Bioorganic Chemistry 93 (2019) 103314

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Exploration of Pd-catalysed four-component tandem reaction for one-pot assembly of pyrazolo[1,5-*c*]quinazolines as potential EGFR inhibitors

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ARTICLE INFO

Keywords:
EGFR
Pyrazolo[1,5-*c*]quinazolines
Catalysis
Anticancer
Drug discovery
Molecular modelling

ABSTRACT

A series of pyrazolo[1,5-*c*]quinazolines as EGFR inhibitors was designed and synthesized by highly efficient and novel multicomponent route involving Pd-catalyzed tandem one-pot four-component reaction. The reaction proceeds with good functional group tolerance under a simple condition with excellent regioselectivity and high efficiency. Target compounds were screened against cancer cell lines MDA-MB-231, A549 and H1299. Of these, **9b** and **10b** exhibited superior anticancer activity (IC_{50} < 2.5 μM) to erlotinib and gefitinib. Synthetics were able to inhibit EGFR mediated kinase activity, induced ROS in cancer cells promoting mitochondrial mediated apoptosis via halting cell cycle progression at G1 phase.

1. Introduction

Epidermal Growth Factor Receptor (EGFR) protein belonging to tyrosine kinase family is highly overexpressed in various cancers such as lung, head and neck, breast, gastric, colon, esophageal, prostate, bladder, renal, etc., and has been well exploited as a druggable oncological target [1–3]. Quinazoline-based first-generation EGFR inhibitors such as gefitinib (2002) and erlotinib (2004), were found to be effective during the initial stages of treatment [4]. However, the point mutations (T769M and L837R) observed made them less effective and led to development of irreversible second (canertinib) and subsequently third (osimertinib, WZ4002) generation inhibitors able to make covalent bond with CYS773 [5]. But, due to their toxicity [6], the search for EGFR inhibitors with alternate scaffold took place. More recently, fourth generation EGFR inhibitor EA1045 was discovered [7,8] and is under clinical trial and its side effects are yet to be observed (Fig. 1).

2. Drug design

In continuous strive for the drug development by our group, recently we reported a four-component reaction [9] promoted by Pd(II)/Ag(I) binary catalysis for the synthesis of an interesting polyheterocycles with immense biological significance 'pyrazolo[1,5-c]quinazolines' [10–15], which emerged as effective EGFR inhibitors [9]. The study also revealed that smaller and polar substitutions on pyrazoles were essential for binding with EGFR [9]. Based on this, new series of compounds (**9** or **10**; Fig. 2) was designed replacing either one of -COOMe groups of **5fb** [9] (Fig. 2) with a small and polar substituent such as $-NH_2$ or both the groups with $-CN$ or $-NH_2$ (**9**) or by an aryl ketone and other with $-NH_2$ (**10**). Considering the *in silico* binding of **9** or **10** into the EGFR-ATP pocket, the hydrogen of NH_2 was found to interact with lone pair of *N*H in MET769, which is important for the inhibition of EGFR. The hinge residues region starts from the LYS764 residue and ends with CYS773. THR766 (a gatekeeper residue) does not participate in ATP binding but it is important for entry of inhibitor to the active site of EGFR. The activation loop contains DFG motif (ASP831-PHE832-GLY833) that runs from ASP831 and ends with PRO853 and activates the kinase domain for binding of ATP to the active site [15,16]. The co-crystallized erlotinib and **5fb** [9] was considered for the design of EGFR inhibitors (PDB ID: 1 M17) [17].

3. Results and discussion

3.1. Chemistry

Being actively engaged in the synthetic methodologies of pyrazolo [1, 5-*c*]quinazoline [9,18] (Fig. 3A), we further planned to develop a

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https://doi.org/10.1016/j.bioorg.2019.103314
Received 15 May 2019; Received in revised form 12 August 2019; Accepted 24 September 2019
Available online 26 September 2019

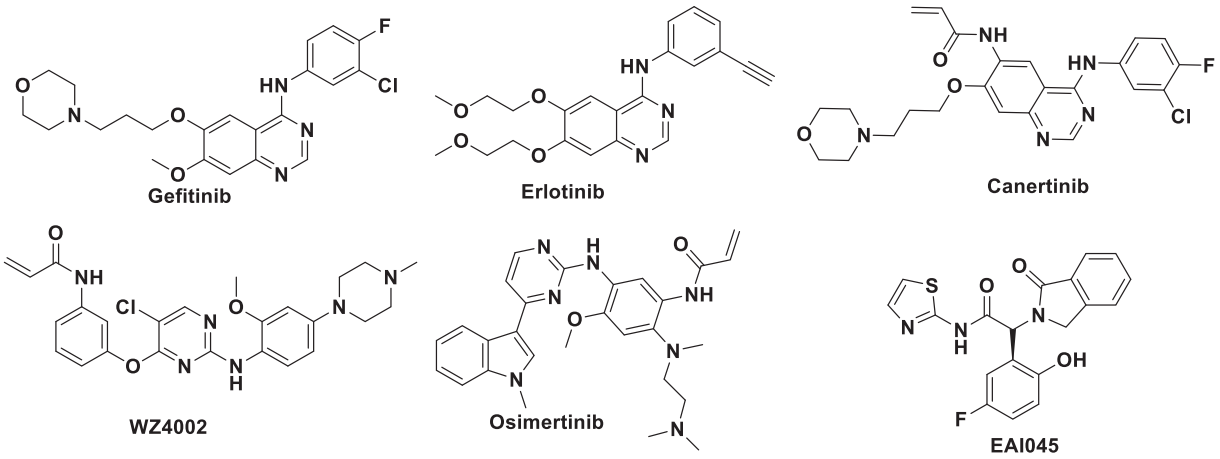


Fig. 1. Chemical structures of reported EGFR inhibitors.

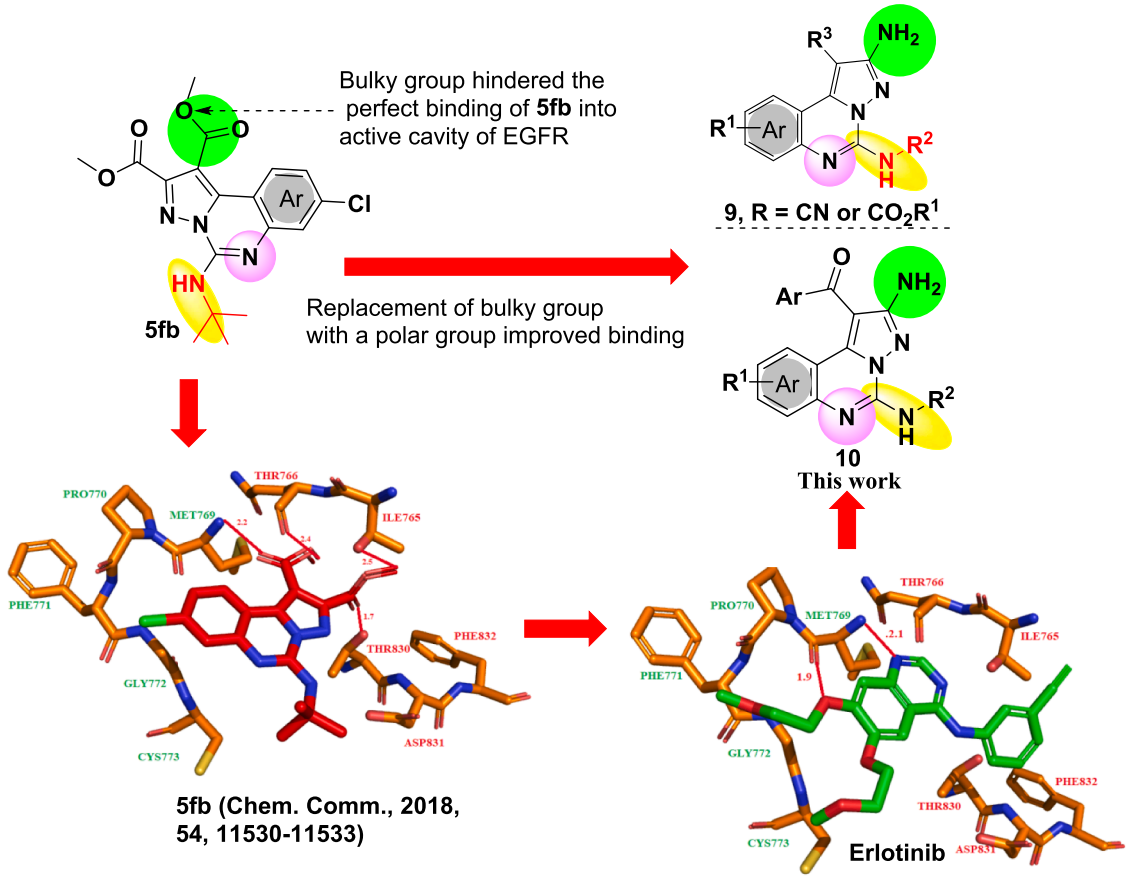


Fig. 2. Design of **9** and **10** on the basis of pharmacophoric features (PDB ID: 1M17) of reported inhibitors erlotinib (green color) and **5fb** (red color).

novel protocol for accessing target compounds. We envisaged that a range of acetonitrile could be reacted with azomethine imine **4**, based on the report of Wu et al., on 2-alkenylbenzaldehyde [19] (Fig. 3B). Such strategy could be elegantly transformed into a four component reaction to generate pyrazolo[1,5-*c*]quinazolines with polar substitutions on the pyrazole ring (Fig. 3C).

