Structure-based design of a new class of highly selective pyrazolo[3,4-*d*]pyrimidines based inhibitors of cyclin dependent kinases

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Abstract
Structure-based design approach was successfully used to guide the evolution of a pyrazolo[3,4-*d*]pyrimidine scaffold yielding a new structural class of highly selective inhibitors of cyclin dependent kinases capable to interact with an identified part of the protein. Several compounds from this series displayed potent and selective activity against CDK2.

Keywords: Pyrazolo[3,4-*d*]pyrimidine derivatives, design, docking, conformational analysis, CDK2/Cyclin A

Introduction

The normal orderly progression through the cell division cycle is regulated by the sequential activation and deactivation of several members of the cyclin-dependent kinase (CDK) family[1]. Virtually all cancers exhibit at least one alteration in CDK function, either through upregulation of positive effectors such as cyclins D and E, loss of negative regulators such as p16 and p27, or genetic mutations to CDK substrates[2,3]. Consequently, there is much interest in the development of CDK inhibitors that might offer selective and tolerable treatment for cancer.

Particular attention has been focused on CDK4 and CDK2 as potential targets for small-molecule inhibition of cell cycling, although there is some controversy over which is the more important of the two. Phosphorylation of the retinoblastoma protein (pRb) by CDK4 and/or CDK6 in combination with cyclin D drives progression through the G1 phase[4]. However, inhibition of CDK4/CDK6 in cells lacking pRb does not lead to cell cycle arrest[3], suggesting that some tumors may be insensitive to specific inhibition of CDK4. Complex formation between CDK2 and cyclin E sustains pRb phosphorylation to enable the G1-S-phase transition and activate the transcription factor E2F, leading to transcription of the genes responsible for completing DNA synthesis[4]. CDK2 then associates with cyclin A, promoting uninterrupted passage through the S phase and appropriately timed deactivation of E2F to complete this phase[3].

Because persistence of E2F activity during the S phase results in apoptosis,[3,5] selective inhibition of CDK2 activity may cause activation of apoptotic pathways as opposed to cell cycle arrest. Encouragingly, there is also evidence to suggest that inhibition of CDK2 selectively kills tumor cells with deregulated E2F activity.[6-9]

Due to the known biological activities of pyrazolo[3,4-d]pyrimidines as CDK2 inhibitors[10-12] and As a part of our continuing search for potential anticancer drug candidates[13], we describe herein the rational design, synthesis and biological profile of a new class of compounds containing pyrazolo[3,4-d]pyrimidine nucleus as a highly potent and selective CDK2-R PTK inhibitors.

