjected to a vehicle (0.1% DMSO) or 2.04 µM **12b** compoundfor 24hours.Upon adding annexin V-FITC and PI to the binding buffer (10 mM of HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ at pH= 7.4), the cells were then collected and washed using PBS then it were stained for 15 min at room temperature in the dark depending on the flow cytometer apparatus [30].

4.2.5.2. Determination of the active Caspase-3

Quantikine-Human active Caspase-3 Immunoassay (R&D Systems, Inc. Minneapolis, USA)was applied to detect the active Caspase-3 level. The cells were washed with PBS, collected and lysed through the addition of the protease-inhibiting extraction buffer (1 mL per 1 x 107 cells), then the lysate was diluted instantelybefore the study. Upon using a microplate reader set at 450 nm, the optical density of each well was measured within 30 min when the assay has finished.

4.2.5.3. Determination of the gene expression of some apoptosis keymarkers (BAX and Bcl-2)

Cells were obtained from the American Type Culture Collection and the cells were cultured in RPMI 1640 containing 10 percent bovine fetal serum at 37 °C inducted with BAX or Bcl-2 validated compound and lysed using the Cell Extraction Buffer. Standard Diluent Buffer which was used to dilute this lysate along the assay and measured in comparable to human active BAX or Bcl-2 content. Cells are plated at a density of 1.2 – 1.8x 10,000 cells / well in a volume of 100 µL full growth medium plus 100 µL of the compound tested per well in a 96-well plate for 24 hours before measurement for human active BAX or Bcl-2.

4.3. In silico physicochemical and ADME propertiesstudy

Using Chemdraw 12.0 the chemical structures were translated to SMILES databank. Then, owing to predict the physicochemical descriptors, lipophilicity, pharmacokinetics characters, ADME parameters, and medicinal chemistry friendliness, these SMILES were inserted in SwissADME website.

4.5.Molecular modeling study

Molecular Operating Environment (MOE) 2015.10 was utilized to perform all the molecular modeling calculations and docking simulation studies. Protein Data Bank (PDB) was utilized to getEGFR (PDB code: 1M17) 3D X-ray structures.

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