Azomethine imine **4** was readily generated from three component reaction of 2-azidobenzaldehydes **1**, isocyanides **2** and tosyl hydrazides **3** as reported earlier [9]. With an assortment of azomethine imine **4** in hand, we carried out optimization studies (See SI, Section 1) on the reaction of azomethine imine **4** and malononitrile **7** for the synthesis of **9a** (Table 1). Solvent studies revealed reaction carried out in toluene using DABCO (2 equiv) as a base furnished the best yield (entry 1). Other solvents such as THF, dioxane, MeCN and DCE were ineffective for this transformation (entry 2–5). Exploration with various bases such as Et_3N, NMP, DBU, and pyrrolidine furnished inferior results (entry 6–9). Reaction failed to initiate in the absence of a base (entry 10). Lowering the temperature of reaction furnished poor yields of title compounds (entry 11). We next attempted to extend the methodology for α-ketonitrile **8**. Our initial exploration with azomethine **4** and β-ketonitrile **8** in the presence of DABCO as base in toluene at 100 °C, failed to produce desired product **10a** (entry 12). Interestingly, the addition of iodine as Lewis acid facilitated the cyclocondensation of azomethine imine and β-ketonitrile to afford **10a** in good yield, 87%

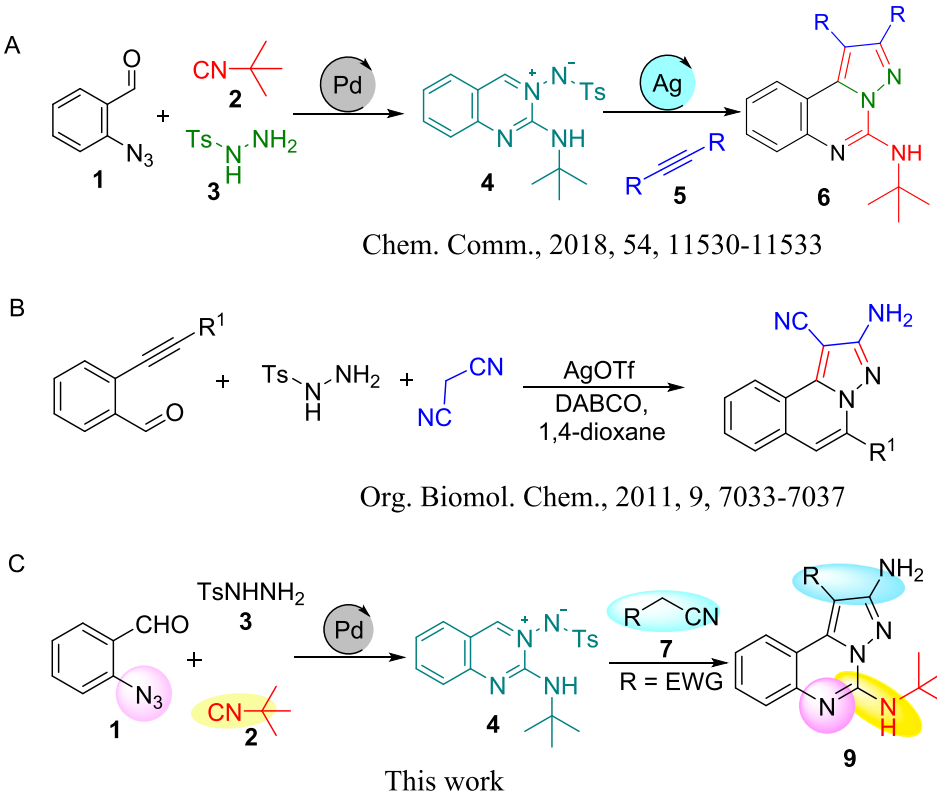
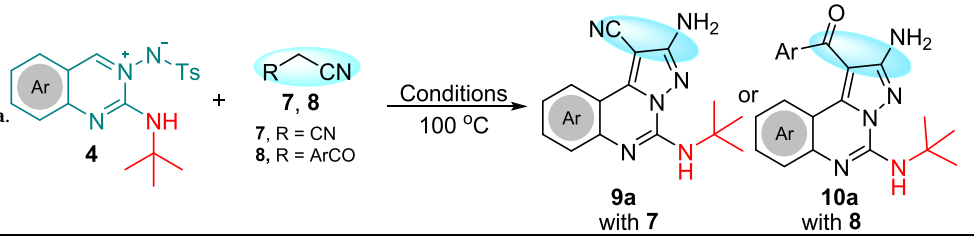


Fig. 3. Traditional approaches (A and B) to the synthesis of pyrazolo[1,5-*c*]quinazoline; C. Current methodology.

Table 1

Optimization of reaction condition for **9a** or **10a**.



9a with **7** **10a** with **8**

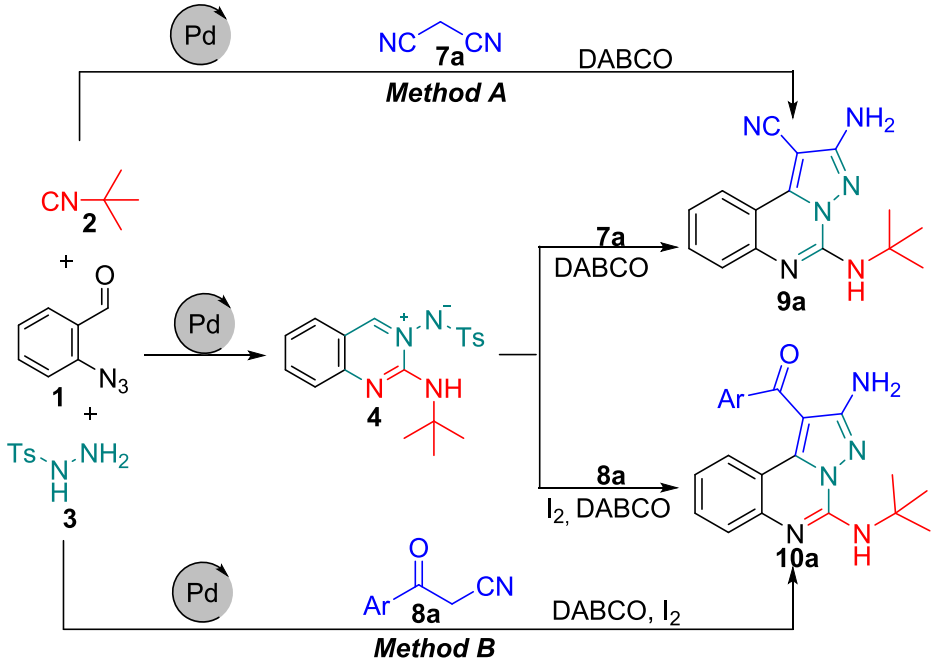
Entry	Substrate/ Product	Solvent	Base	Additive	Product[a] (**9a** or **10a**)
1.	**7/9a**	Toluene	DABCO	–	93
2.	**7/9a**	THF	DABCO	–	75[b]
3.	**7/9a**	Dioxane	DABCO	–	60
4.	**7/9a**	MeCN	DABCO	–	NR
5.	**7/9a**	1,2-DCE	DABCO	–	5
6.	**7/9a**	Toluene	Et_3N	–	55
7.	**7/9a**	Toluene	NMP	–	26
8.	**7/9a**	Toluene	DBU	–	56
9.	**7/9a**	Toluene	pyrolidine	–	56
10.	**7/9a**	Toluene	–	–	–
11.	**7/9a**	Toluene	DABCO	–	65[b]
12.	**8/10a**	Toluene	DABCO	–	–
13.	**8/10a**	Toluene	DABCO	Iodine	87

Reaction conditions: Method A: **4** (1 equiv.) **7**/**8** (2 equiv.) base (3 equiv.) and solvent for 2–3 h.
[a] Isolated yield.
[b] Temperature reduced to 70 °C.

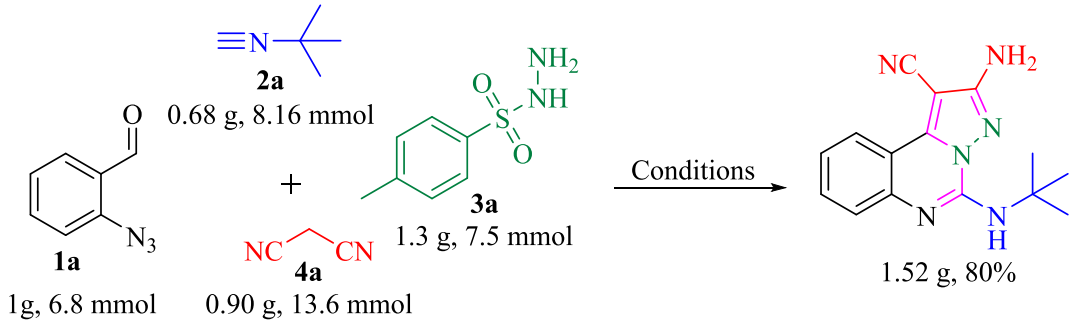
[20–22] (Table 1, entry 13).

Next, in order to check the suitability of this new synthetic method in an enlarged scale, 6.8 mmol of **1a** was treated with 8.16 mmol of **2a** and 7.5 mmol **3a** under standard reaction conditions followed by 13.6 mmol of **4a**. From this reaction, **9a** was obtained in a yield of 80% (Scheme 2).

Encouraged by the results, we next focused our attention on the development of a one-pot four-component protocol for the synthesis of pyrazolo[1,5-*c*]quinazolines from 2-azidobenzaldehyde **1**, isocyanide **2**, tosylhydrazide **3** and derivatives of acetonitrile **7**/**8** (Scheme 1). A mixture of **1**, **2**, and **3** in the presence of palladium acetate in toluene was stirred at rt for 15 min to generate azomethine imine **4** *in situ*, then



Scheme 1. [1]Development of four-component one-pot protocol.