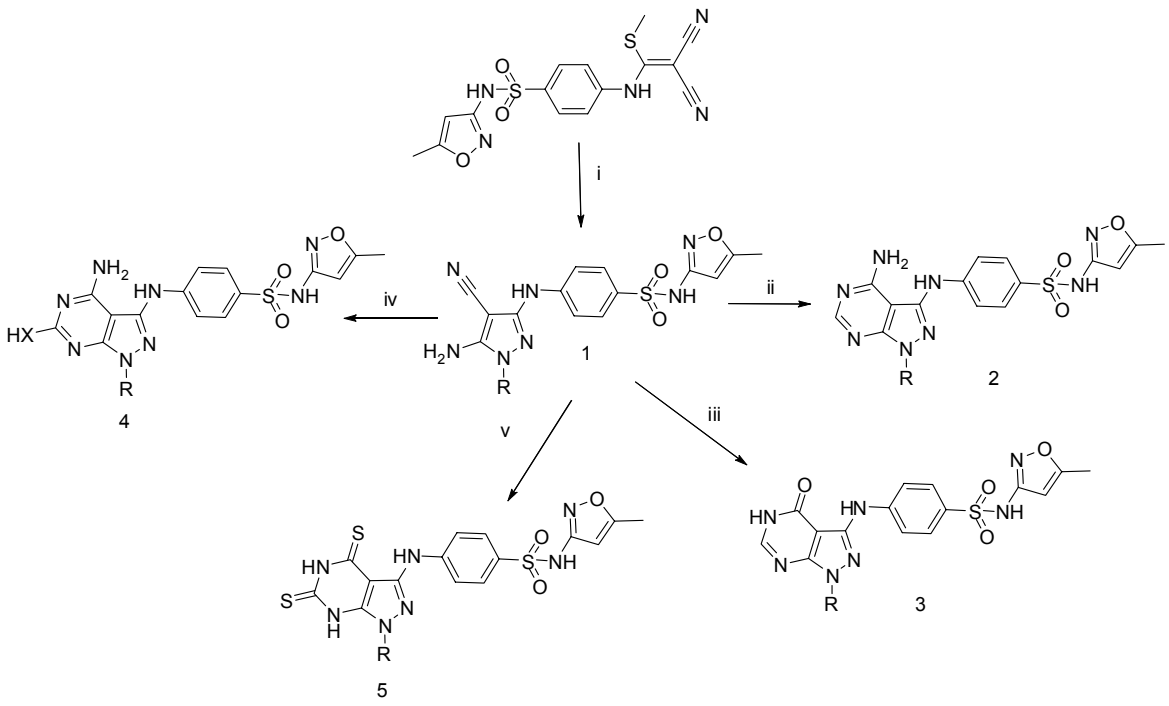
Results and Discussion

Inhibitors design

We made a library from our proposed compounds that contain pyrazolo[3,4-d]pyrimidine derivatives then these compounds were optimized at Semi-empirical level (AM1 method) and docked into the active pocket of CDK2 protein (PDB code 2c6i) in order to make a pre-selection of promising compounds. The pre-selected compounds were optimized at the Hartree-Fock (6-31G), re-docked and according to their docking scores and their interaction with CDK2 receptor we selected the compounds that will be synthesized. In principle all docking applications include four steps, i.e. identification and preparation of the receptor site, preparation of the ligands, docking the ligands and evaluation of the docked orientations. The internal coordinates, charges and hydrogen atoms were adjusted and added to the protein using the Amber Force field. The CDK2 glycine-rich loop region (Gly11, Glu12, Gly16 and Val17) and Glu8 were treated as partially flexible, Lys33, Lys89, Tyr15 and Gln85 as completely flexible. The MolDock scoring function was used, whereas terms such as Van Der Waals and Columbic terms are included. Finally, we carried out conformational analysis using MONTECARLO to the docked compounds and we calculated the strain energy (the difference in energy between the best conformer and the docked conformer). We used Spartan06[14] for molecular modeling calculations and Molegro Virtual Docker[15] for docking.

Chemistry

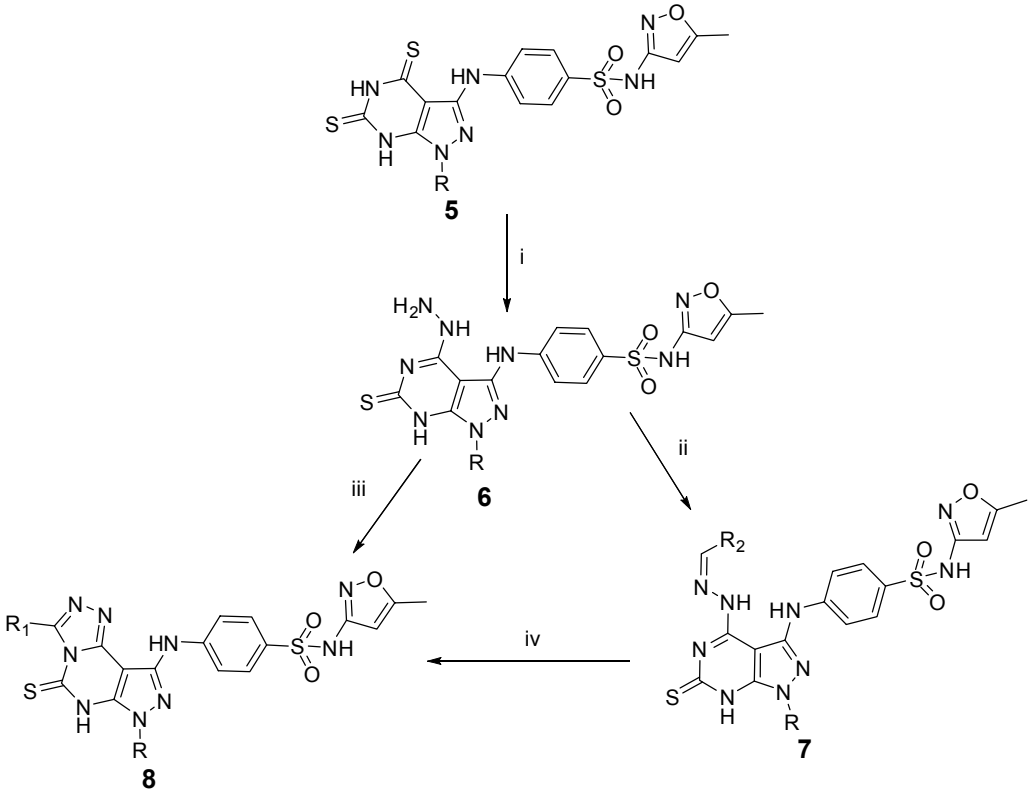
The synthesis of novel pyrazolo[3,4-*d*]pyrimidine derivatives having different substituents is shown in scheme 1,2. The 4-cyano-5-aminopyrazole derivatives (1a-c) serving as key intermediates were prepared according to the method developed previously[16]. This requisite starting material when refluxed with formamide and/ or formic acid for several hours afforded 4-aminopyrazolo-[3,4-*d*]pyrimidine derivatives (2a-c) and pyrazolo[3,4-*d*] pyrimidine-4-one derivatives (3a-c), respectively. The reaction of aminocyanopyrazoles (1a-c) with urea and thiourea at 180-200^{o} for 20 minutes afforded the corresponding 4-aminopyrazolo[3,4-*d*]pyrimidine derivatives (4a-f) in 77-85% yield.