Scheme 2. Scale up reaction for **9a.**

acetonitrile derivative **7** and DABCO as a base were added, and reaction was stirred at 100 °C for 2 h. This 4-CR produced pyrazolo[1,5-*c*]quinazolines **9a** in 85% yield. For the substrate **8,** similar methodology was developed for the synthesis of **10a** with the addition of Iodine as catalyst.

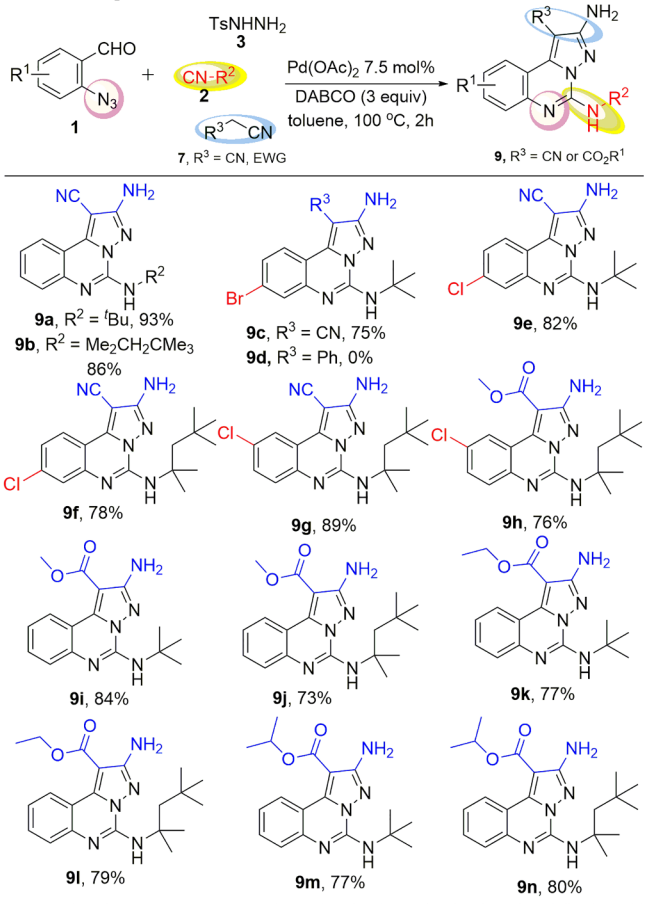
With the optimized reaction condition in hand, the substrate scope of 4-CR was scrutinized under the standard reaction condition (Tables 2 and 3). Various substitutions on 2- azidobenzaldehyde such as Br and Cl were well tolerated (**9c**-**9h**). The presence of electron withdrawing groups on α–position of acetonitrile such as CN, CO_2R, and COAr were essential for their participation in 4-CR. Absence of such group led to the failure of 4-CR (**9d**). Various substitution on the phenyl ring of β-ketonitrile were also well tolerated (**10a**-**10e)** with the only exception of pyridyl substitution in **10f** (Table 3). Pleasingly, the methodology (Tables 2 and 3) has generated a library of pyrazolo[1, 5-*c*]quinazolines having small polar substitutions such as amino, cyano, carboxylate and arylketones on the pyrazole ring.

3.2. Investigational compounds exhibited selective anticancer potential with excellent EGFR inhibitory activity

Encouraged by the results, we envisaged that compound collection of pyrazolo[1,5-*c*]quinazolines with polar substitutions could produce better and potent inhibition of EGFR. To test our hypothesis, we carried out screening of pyrazolo[1,5-*c*]quinazolines for their antiproliferative potential on highly aggressive, invasive and triple negative breast cancer (TNBC) MDA-MB-231 cell, lung cancer A549 (p53 wild-type) and H1299 (p53 null type) cells using MTT assay. Treatment was made with investigational compounds at concentration of 1, 5 and 25 μM for 48 h [15,23–25]. Three compounds **9b**, **10b** and **10d** were shortlisted by their performances in all the three cell lines (IC_{50} < 2.5 μM) employed and in comparison, to positive controls erlotinib and gefitinib (Table 4). The results are significant since investigational compounds were able to exhibit the growth inhibitory potential irrespective of the nature (TNBC) and p53 mutational status of the cancer cell that is found to be implicated in more than 50% of cancer [26]. Next, we quantified the effect of **9b**, **10b** and **10d** in comparison to erlotinib, toward the inhibition of ATP-dependent phosphorylation of EGFR [9,16,23]. The assay was performed at concentration 100, 250 and 500 nM. The results suggested **9b** and **10b** possess the most potent EGFR inhibition with

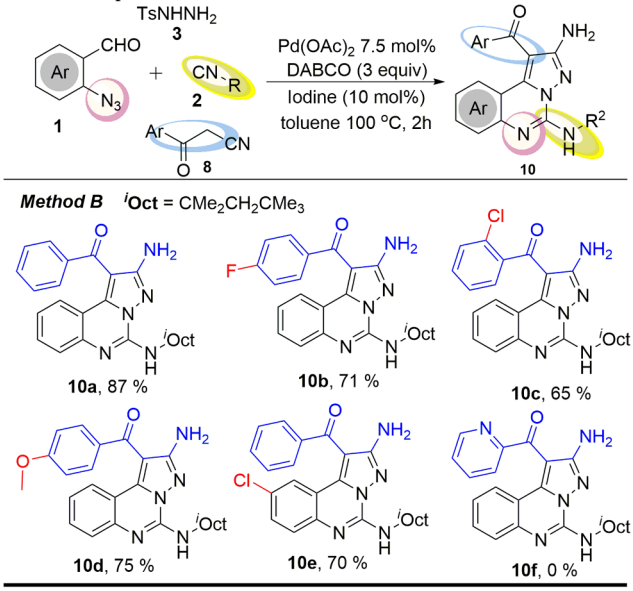
[1] Representative procedure: Method A: 2-Azidobenzaldehyde (0.10 mmol), isocyanide (0.12 mmol), tosylhydrazide (0.11 mmol), Pd catalyst (0.075 mmol), 4 Å MS (300 mg), nitrile (0.12 mmol), DABCO (0.30 mmol), and toluene as solvent at 100 °C stirred for 1–2 h followed by aqueous workup; isolated yields after chromatography. Method B: Same as method A with the addition of iodine (10 mol%).

Table 2
Substrate scope of 4-CR with method A.[a]



[a] Representative procedure: Method A of Scheme 1, Isolated yields are referred after chromatographic purification.

Table 3
Substrate scope of 4-CR with method B.[a]



[a] Representative procedure: Method B of Scheme 1, Isolated yields are referred after chromatographic purification.

Table 4
Antiproliferative potential of investigational Molecules against three human cancer cells.

IC_{50} (μM) ± SD[a]				
Entry	Code	MDA-MB-231 (Breast Cancer)	A549 (Lung Cancer)	H1299 (Lung Cancer)
1.	**9a**	6.86 ± 0.32	3.98 ± 0.39	4.97 ± 0.31
2.	**9b**	1.96 ± 0.23	2.87 ± 0.29	2.02 ± 0.34
3.	**9c**	5.89 ± 0.25	4.98 ± 0.19	6.35 ± 0. 27
4.	**9e**	7.98 ± 0. 51	5.93 ± 0.42	4.86 ± 0. 38
5.	**9f**	5.65 ± 0.33	4.56 ± 0.40	4.93 ± 0.42
6.	**9g**	8.54 ± 0.52	10.44 ± 0.51	9.34 ± 0.51
7.	**9h**	7.83 ± 0.24	5.27 ± 0.31	5.01 ± 0.22
8.	**9i**	7.90 ± 0.41	8.33 ± 0. 36	6.65 ± 0.39
9.	**9j**	4.69 ± 0.15	2.78 ± 0.29	4.94 ± 0.42
10.	**9k**	8.83 ± 0.39	4.89 ± 0.45	6.99 ± 0.64
11.	**9l**	7.82 ± 0.37	5.89 ± 0.43	4.75 ± 0. 47
12.	**9m**	4.76 ± 0.38	3.29 ± 0.45	3.47 ± 0.52
13.	**9n**	9.36 ± 0.45	6.35 ± 0.54	5.96 ± 0.32
14.	**10a**	5.28 ± 0.36	6.84 ± 0.39	7.22 ± 0.34
15.	**10b**	1.93 ± 0.55	1.06 ± 0.48	1.32 ± 0.56
16.	**10c**	5.97 ± 0.19	6.45 ± 0.32	7.84 ± 0.36
17.	**10d**	2.45 ± 0.31	1.74 ± 0.23	2.04 ± 0.33
18.	**10e**	3.49 ± 0.31	2.69 ± 0.36	3.22 ± 0.53
19.	Erlotinib	4.56 ± 0.46 7.0 [27]	2.98 ± 0.39 2.8 ± 0.32 [16]	3.33 ± 0.31 2.09 ± 0.44 [28]
20.	Gefitinib	6.85 ± 0. 18 16.5 [27]	2.65 ± 0.32 5 ± 1 [29]	3.02 ± 0.29 > 10 [30]

[a] Assay was performed in triplicate and data was compiled for 48 h incubation.

Table 5
EGFR inhibitory activity of **9b**, **10b** and **10d**.