Scheme 1. Reagent and condition: (i) $RNHNH_2$, EtOH, reflux; (ii) $HCONH_2$, reflux 10 h; (iii) HCOOH, reflux 10 h; (iv) $(NH_2)_2C=X$, heat; (v) CS_2, pyridine, reflux 8h.

For the synthesis of compound (7a,b), 5-amino-4-cyano-1H-pyrazoles were refluxed with carbon disulfide in pyridine for 10 hours to give the corresponding 4,6-dithiopyrazolo[3,4-d]pyrimidine derivatives (5), which were converted to hydrazinyl derivative (6) by refluxing with hydrazine hydrate for 30 minutes. Reaction of hydrazinyl derivative with aromatic aldehydes in dimethyl formamide at room temperature gave the hydrazone derivatives (7a,b).

Finally, the hydrazinyl derivative was converted to the triazolo derivatives (8a-c) by reaction of the hydrazinyl derivative with triethyl orthoformate, acetic anhydride and carbon disulfide, also the hydrazone derivatives were converted to the triazolo derivatives by treatment with nitric acid as depicted in scheme 2.



Scheme 2. Reagent and condition: (i) NH_2NH_2, reflux 30 min.; (ii) R_2CHO, stir at R.T for 12 h; (iii) $CH(OEt)_3(AC_2O)(CS_2)$; (iv) HNO_3, DMF, reflux.

Biological activities

Among several classes of potential CDK2/Cyclin A inhibitors, we focused on pyrazolo[3,4-*d*]pyrimidine derivatives because of their known biological activities as CDK2 inhibitors, low molecular weight of the scaffold and amenability for easy structural modification. Initial hits 2,3 were selected and docked into the active site of the CDK2 receptor (PDB code 2c6i). Subsequent analysis of the docking results modifying the remaining proposed compounds by working in positions 1, 4 and 6 in the pyrazolo[3,4-*d*] pyrimidine moiety was directed in order to obtain maximum fit to the receptor. Finally, we carried out conformational analysis for the docked compounds to calculate the difference in energy between the best conformer and the docked one (strain energy).

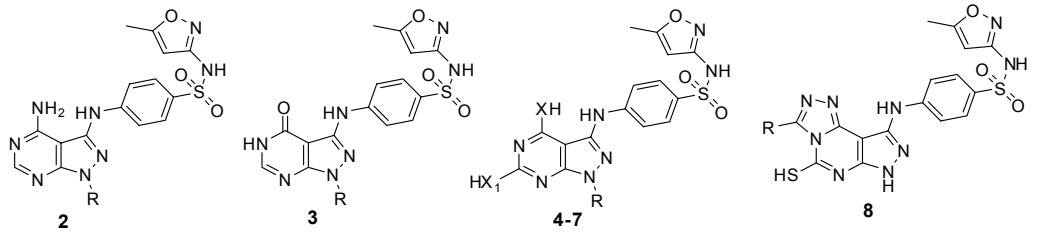
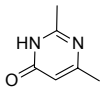
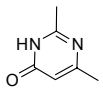
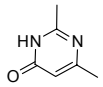
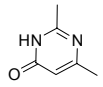
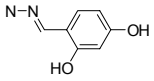
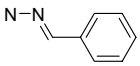
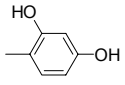


Table 1. CDK2-R Tyrosine kinase activity, docking scores and strain energy of newly synthesized derivatives

Compd	R	X	X_1	CDK2/cyclin A inhibition $IC_{50\ (\mu M)}$[a]	Docking Score	Strain Energy (KJ/mol)
2a	H			0.27	-170.91	3.692
2b	Ph			0.25	-192.74	0.997
2c				0.67	-208.83	2.304
3a	H			0.40	-163.02	14.561
3b	Ph			0.54	-187.55	24.911
3c				>20	-201.77	79.43
4a	H	NH	O	0.24	-177.48	18.805
4b	Ph	NH	O	1.63	-192.73	16.451
4c		NH	O	0.98	-204.82	14.487
4d	H	NH	S	0.77	-176.22	3.556
4e	Ph	NH	S	2.86	-184.36	1.065
4f		NH	S	0.82	-209.69	5.796
5	H	S	S	1.65	-176.59	33.92
6	H	NNH_2	S	5.63	-171.65	1.312
7a	H		S	>20	-201.84	62.118
7b	H		S	>20	-204.41	43.449
8a	H			3.25	-181.98	18.734
8b	CH_3			4.64	-188.12	21.866
8c	SH			2.66	-185.35	0.487
8d	Ph			10.6	-213.83	16.943
8e				>20	-207.92	49.544

[a]Values are means of three experiments; see Ref. 17 for assays protocol.

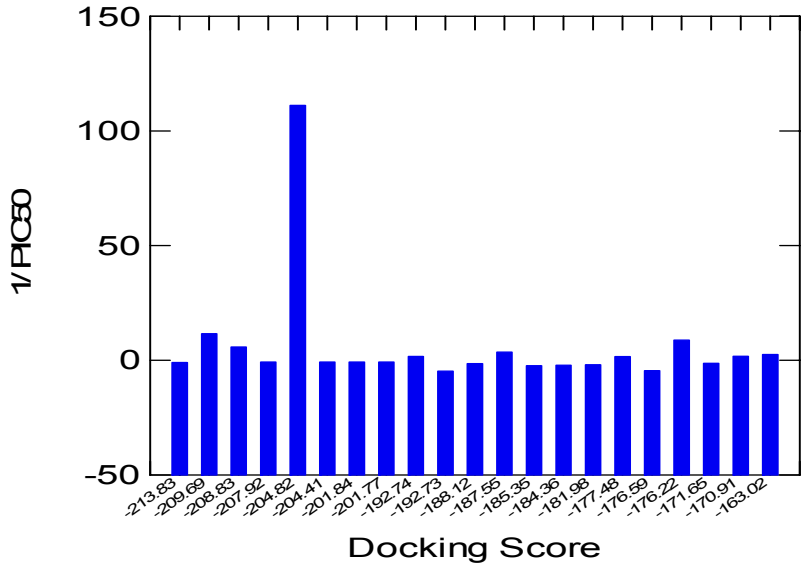


Figure 1. Docking scores against 1/ PIC_{50}.

From the data depicted in table (1), we found that compound (3b) had the highest docking score but not the highest biological activity, this may be explained by the high strain energy (> 20 KJ/mol). The most active compound was (4a), which had IC_{50} 0.24 and had a high docking score and low strain energy. Also compounds 2a, 3a, 2c, 4c, 4d and 4f showed high inhibitory activity (0.27, 0.4, 0.67, 0.98, 0.77 and 0.82 μM) against CDK2 receptor and displayed both high docking score and low strain energy. Compounds 3c, 7a,b and 8e were found to be inactive although they had a high docking score that because of the very high strain energy of these compounds as compared to the most stable conformer.

Overall, the remaining of the newly synthesized compounds showed a good correlation between the biological activity and both the docking score and the strain energy.