Code	EGFR Inhibitory Activity IC_{50} ± SD (nM)[a]
9b	165.38 ± 0.29
10b	157.63 ± 0.34
10d	216.35 ± 0.22
5fb	239.10 ± 0.21 (Lit: 235) [9]
Erlotinib	201.34 ± 0.39

[a] Assay was performed in duplicate.

IC_{50} of 165.38 and 157.63 nM, respectively, in comparison to erlotinib (IC_{50} = 201.34 nM) (Table 5). The presence of amino group on pyrazole ring increases the overall potency of pyrazolo[1,5-*c*]quinazolines for the inhibition of EGFR.

3.3. Molecular modelling corroborated biological response of 10b

Further, the molecular docking studies of most potent EGFR inhibitor **10b** within the active site of EGFR protein (PDB ID: 1M17) revealed that compound perfectly fits into the ATP domain of EGFR and have much better docking score (−9.10) than erlotinib (−7.20) (Fig. 4, Table 6). The compound therefore was found much potent in comparison to previously reported lead (**5fb**) [9] thus, revealing that smaller and polar substitutions on pyrazole ring are essential for binding with EGFR.

The thorough investigation on EGFR inhibitory activity and molecular docking studies (Fig. 4A–D) provided Structure–activity relationships (SAR): (a) the *N*-substituted bulkier alkyl group (**10b**, **10d**, **9b**) enhanced the hydrophobic interactions, occupying the active space between Val and Phe residues; (b) Among **10b** and **10d**, replacement by -F group (**10b**) increased the electronegativity that might enhanced the electrostatic interaction with lone pair of $-NH_2$ at Lys721 residue (**10d**); the replacement at 4th position of phenylmethanone with −CN (**9b**) was found to be tolerable in enhancing the kinase activity. The

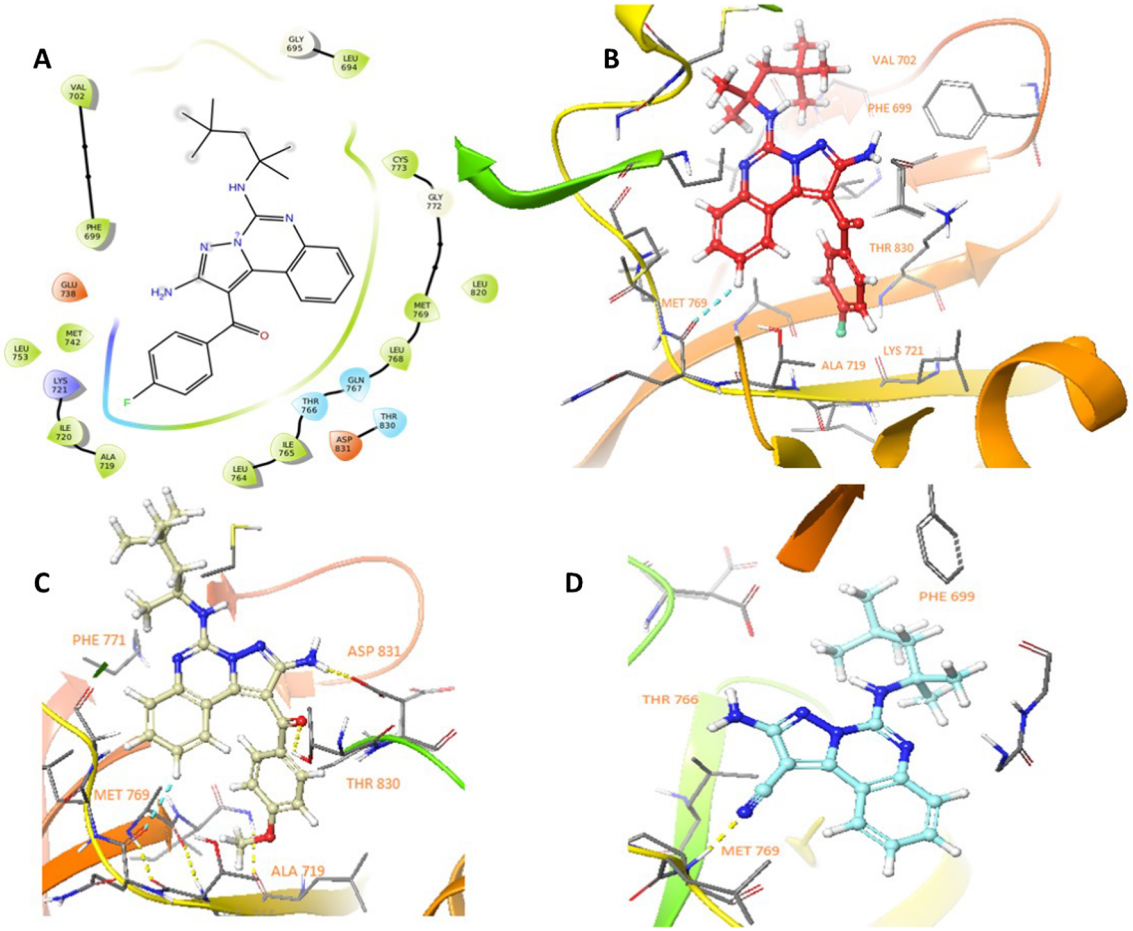


Fig. 4. Compounds within active ATP domain of EGFR (PDB: 1M17). (**A**) 2D interaction of **10b** with residual amino acids; (**B**) 3D illustration depicting **10b** bounds within ATP domain of EGFR; (**C**) 3D docking pose of **10d** and (**D**) 3D docking pose of **9b.**

Table 6
Docking score (kcal/mol) and interacting amino acid during docking against the target kinase enzyme (PDB ID: 1M17).

Chemical	Docking score	Interacting amino acid
Erlotinib	−4.29	PHE 699, VAL 702, LYS 721, MET 769, LEU 820, ASP 831
10b	−6.40	PHE 699, VAL 702, THR 830, LYS 721, ALA 719, MET 769

Table 7
ADME properties of **9b, 10b** and **10d.**

Entry	QPlogP o/w[a]	QPlogS[b]	QPP Caco[c]	Lipinski Violations[d]
9b	3.208	−5.379	450.082	0
10b	5.743	−5.626	1232.568	1
10d	5.614	−6.197	1239.068	1

[a] Predicted partition coefficient (QlogPo/w) (acceptable range: −2.0 to 6.5).
[b] Predicted aqueous water solubility (QPlogS) (acceptable range: −6.5 to 0.5).
[c] Predicted cell permeability (QPPCaco) (acceptable range: < 25 poor, > 500 great).
[d] Predicted Lipinski Violation (Maximum is 4).

−CN group (π electrons cloud) participated in π-cationic interaction with the backbone *N*H of Met769 in the Hinge region which is considered as an important interaction for EGFR inhibition; (c) The free $-NH_2$ of pyrazole ring was also found substantial for optimal activity. The docking score of compounds was found in accordance with the EGFR inhibitory activity obtained i.e. **10b** (−6.40) > **9b** (−5.32) > **10d** (−3.95).

Further to explore drug likeability of **10b**, **10d**, and **9b**, ADME *in silico* studies were performed to predict the partition coefficient (QlogPo/w), aqueous water solubility (QPlogS), cell permeability (QPPCaco) and Lipinski violations. The analysis revealed (Table 7) that values were found to be in acceptable range of an ideal drug.

3.4. Selected compounds were found to increase ROS, altered the mitochondrial permeability, induced apoptosis and halt cell cycle progression at G1 phase

Next, as EGFR inhibitors are associated with cardiotoxicity [31] we screened selected pyrazolo[1,5-*c*]quinazolines toward their effect on cardiac cells H9c2. The MTT results revealed that compounds were non-toxic towards H9c2 cell at a concentration of up to 10 μM (although erlotinib possess slight toxic effects) (See SI, Section S3). The plausible explanation for the non-toxic nature could be attributed to an unaltered mitochondrial membrane permeability as analyzed by JC-1 assay. Similarly, pyrazolo[1,5-*c*]quinazolines were found to exhibit no toxicity toward HBL-100 (normal breast) cells at similar concentration (10 μM) for 48 h thus conferring their selectivity towards cancer cells only. To note, reactive oxygen species play important role in various