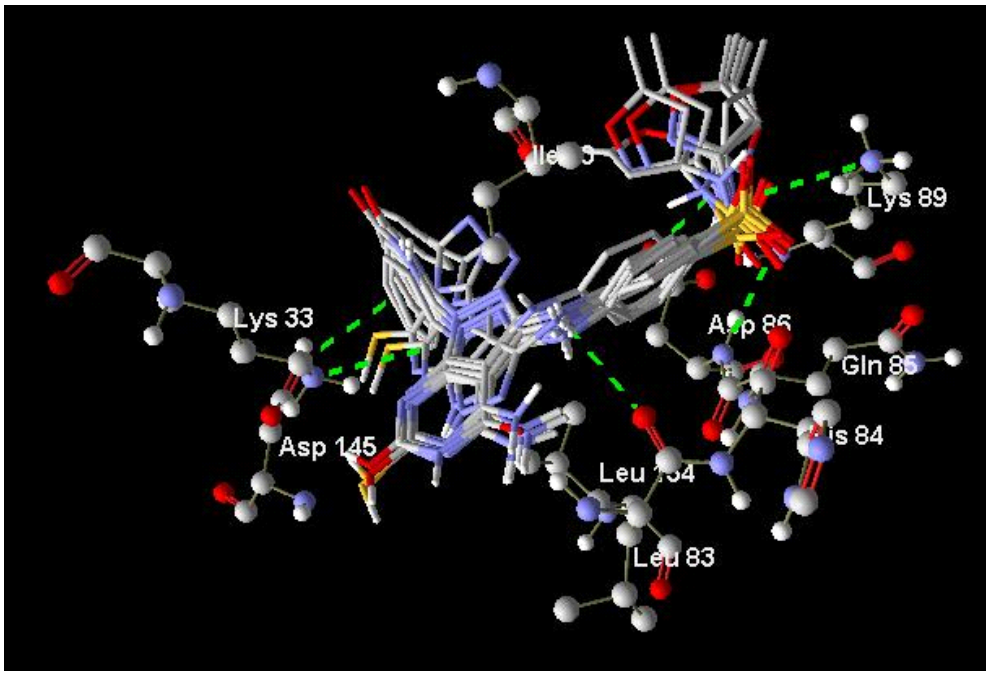


Figure 2. Binding of Pyrazolo[3,4-d]pyrimidine derivatives (that have high biological activity) with CDK2 highlighting the surrounding residues and hydrogen bond interactions (green doted lines).