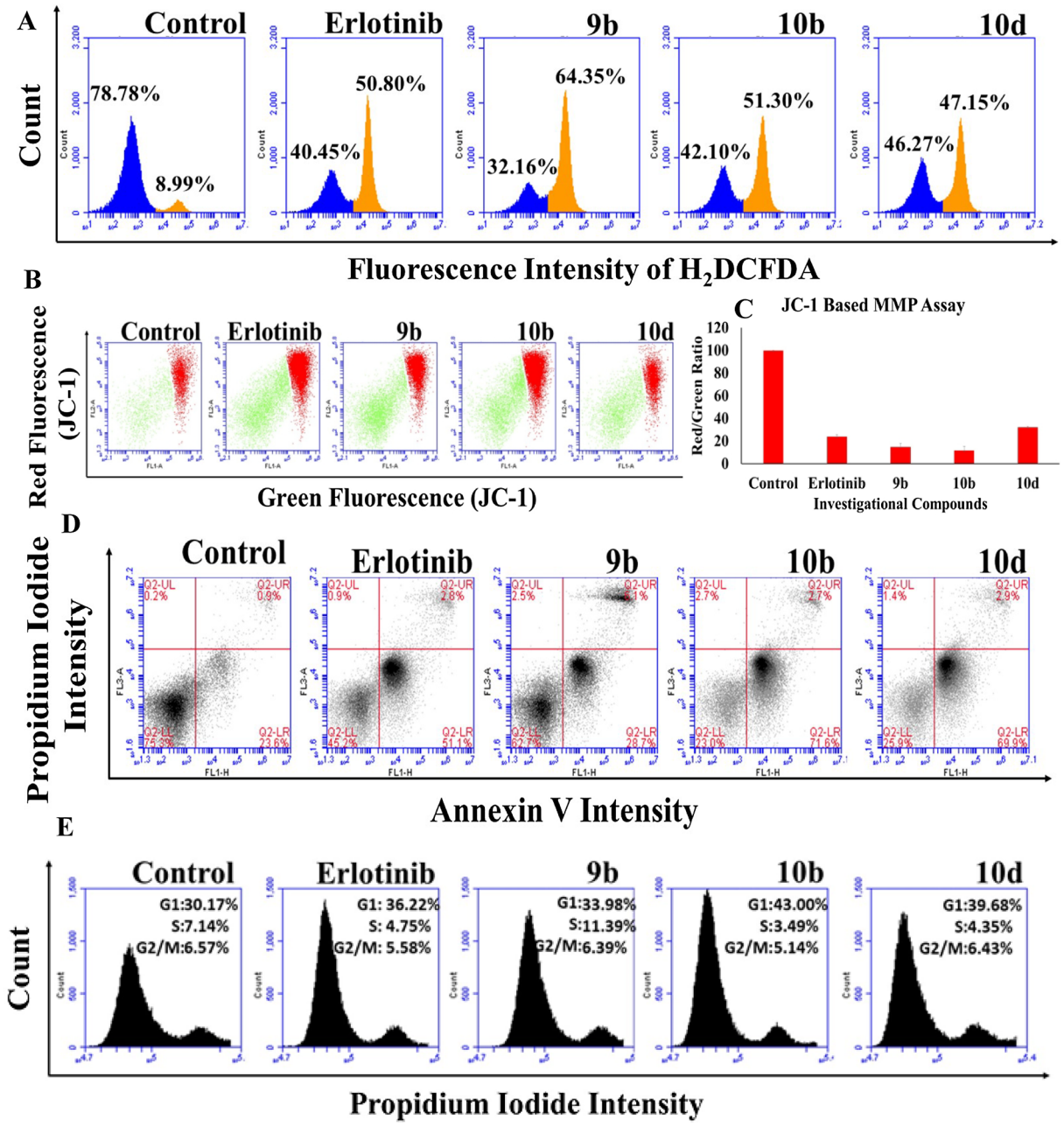


Fig. 5. (**A**) Relative fluorescence altered by investigational compounds under the influence of ROS generation as conferred by H_2DCFDA based assay; (**B**) Mitochondrial membrane permeability detected by JC-1 based mitochondrial permeability assay; (**C**) Relative ratio of change in RED/GREEN (J-aggregates Vs Monomers), higher red/green ratio suggest healthy condition of mitochondria, whereas lower red/green ratio suggests reduced mitochondrial potential (**D**) Annexin V vs PI assay, suggesting the apoptotic mode of cell death by investigational molecules; (**E**) Cell cycle analysis using propidium iodide suggesting percent cell count (DNA) at various stages of cell cycle. All the experiments were performed in A549 cells treated at subIC_{50} concentration previously treated for 48 h.

physiological as well as pathological processes. Many anticancer compounds imbalance the oxidant-antioxidant levels by altering the ROS concentrations [15,23–25]. The selected compounds **9b**, **10b** and **10d** were found to increase the ROS levels (indicated via H_2DCFDA assay) (Fig. 5A) in A549 cells at sub IC_{50} concentrations treated previously for 48 h. Generation of ROS by anticancer compounds initiate mitochondria mediated cancer cell death by altering the mitochondrial membrane permeability [15,23–25]. The pyrazolo[1,5-*c*]quinazolines **9b**, **10b** and **10d** induced mitochondria membrane depolarization (inferred by JC-1 assay) (Fig. 5B–C) in A549 cells at sub IC_{50} concentrations treated previously for 48 h [15,23–25]. Results suggested compounds depolarized mitochondria as the red to green ratio decreased significantly as compared to control (Fig. 5B–C). Further the compounds were found to exhibit cell death via apoptosis as indicated by Propidium Iodide (PI) vs Annexin V assay in A549 cells at sub IC_{50} concentrations treated previously for 48 h [15,23–25]. Compound **10b** was found to possess most potent apoptotic profile (74.3%) as compared to erlotinib (53.9%) (Fig. 5D). Next, we were interested the exact phase of cell cycle inhibited by investigational compounds. PI based cell cycle assay [15,23–25] revealed the halt of cell cycle progression at G1 phase. **10b** portrayed highest accumulation of cells in G1 phase (43%). All compounds suggested halt in cell cycle in accordance to erlotinib (Fig. 5E). The results are thus significant suggesting inhibition of mitogen dependent cell cycle progression from G_0 to G_1 phase [32].

4. Conclusion

In summary, we have developed four-component reaction for the synthesis of pyrazolo[1, 5-*c*]quinazolines with small polar substitution of pyrazole ring for the exploration of EGFR inhibitors. The 4-CR typically involves reaction of azomethine imine **4** generated *in situ* and acetonitrile derivative **7**/**8**. Acetonitrile with EWG at α-position such as malononitrile, α-cyanocaboxyalate and β-ketonitrile participated in the reaction. Pyrazolo[1,5-*c*]quinazolines exhibited antiproliferative activity against MDA-MB 231, A549 and H1299 cell lines. Of these, **9b** and **10b** were found more potent than erlotinib and gefitinib as potential EGFR inhibitors. Pleasingly, these substrate **9b** and **10b** did not show any effect on cardiac cells which is generally associated with EGFR inhibitors. Compound **9b**, **10b** and **10d** elevated ROS level and altered mitochondrial potential resulting in apoptosis via G1 phase. In a nutshell, presence of amino group on pyrazole ring of pyrazolo[1,5-*c*]quinazolines made them potent as compared to the previously identified lead (**5fb**) [9].

5. Experimental section

5.1. General considerations

All the reactions were carried out in dried reaction vessel with Teflon screw caps. THF was freshly dried and distilled over Na-benzophenone and kept under an inert atmosphere. All other solvent was used in synthesis purchased from CDH. Aliphatic isocyanides and acetonitrile derivative were purchased from Sigma-Aldrich, TCI, and Alfa Aesar. All starting reagents were synthesized by the following literature. Other reagents were purchased from Aldrich or TCI used as such without purification. Analytical TLC was performed using 2 × 4 cm plate coated with the 0.25 mm thickness of silica gel (60F-254 Merck), and visualization was accomplished with UV light or I_2/$KMnO_4$ staining. Melting points were uncorrected. ^{1}H, ^{13}C NMR, recorded on Bruker's Ascend 500 MHz spectrophotometer operating at 500.3 MHz for 1H and 125.8 MHz for 13C experiments; spectra were recorded at 295 K in $CDCl_3$; chemical shifts were calibrated to the residual proton and carbon resonance of the solvent: $CDCl_3$ (1H δ 7.269; 13C δ 77.0). Mass spectra were recorded on electrospray ionization quadrupole time of flight (ESI-QTOF-MS). The abbreviations used: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet, br s = broad singlet & br = broad signal.

5.1.1. General procedure for the ortho-Azidobenzaldehydes **1** [33]

Substituted 2-azidobenzaldehydes were prepared by using a protocol reported by Driver et al. in 2011 in one step using the reaction of commercially available 2-nitrobenzaldehydes with sodium azide in HMPA. In a reaction vial dissolved 2-nitrobenzaldehyde (1.0 g, 6.62 mmol) in HMPA (10 mL) and sodium azide (0.90 g, 13.9 mmol) was added at 0 °C. The reaction was stirred at ambient temperature for overnight. After completion of the reaction, the mixture was diluted with ice water and extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with water (3 × 30 mL) and then dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography as eluent EtOAc: hexanes to afford the desired product.

5.1.2. General procedure for the aryl sulphonyl hydrazide **3** [34]

Aryl sulfonyl hydrazides were prepared according to reported literature [34]. To a solution of an aryl sulfonyl chloride (2.0 mmol) in tetrahydrofuran (10 mL), was added hydrazine monohydrate (10 mmol) drop wise under nitrogen at 0 °C. After vigorous stirring for 30 min at 0 °C, the reaction mixture was diluted with ethyl acetate (40 mL) and washed with saturated brine (3 × 10 mL). The organic layer was dried over sodium sulfate, concentrated and added to hexane (12 mL) over 5 min. The mixture was filtered, and the collected solid was dried in vacuum and used as such without further purification.

5.1.3. General procedure for the synthesis of **4** [9]

Azomethine **4** were prepared by using recently reported literature [9]. To a 10 mL reaction vial was charged with 2-azidobenzaldehyde (1.0 equiv), isocyanide (1.2 equiv), $Pd(OAc)_2$ (7.5 mol %,), 4 Å MS, $TsNHNH_2$ (1.1 equiv) in toluene at room temperature. After stirring for 30 min at room temperature, starting material was completely disappeared and a yellow suspension was obtained. The suspension was then quenched by water and extracted with EtOAc (3 × 15 ml). After removal of solvents in vacuo, the residue was subjected to column chromatography on silica gel (100–200 mesh) using 20:80 EtOAc and hexane as eluent to give desired product **4**.

5.1.4. General procedure for the synthesis of **9** (method A)

A mixture of 2-Azidobenzaldehyde (1.0 equiv), isocyanide (1.2 equiv), $Pd(OAc)_2$ (7.5 mol%), 4 Å MS, $TsNHNH_2$ (1.0 equiv), in 1.0 mL of toluene stirred at 100 °C for 10 min then (2 equiv) malononitrile **7**, and DABCO (3 equiv) were added and stirred at 100 °C for 3 h. The reaction mixture was diluted with ethyl acetate (15 mL) and washed with water (10 mL). The organic layer was separated, dried on Na_2SO_4 and evaporated under vacuum. The crude product, so obtained, was purified by column chromatography to afford the desired product.