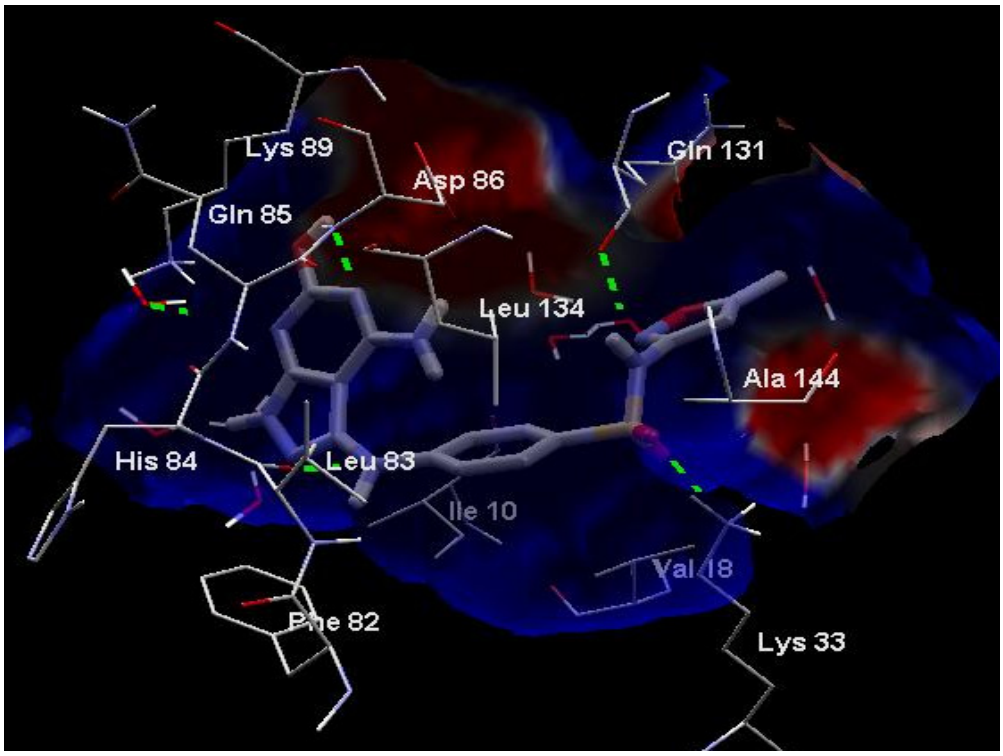


Figure 3. Binding of pyrazolo[3,4-d]pyrimidine 4a to CDK2 highlighting the surrounding residues, electrostatic interactions and hydrogen bond interactions (green doted lines).

Conclusions

In conclusion, our results demonstrate that docking is a useful technique for designing potent and selective CDK2 inhibitors, also a simple, mild and efficient method for the synthesis of novel functionalized pyrazolo[3,4-d]pyrimidine derivatives was demonstrated. Finally, our results have showed that strain energy is an important factor to explain the difference between the docking score and biological activity.

Experimental Section

General Procedures. Melting points were determined with an electrothermal capillary melting point apparatus and are uncorrected. IR spectra (KBr disks) were recorded on a Perkin-Elmer 1430 spectrometer. ^{1}H-NMR and ^{13}C-NMR were measured with a Varian GEMINI 200 spectrometer (200 MHz for ^{1}H-NMR; 500MHz for ^{13}C-NMR). Mass spectra were recorded on a GCMS - QP 1000 EX (70EV) spectrometer. Elemental analyses were carried out at the Microanalytical Center, Cairo University. Anticancer screening of the newly synthesized compounds was carried out at the National Cancer Institute.

4-(5-Amino-4-cyano-1-substituted pyrazol-3-ylamino)-*N*-(5-methyliso-xazol-3-yl) benzene-sulfonamide derivatives (1). General procedure

A solution of {[(4 – methylisoxazol -3 -yl) amino] sulfonyl} phenyl) amino] methyl-thiomethylene} methan-1, 1-dicarbonitrile[16] 37.5g (0.1 mol) and hydrazine derivatives (0.1 mol) in methanol (300 ml) was refluxed for 4 hrs. After evaporating the solvent, the residue was recrystallized from methanol to give the appropriate product (1) in good yield.

4-(5-Amino-4-cyano-1*H*-pyrazol-3-ylamino)-*N*-(5-methylisoxazol-3-yl) benzenesulfonamide (1a). Using hydrazine hydrate. Mp (^{o}C) 172-175; IR (KBr) (cm^{-1}) 3465 (NH), 3360 (NH), 3330, 3350 (NH_2), 3260 (NH), 2220 (C≡N), 1595 (C=C/C=N); ^{13}C (DMSO-d_6) (δ ppm) 12.8, 65, 99, 113, 120, 129, 135, 144, 150, 155, 159, 170. Anal. Calcd for $C_{14}H_{13}N_7O_3S$: C, 46.79; H, 3.65; N, 27.28. Found: C, 47.2; H, 4.2; N, 26.6

4-(5-Amino-4-cyano-1-phenyl-1*H*-pyrazol-3-ylamino)-*N*-(5-methyl-isoxazol-3-yl) benzene-sulfonamide (1b). Using phenylhydrazine. Mp (^{o}C) 140-142; IR (KBr) (cm^{-1}) 3360 (NH), 3300, 3350 (NH_2), 3215 (NH), 2210 (C≡N), 1600 (C=C/C=N); ^{1}HNMR (DMSO-d_6) (δ ppm) 2.2 (s, 3H, CH_3), 6.3 (s, 1H, Isoxazole-H4), 6.9 (s, 2H, NH_2), 7.2-7.9 (m, 9H, aromatic ring), 9.2 (s, 1H, Ar-NH), 12 (s, 1H, SO_2-NH). Anal. Calcd for $C_{20}H_{17}N_7O_3S$: C, 55.16; H, 3.93; N, 22.52. Found: C, 55.6; H, 4.3; N, 22.6.