5.1.5. Analytical data

5.1.5.1. 9a. 2-amino-5-(tert-butylamino)pyrazolo[1,5-c]quinazoline-1-carbonitrile. The general procedure was followed using (0.100 g, 0.68 mmol) of 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.068 mg, 0.81 mmol) of *t*-butyl isocyanide **2a**, and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.089 g, 1.36 mmol) Malononitrile **7a**, DABCO (3 equiv) was added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9a** as a white solid (0.171 g, 90%), mp 196–198 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 8.28 (d, 1H, *J* = 8.0 Hz), 7.65(d, 1H, *J* = 8.2 Hz), 7.61 (t, 1H, *J* = 8.0 Hz), 7.33 (t, 1H, *J* = 8.1 Hz), 6.08 (br s, 1H, N—*H*), 4.54(br s, 2H, N—*H*), 1.61 (s, 9H) ^{13}C NMR (125 MHz, $CDCl_3$): 157.7, 143.2, 141.4, 131.7, 126.4, 123.3, 123.2, 114.6, 113.5, 72.5, 52.2, 28.9. HRMS (EI) calcd for $C_{15}H_{17}N_6$ ($M+H^+$) 281.1509 found 281.1405.

5.1.5.2. 9b:2-amino-5-((2,4,4-trimethylpentan-2-yl)amino)pyrazolo[1,5-c]quinazoline-1-carbonitrile. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2a,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.089 g, 1.36 mmol) Malononitrile **7a**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9b** as a white solid (0.197 g, 86%), mp 224–226 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). 1H NMR (δ ppm): (500 MHz, $CDCl_3$), 8.28 (d, 1H, *J* = 7.8 Hz), 7.65–7.58 (m, 2H), 7.33–7.28 (m, 1H), 6.18 (br s, 1H, N—*H*), 4.53 (br s, 2H, N—*H*), 2.01 (s, 2H), 1.66 (s, 6H), 1.02 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 157.7, 143.4, 143.1, 141.3, 131.7, 126.4, 123.3, 123.2, 114.5, 113.5, 72.5, 55.9, 51.8, 31.7, 31.5, 29.3; HRMS (EI) calcd for $C_{19}H_{25}N_6$ ($M+H^+$) 337.2135 found 337.2121.

5.1.5.3. 9c. 2-amino-8-bromo-5-(tert-butylamino)pyrazolo[1,5-c]quinazoline-1-carbonitrile. The general procedure was followed using of (0.100 g, 0.44 mmol) 2-azido 4-bromobenzaldehyde **1b**, (7.4 mg, 0.033 mmol) Pd $(OAc)_2$, (0.043 mg, 0.52 mmol) *t*-butyl isocyanide **2a**, and 200 mg of powdered 4 Å molecular sieves followed by (0.085 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then

add (0.058 g, 0.88 mmol) malononitrile **7a**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9c** as a white solid (0.118 g, 75%), mp 236–238 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 8.13 (d, 1H, J = 8.5 Hz), 7.85 (s, 1H), 7.42 (d, 1H, J = 8.2 Hz), 6.17 (br s, 1H, N—*H*), 4.59 (br s, 2H, N—*H*), 1.62 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 157.7, 144.4, 142.7, 141.8, 128.9, 126.5, 125.8, 124.4, 114.3, 112.2, 72.8, 52.4, 28.9; HRMS (EI) calcd for $C_{15}H_{16}BrN_6$ (M + H^+) 359.0615 found 359.0607.

5.1.5.4. 9e. 2-amino-5-(tert-butylamino)-8-chloropyrazolo[1,5-c]quinazoline-1-carbonitrile. The general procedure was followed using (0.100 g, 0.55 mmol) 2-azido 4-chlorobenzaldehyde **1c**, (9.2 mg, 0.041 mmol) Pd $(OAc)_2$, (0.54 mg, 0.66 mmol) of *t*-butyl isocyanide **2a**, and 200 mg of powdered 4 Å molecular sieves followed by(0.106 g, 0.60 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.072 g, 1.1 mmol) Malononitrile **7a**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9e** as a off white solid (0.181 g, 89%), mp 210–212 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 8.21 (d, 1H, J = 8.6 Hz), 7.67 (s, 1H), 7.29 (s, 1H), 6.17 (br s, 1H, N—*H*), 4.57 (br s, 2H, N—*H*), 1.61 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 157.7, 144.4, 142.7, 141.9, 137.5, 125.8, 124.3, 123.8, 114.3, 111.9, 72.6, 52.4, 28.9; HRMS (EI) calcd for $C_{15}H_{16}ClN_6$ (M + H^+) 315.112 found 315.1108.

5.1.5.5. 9f:2-amino-8-chloro-5-((2,4,4-trimethylpentan-2-yl)amino) pyrazolo[1,5-c]quinazoline-1-carbonitrile. The general procedure was followed using (0.100 g, 0.55 mmol) 2-azido 4-chlorobenzaldehyde **1c**, (9.2 mg, 0.041 mmol) $Pd(OAc)_2$, (0.091 mg, 0.66 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b**, and 200 mg of powdered 4 Å molecular sieves followed by (0.106 g, 0.60 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.072 g, 1.1 mmol) Malononitrile **7a**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9f** as a dull white solid (0.171 g, 84%), mp 240–242 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 8.20 (d, 1H, J = 8.6 Hz), 7.65 (s, 1H), 7.28 (d, 1H, J = 9.1 Hz), 6.26 (br s, 1H, N—*H*), 4.59 (br s, 2H, N—*H*), 2.01 (s, 2H), 1.66 (s, 6H), 1.04 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 157.7, 144.3, 142.6, 141.7, 137.5, 125.7, 124.3, 123.7, 114.3, 111.8, 72.8, 56.2, 51.7, 31.8, 31.5, 29.3; HRMS (EI) calcd for $C_{19}H_{24}ClN_6$ (M + H^+) 371.1746 found 371.1755.

5.1.5.6. 9g:2-amino-9-chloro-5-((2,4,4-trimethylpentan-2-yl)amino) pyrazolo[1,5-c]quinazoline-1-carbonitrile. The general procedure was followed using (0.100 g, 0.55 mmol) 2-azido 5-chlorobenzaldehyde **1d**, (9.2 mg, 0.041 mmol) $Pd(OAc)_2$, , (0.091 mg, 0.66 mmol)of 1,1,3,3-tetramethylbutylisocyanide **2b**, and 200 mg of powdered 4 Å molecular sieves followed by (0.106 g, 0.60 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.072 g, 1.1 mmol) Malononitrile **7a**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9 g** as a Light yellow solid (0.181 g, 89%), R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 8.22 (s, 1H, C—*H*), 7.60 (d, 2H, C—*H*, J = 8.8 Hz), 7.55 (dd, 1H, C—*H*, J = 8.9, 1.7 Hz), 6.24 (br s, 1H, N—*H*), 4.59 (br s, 2H, N—*H*), 2.01 (s, 2H), 1.66 (s, 6H), 1.03 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$):157.7, 141.9, 141.9, 141.3, 132.2, 128.3, 127.9, 122.2, 114.1, 108.7, 72.7, 56.1, 51.7, 31.5, 29.7, 29.2; HRMS (EI) calcd for $C_{19}H_{24}ClN_6$ (M + H^+) 371.1746 found 371.1755.

5.1.5.7. 9h:Methyl-2-amino-9-chloro-5-((2,4,4-trimethylpentan-2-yl) amino)pyrazolo[1,5-c]quinazoline-1-carboxylate. The general procedure was followed using (0.100 g, 0.55 mmol) 2-azido 5-chlorobenzaldehyde **1d**, (9.2 mg, 0.041 mmol) $Pd(OAc)_2$, (0.091 mg, 0.66 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b**, and 200 mg of powdered 4 Å molecular sieves followed by (0.106 g, 0.60 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.072 g, 1.1 mmol) methylcyanoacetate **7b**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 5% EtOAc and hexane as eluent afforded **9 h** as a Light yellow solid (0.181 g, 89%), mp 121–123 °C, R_f = 0.5 (10:90 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 9.31 (s, 1H), 7.58 (d, 1H, J = 8.8 Hz), 7.52 (d, 1H, J = 8.8 Hz), 6.41 (br s, 1H, N—*H*), 5.28 (br s, 2H, N—*H*), 4.03 (s, 3H,), 2.03 (s, 2H), 1.68 (s, 6H), 1.04 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 164.5 158.0, 142.7, 141.8, 140.3, 131.1, 127.5, 127.4, 126.0, 115.4, 94.3, 55.8, 51.8, 51.5, 31.6, 31.5, 29.4; HRMS (EI) calcd for $C_{20}H_{27}ClN_5O_2$ (M + H^+) 404.1848 found 404.1842.