4-(5-Amino-4-cyano-1-(4-methyl-6-oxo-1,6-dihydropyrimidin-2-yl)-1*H*-pyrazol-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (1c). Using 2-hydrazinyl-6-methylpyrimidin-4(3H)-one. Mp (^{o}C) 120-122; IR (KBr) (cm^{-1}) 3420 (NH), 3360 (NH), 3300, 3350 (NH_2), 3215 (NH), 2210 (C≡N), 1685 (C=O amide), 1580 (C=C/C=N); ^{1}HNMR (DMSO-d_6) (δ ppm) 2.4 (s,

3H, CH_3), 2.2 (s, 3H, CH_3), 6.1 (s, 1H, Isoxazole-H4), 6.2 (s, 1H, H5 pyrimidine), 6.7 (s, 2H, NH_2), 7.2-7.6 (m, 4H, aromatic ring), 9.2 (s, 1H, Ar-NH), 12 (s, 1H, SO_2-NH), 13.2 (s, 1H, NH-pyrimidine). Anal. Calcd for $C_{19}H_{17}N_9O_4S$: C, 48.82; H, 3.67; N, 26.97. Found: C, 49.6; H, 4.1; N, 26.1.

4-(4-Amino-1-substituted pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl) benzenesulfonamide derivatives (2). General proceduure

A mixture of 5-amino-4-cyano-1-substituted pyrazole derivatives (0.01mol), formamide (15 ml), dimethyl formamide (5 ml) and formic acid (2 ml) was heated under reflux for 10 hrs. The reaction mixture was concentrated, allowed to cool to room temperature and triturated with water. The solid obtained was filtered, washed with cold ethanol dried and recrystallized from a mixture of dimethylformamide and ethanol. It yielded the desired product (2).

4-(4-Amino-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzene-sulfonamide (2a). Mp (^oC) 160-162; IR (KBr) (cm^{-1}) 3460 (NH), 3300, 3350 (NH_2), 3215 (NH ring), 1680 (C=O), 1600 (C=C/C=N); ^{1}HNMR (DMSO-d_6) (δ ppm) 2.4 (s, 3H, CH_3), 6 (s, 1H, Isoxazole-H4), 6.9 (s, 2H, NH_2), 7.2-7.7 (m, 4H, aromatic ring), 8.3 (s, 1H, pyrimidine), 9.5 (s, 1H, Ar-NH), 11 (s, 1H, SO_2-NH), 13 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{15}H_{14}N_8O_3S$: C, 46.63; H, 3.65; N, 29.00. Found: C, 45.9; H, 3.95; N, 28.6.

4-(4-Amino-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl) benzenesulfonamide (2b). Mp (^oC) 210-212; MS m/z 462 $(M)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.2 (s, 3H, CH_3), 6.1 (s, 1H, Isoxazole-H4), 6.5 (s, 2H, NH_2), 7.2-7.5 (m, 4H, aromatic ring), 7.6-8.2 (m, 5H, N-Ph), 8.4 (s, 1H, pyrimidine), 9.5 (s, 1H, Ar-NH), 11.1 (s, 1H, SO_2-NH). Anal. Calcd for $C_{21}H_{18}N_8O_3S$: C, 54.54; H, 3.92; N, 24.23. Found: C, 54.2; H, 3.1; N, 25.1.

4-(4-Amino-1-(4-methyl-6-oxo-1,6-dihydropyrimidin-2-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (2c). Mp (^oC) 190-192; MS m/z 495 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.3 (s, 3H, CH_3), 2.1 (s, 3H, CH_3-Pyrimidinone), 6.1 (s, 1H, CH-Pyrimidinone), 6.3 (s, 1H, Isoxazole-H4), 6.9 (s, 2H, NH_2), 7.1-7.6 (m, 4H, aromatic ring), 8.1 (s, 1H, pyrimidine), 8.9 (s, 1H, Ar-NH), 10.8 (s, 1H, SO_2-NH), 11.5 (s, 1H, NH-Pyrimidinone). Anal. Calcd for $C_{20}H_{18}N_{10}O_4S$: C, 48.58; H, 3.67; N, 28.33. Found: C, 47.9; H, 3.3; N, 27.8.