5.1.5.8. 9i: Methyl 2-amino-5-(tert-butylamino)pyrazolo[1,5-c] quinazoline-1-carboxylate. The general procedure was followed using (0.100 g, 0.68 mmol) of 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.068 mg, 0.81 mmol) of *t*-butyl isocyanide **2a**, and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add **7b** (0.134 g, 1.36 mmol) methyl cyanoacetate, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9i** as a Yellow solid (0.178 g, 84%), mp 108–110 °C, R_f = 0.6 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 9.26 (d, 1H, J = 8.25 Hz), 7.65 (d, 1H, J = 8.1 Hz),7.58 (t, 1H, J = 7.9 Hz), 7.30 (t, 1H, J = 7.95 Hz), 6.26 (br s, 1H, N—*H*), 5.25 (s, 2H, N—*H*), 4.00 (s, 3H), 1.62 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 164.7, 157.9, 144.2, 141.8, 141.5, 130.9, 126.8, 126.2, 122.5, 114.8, 94.0, 51.9, 51.5, 29.0; HRMS (EI) calcd for $C_{16}H_{20}N_5O_2$ (M + H^+) 314.1612 found 314.1601.

5.1.5.9. 9j. Methyl 2-amino-5-((2,4,4-trimethylpentan-2-yl)amino) pyrazolo[1,5-c]quinazoline-1-carboxylate. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.134 g, 1.36 mmol) methyl cyanoacetate **7b**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9j** as a Yellow solid (0.183 g, 73%), mp 160–162 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 9.28 (d, 1H, J = 8.3 Hz), 7.67 (d, 1H, J = 8.1 Hz),7.60 (dt, 1H, J = 6.9, 1.3 Hz), 7.32–7.28 (m, 1H), 6.38 (br s, 1H, N—*H*), 5.27 (br s, 2H, N—*H*), 4.02 (s, 3H), 2.06 (s, 2H), 1.69 (s, 6H), 1.04 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 164.7, 157.9, 144.2, 141.6, 141.4, 130.9, 126.8, 126.2, 122.3, 114.7, 94.0, 55.7, 51.6, 51.5, 31.8, 31.5, 29.5, HRMS (EI) calcd for $C_{20}H_{28}N_5O_2$ (M + H^+) 370.2238 found 370.2229.

5.1.5.10. 9k. Ethyl 2-amino-5-(tert-butylamino)pyrazolo[1,5-c] quinazoline-1-carboxylate. The general procedure was followed using (0.100 g, 0.68 mmol) of 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.068 mg, 0.81 mmol) of *t*-butyl isocyanide **2a**, and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.152 g, 1.36 mmol) ethyl cyanoacetate **7c**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9 k** as a Yellow solid (0.177 g, 77%), mp

100–102 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 9.32 (d, 1H, J = 8.3 Hz), 7.67 (d, 1H, J = 8.25 Hz), 7.60 (dt, 1H, J = 7.1, 1.3 Hz), 7.31–7.29 (m, 1H), 6.28 (br s, 1H, N—*H*), 5.29 (br s, 2H, N—*H*), 4.52 (q, 2H, C—*H*, J = 7.15 Hz), 1.64 (s, 9H), 1.53 (t, 3H, J = 7.1 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): 164.1, 158.0, 144.2, 141.5, 130.9, 126.9, 126.2, 122.3, 114.8, 94.1, 60.6, 52.1, 29.0, 14.5. HRMS (EI) calcd for $C_{17}H_{22}N_5O_2$ (M + H^+) 328.1768 found 328.1752.

5.1.5.11. 9l. Ethyl 2-amino-5-((2,4,4-trimethylpentan-2-yl)amino) pyrazolo[1,5-c]quinazoline-1-carboxylate. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2a,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.152 g, 1.36 mmol) ethyl cyanoacetate **7c**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9 l** Yellow oil (0.205 g, 77%), R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 9.31 (dd, 1H, J = 8.3, 1.0 Hz), 7.66 (d, 1H, J = 8.2 Hz), 7.59 (dt, 1H, J = 7.0, 1.3 Hz), 7.31–7.27 (m, 1H), 6.37 (br s, 1H, N—*H*), 5.28 (br s, 2H, N—*H*), 4.52 (q, 2H, J = 14.25, 7.1 Hz), 2.06 (s, 2H), 1.68 (s, 6H), 1.52 (t, 3H, J = 7.2 Hz), 1.03 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 164.4, 157.9, 144.2, 141.7, 141.3, 130.8, 126.9, 126.2, 122.2, 114.8, 94.3, 60.6, 55.7, 51.5, 31.8, 31.5, 29.5, 14.5; HRMS (EI) calcd for $C_{21}H_{30}N_5O_2$ (M + H^+) 384.2394 found 384.2402.

5.1.5.12. 9m. Isopropyl 2-amino-5-(tert-butylamino)pyrazolo[1,5-c] quinazoline-1-carboxylate. The general procedure was followed using (0.100 g, 0.68 mmol) of 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.068 mg, 0.81 mmol) of *t*-butyl isocyanide **2a**, and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.172 g, 1.36 mmol) isopropylcyanoaceate **7d**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9 m** as a light Yellow solid (0.185 g, 80%), mp 113–114 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 9.32 (d, 1H, J = 8.15 Hz), 7.64 (d, 1H, J = 8.0 Hz), 7.57 (t, 1H, J = 7.6 Hz), 7.28–7.26 (m, 1H), 6.26 (br s, 1H N—*H*), 5.39–5.36 (m, 1H), 5.27 (br s, 2H, N—*H*), 1.62 (s, 9H), 1.48 (d, 6H, sp^3, J = 6.3 Hz), ^{13}C NMR (125 MHz, $CDCl_3$): 163.9, 158.1, 144.2, 141.8, 141.3, 130.8, 127.1, 126.1, 122.2, 114.7, 94.6, 68.3, 51.9, 29.1, 22.2, 21.6; HRMS (EI) calcd for $C_{18}H_{24}N_5O_2$ (M + H^+) 342.1925 found 342.1918.

5.1.5.13. 9n. Isopropyl 2-amino-5-((2,4,4-trimethylpentan-2-yl)amino) pyrazolo[1,5-c]quinazoline-1-carboxylate. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.172 g, 1.36 mmol) isopropyl cyanoacetate **7c**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9n** as a light yellow oil (0.207 g, 77, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 9.34 (d, 1H, C—*H*, J = 8.3 Hz), 7.66 (d, 1H, J = 8.2 Hz), 7.59 (dt, 1H, J = 8.0, 1.0 Hz), 7.31 (dt, 1H, J = 8.0, 0.85 Hz), 6.37 (br s, 1H, N—*H*), 5.42–5.37 (m, 1H), 5.29 (br s, 2H, N—*H*), 2.06 (s, 2H), 1.68 (s, 6H), 1.50 (d, 6H, J = 6.3 Hz), 1.03 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 164.0, 158.0, 144.2, 141.7, 141.2, 130.7, 127.0, 126.1, 122.0, 114.8, 94.6, 68.3, 55.6, 51.9, 31.8, 31.5, 29.5, 22.3; HRMS (EI) calcd for $C_{22}H_{32}N_5O_2$ (M + H^+) 398.2551 found 398.2553.

*5.1.6. General procedure for the synthesis of **10** (method B)*

The general procedure was followed using (1 equiv) 2-azidobenzaldehyde **1**, (7.5 mol%) of $Pd(OAc)_2$, (1.2 equiv) of 1,1,3,3-tetramethylbutylisocyanide **2b**, and 200 mg of powdered 4 Å molecular sieves followed by (1.1 equiv) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (2 equiv) Benzoylacetonitrile **8a**, DABCO (3 equiv) added followed by iodine (10 mol%) and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **10.**

*5.1.7. Analytical data of (**10a-10e**)*

5.1.7.1. 10a.(2-amino-5-((2,4,4-trimethylpentan-2-yl)amino)pyrazolo [1,5-c]quinazolin-1-yl)(phenyl)methanone. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.197 g, 1.36 mmol) Benzoylacetonitrile **8a**, DABCO (3 equiv) added followed by 10 mol% iodine and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **10a** as a yellow oil (67%), Rf = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light).^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 7.81 (d, 2H, , J = 7.7 Hz), 7.59–7.55 (m, 2H), 7.44–7.38 (m, 3H), 7.09 (d, 1H, J = 8.3 Hz), 6.78 (t, 1H, J = 7.9 Hz) 6.40 (br s, 1H, N—*H*), 5.14 (br s, 2H, N—*H*), 2.07 (s, 2H), 1.71 (s, 6H), 1.08 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 192.1, 158.1, 144.3, 141.8, 140.3, 139.9, 130.8, 129.6, 128.8, 126.5, 126.2, 121.7, 113.9, 102.7, 55.8, 55.0, 31.7, 31.5, 29.4; HRMS (EI) calcd for $C_{25}H_{30}N_5O$ (M + H^+) 416.2445 found 416.2455.

5.1.7.2. 10b.(2-amino-5-((2,4,4-trimethylpentan-2-yl)amino)pyrazolo[1,5-c] quinazolin-1-yl)(4-fluorophenyl)methanone. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.221 g, 1.36 mmol) 4-flurobenzoylacetonitrile **8b**, DABCO (3 equiv) added followed by 10 mol% iodine and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **10b** yellow oil (71%), Rf = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light). ^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 7.88–7.83 (m, 2H), 7.61 (d, 1H, J = 8.3 Hz), 7.43 (t, 1H, J = 8.2 Hz), 7.11–7.06 (m, 3H), 6.83 (t, 1H, J = 8.0 Hz) 6.40 (br s, 1H, N—*H*), 5.13 (br s, 2H, N—*H*), 2.07 (s, 2H), 1.71 (s, 6H), 1.08 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 192.4, 158.0, 144.4, 141.8, 140.2, 136.0, 131.9, ($J_{C\text{-}f}$ = 12.5 Hz), 130.5, 126.4, 121.8, 116.0, 113.8, 102.4, 55.9, 51.1, 31.8, 31.6, 29.4. HRMS (EI) calcd for $C_{25}H_{28}FN_5O$ (M + H^+) 434.2318 found 434.2323.

5.1.7.3. 10c.(2-amino-5-((2,4,4-trimethylpentan-2-yl)amino)pyrazolo[1,5-c] quinazolin-1-yl)(2-chlorophenyl)methanone. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.243 g, 1.36 mmol) 2-chlorobenzoylacetonitrile **8c**, DABCO (3 equiv) added followed by 10 mol % iodine and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **10c** light yellow oil (65%), Rf = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light).^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 7.60 (d, 1H, J = 8.2 Hz), 7.51 (d, 1H, J = 7.9 Hz), 7.48–7.41 (m, 3H), 7.32–7.27 (m, 2H), 6.81 (t, 1H, J = 7.4 Hz) 6.43 (br s,

1H, N—*H*), 5.30 (br s, 2H, N—*H*), 2.05 (s, 2H), 1.70 (s, 6H), 1.06 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 189.3, 158.4, 144.6, 141.7, 140.9, 140.3, 139.3, 130.9, 130.7, 129.8, 127.4, 126.3, 125.8, 122.0, 114.1, 114.0, 103.9, 55.8, 51.8, 31.8, 31.5, 29.7; HRMS (EI) calcd for $C_{25}H_{29}ClN_5O$ (M $+H^+$) 450.2055 found 450.2040.

5.1.7.4. 10d. (2-amino-5-((2,4,4-trimethylpentan-2-yl)amino)pyrazolo[1,5-c]quinazolin-1-yl)(4-methoxyphenyl)methanone. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azidobenzaldehyde **1a**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b,** and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.238 g, 1.36 mmol) 4-methoxybenzoylacetonitrile **8d**, DABCO (3 equiv) added followed by 10 mol% iodine and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **10d** yellow oil (0.207 g, 75%), Rf = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light).^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 7.84 (d, 2H, C—*H*, *J* = 8.6 Hz), 7.60 (d, 1H C—*H,* *J* = 8.3 Hz), 7.42 (t, 1H, *J* = 7.2 Hz), 7.26 (d, 1H, *J* = 8.3 Hz), 6.91 (d, 2H, *J* = 8.6 Hz), 6.86 (t, 1H, *J* = 7.2 Hz) 6.38 (br s, 1H, N—*H*), 5.01 (br s, 2H, N—*H*), 3.88 (s, 3H, O-*Me*) 2.08 (s, 2H), 1.71 (s, 6H), 1.08 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 190.7, 163.5, 157.8, 144.2, 141.8, 139.9, 131.7, 130.2, 126.5, 126.2, 121.8, 114.1, 114.0, 102.6, 55.8, 55.5, 52.0, 31.7, 31.5, 29.4; HRMS (EI) calcd for $C_{26}H_{32}N_5O_2$ ($M+H^+$) 446.2551 found.446.2541.

5.1.7.5. 10e:(2-amino-9-chloro-5-((2,4,4-trimethylpentan-2-yl)amino) pyrazolo[1,5-c]quinazolin-1-yl)(phenyl)methanone. The general procedure was followed using of (0.100 g, 0.68 mmol) 2-azido-5-chlorobenzaldehyde **1d**, (11 mg, 0.051 mmol) of $Pd(OAc)_2$, (0.112 mg, 0.81 mmol) of 1,1,3,3-tetramethylbutylisocyanide **2b**, and 200 mg of powdered 4 Å molecular sieves followed by (0.127 g, 0.74 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.160 g, 1.10 mmol) benzoylacetonitrile **8a**, DABCO (3 equiv) added followed by 10 mol% iodine and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **10e** transparent oil (0.207 g, 70%), Rf = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light).^{1}H NMR (δ ppm): (500 MHz, $CDCl_3$), 7.78 (d, 2H, *J* = 7.1 Hz), 7.65 (t, 1H, *J* = 7.1 Hz), 7.51 (d, 1H, *J* = 8.8 Hz), 7.48 (dd, 1H, *J* = *2.3,* 8.8 Hz), 6.88 (d, 1H, *J* = 2.3 Hz), 6.43 (br s, 1H, N—*H*), 5.24 (br s, 2H, N—*H*), 2.04 (s, 2H), 1.69 (s, 6H), 1.06 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$): 192.1, 158.2, 142.8, 141.8, 139.8, 139.2, 132.8, 130.8, 129.04, 129.01, 127.5, 126.7, 125.9, 114.7, 103.0, 55.9, 51.9, 31.8, 31.5.

*5.1.8. Scale up reaction procedure **9a***

The general procedure was followed using (0.68 g, 8.16 mmol) of 2-azidobenzaldehyde **1a**, (0.1 g, 0.51 mmol) of $Pd(OAc)_2$, (0.90 g, 13.6 mmol) of *t*-butyl isocyanide **2a**, and 200 mg of powdered 4 Å molecular sieves followed by (1.3 g, 7.5 mmol) tosylhydrazide **3a** in 1.0 mL of toluene stirred at 100 °C for 10 min then add (0.9 g, 13.6 mmol) Malononitrile **7a**, DABCO (3 equiv) added and stirred at 100 °C for 3 h. Purification by column chromatography on the bed of silica gel using 10% EtOAc and hexane as eluent afforded **9a** as a white solid (1.52 g, 80%), mp 196–198 °C, R_f = 0.5 (20:80 EtOAc:hexanes, visualized by 254 nm UV light).

5.2. Material and methods for biological evaluation

5.2.1. Cell culture

Cancer and Normal cell lines were purchased from National cell repository situated at NCCS, Pune, India. Cell were maintained in DMEM media supplemented with 10% Foetal Bovine serum (FBS) and antibiotic solution (1× Penstrip, Invitrogen) in standard conditioned inside the CO_2 incubator with 5% CO_2 and 95% humidity at 37 °C. Cells were seeded at recommended density for each cell lines, and sub-cultured using trypsin (trypsinisation) when 70–80% confluent [15,16,24].

5.2.2. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell were counted in automated counter and approximately 6×10^3 cells were seeded in the each well of 96 well plate. Incubated the cells in CO_2 incubator overnight and after that these cells were treated with investigational compounds of varied concentration dissolved in biological grade DMSO. After 48 h of incubation period, media were discarded and MTT dissolved in PBS was added to each well and further incubated in CO_2 incubator for 3–4 h. Dissolved the formed formazan in DMSO and absorbance was read on microplate reader at 570 nm. The results were represented as mean ± SD [15,16,24].

5.2.3. Epidermal growth factor receptor (EGFR) inhibitory activity

EGFR Inhibitory activity of the investigational compounds was determined by z-lyte kinase assay kit-tyr4 peptide (catalogue no. PV3193) and as per Manufacturer Protocol. The percentage inhibition of kinase was measured using Microplate reader at absorbance 400, 445 and 520 nm, respectively. Erlotinib was used as a positive control for the inhibitory assay [16,23].

Calculation emission ratio: Emission ratio = Coumarin Emission (445 nm)/fluorescein emission (520 nm). The extent of phosphorylation was calculated by following formula:

$$\%phosphorylation = 1 - \frac{(Emission\ \ ratio \times F100\%) - C100\%}{(C0\% - C100\%) + [Emission\ \ ratio\,(F100\% - F0\%)]}$$

where C0% = Average coumarin emission signal of the 100% Phos. Control; C100% = Average coumarin emission signal of the 0% Phos. Control; F100% = Average Fluorescein emission signal of the 100% Phos. Control; F0% = Average Fluorescein emission signal of the 0% Phos. Control

5.2.4. Molecular modelling studies

For molecular modelling studies protein structure PDB ID: 1M17 was retrieved from the protein data bank. The protein was further refined for missing loops and sidechains. The ligands were prepared using ChemBio Draw software. The docking was performed using Schrodinger Glide Module [35]. ADME properties were predicted using QikProp Module of Schrodinger software.

5.2.5. Reactive oxygen species (ROS) and mitochondria membrane integrative assay

Cells were seeded for ROS and mitochondria membrane potential assay and grown. Cells were trypsinzed centrifuged and washed with PBS and suspended the washed cell again in PBS. H_2DCFDA and JC-1 (Purchased from Sigma Aldrich) were added to suspended cells and incubated for 30 min in dark and analysed by using flow cytometer (BD Accuri) [36].

5.2.6. Apoptosis and cell cycle analysis

Cells were seeded in culture dishes and maintained the growth of cells. Cells were treated with selected investigational compounds at specified concentrations and incubated for desired time periods after that cells were trypsinzed and washed in PBS. For cell cycle analysis cell were fixed in 70% chilled ethanol and incubated at −20 °C for 4 h. After that cell was centrifuged and washed with PBS and added RNAase solution and incubated for 30 min. Further added PI stain and incubated for 30 min in dark. For performing apoptosis assay washed cell was suspended in Annexin binding buffer and followed the protocol as per indicated by manufacturer (ThermoFisher, India). Processed sample were analysed using flow cytometry (BD Accuri) [37,38].

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

Devesh M. Sawant is grateful to the Central University of Rajasthan, DST SERB project No. [EMR/2016/008016] and [EMR/2016/003969] for funds. Arshad J. Ansari is thankful to Indian Council of Medical Research for providing fellowship (File No. 45/20/2018-BIO/BMS). GJ and UPY thanks CSIR (Grant Nos. 05/1051(0011)/2018-EMR-I and 09/1051(0009)/2017, for providing SRF and JRF, respectively). RK and SS thanks Central University of Punjab for providing necessary infrastructure to carry out the research work. RK also acknowledges DST SERB protect No. EMR/2017/002702 for the financial support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103314.

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