***N*-(5-Methylisoxazol-3-yl)-4-(4-oxo-4,5-dihydro-1-substituted pyrazolo[3,4-*d*]pyrimidin-3-ylamino)benzenesulfonamide derivatives (3). General procedure**

A solution of 5-amino-4-cyano-1-substituted pyrazole derivatives (0.01mol) in formic acid (30 ml) was refluxed for 10 hrs. The reaction mixture was cooled, triturated with cold water and the separated solid was filtered, washed with cold water, followed by aqueous methanol, dried and recrystallized from dimethyl formamide to afford the title compound (3).

***N*-(5-Methylisoxazol-3-yl)-4-(4-oxo-4,5-dihydro-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino) benzenesulfonamide (3a).** Mp (^oC) 240-242; MS m/z 388 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.4 (s, 3H, CH_3), 6.2 (s, 1H, Isoxazole-H4), 7.3-7.6 (m, 4H, aromatic ring), 7.8 (s, 1H,

pyrimidine), 10.4 (s, 1H, Ar-NH), 10.9 (s, 1H, SO_2-NH), 12.1 (s, 1H, NH pyrimidine), 13.2 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{15}H_{13}N_7O_4S$: C, 46.51; H, 3.38; N, 25.31. Found: C, 45.8; H, 3.1; N, 25.9.

***N*-(5-Methylisoxazol-3-yl)-4-(4-oxo-1-phenyl-4,5-dihydro-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)benzenesulfonamide (3b).**Mp (^oC) 195-198; MS m/z 464 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.0 (s, 3H, CH_3), 6.2 (s, 1H, Isoxazole-H4), 7.0-7.4 (m, 4H, aromatic ring), 7.5-8.0 (m, 5H, N-Ph), 7.8 (s, 1H, pyrimidine), 10.2 (s, 1H, Ar-NH), 10.9 (s, 1H, SO_2-NH), 11.8 (s, 1H, NH-pyrimidine). Anal. Calcd for $C_{21}H_{17}N_7O_4S$: C, 54.42; H, 3.70; N, 21.15. Found: C, 54.2; H, 3.4; N, 20.6.

4-(1-(4-Methyl-6-oxo-1,6-dihydropyrimidin-2-yl)-4-oxo-4,5-dihydro-1*H*-pyrazolo[3,4-*d*] pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (3c). Mp (^oC) 185-188; MS m/z 495 $(M)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.2 (s, 3H, CH_3), 2.1 (s, 3H, CH_3-Pyrimidinone), 6.2 (s, 1H, CH-Pyrimidinone), 6.0 (s, 1H, Isoxazole-H4), 7.1-7.6 (m, 4H, aromatic ring), 7.8 (s, 1H, pyrimidine), 10.2 (s, 1H, Ar-NH), 11.1 (s, 1H, SO_2-NH), 11.5 (s, 1H, NH- Pyrimidinone), 12.5 (s, 1H NH-fused pyrimidine). Anal. Calcd for $C_{20}H_{17}N_9O_5S$: C, 48.48; H, 3.46; N, 25.44. Found: C, 47.9; H, 2.9; N, 24.8.

4-(4-Amino-6–hydroxy(thiol)-1-substituted pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide derivatives (4). General procedure

5-amino-4-cyano-1-substituted pyrazole derivatives (0.025 mol) were heated with ureas or thioureas respectively (6g) at 180-200^0 for 20 minutes at which time the boiling liquid changes to a semi-solid mass. The cooled solid was dissolved in 2N sodium hydroxide and heated till boiling. The hot solution was acidified with glacial acetic acid and filtered. The crude product was re-precipitated twice and washed with water to give pure product (4).

4-(4-Amino-6-hydroxy-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (4a). Mp (^oC) 200-203; IR (KBr) (cm^{-1}) 3760 (OH), 3460 (NH), 3300, 3350 (NH_2), 3215 (NH ring), 1680 (C=O), 1600 (C=C/C=N); MS m/z 403 $(M+H)^{+\cdot}$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.3 (s, 3H, CH_3), 6.2 (s, 1H, Isoxazole-H4), 6.5 (s, 2H, NH_2), 7.0 -7.5 (m, 4H, aromatic ring), 9.2 (s, 1H, Ar-NH), 11 (s, 1H, SO_2-NH), 11.4 (s, 1H, OH), 12.1 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{15}H_{14}N_8O_4S$: C, 44.77; H, 3.51; N, 27.85. Found: C, 45.1; H, 3.6; N, 27.1.

4-(4-Amino-6-hydroxy-1-phenyl-1*H*-pyrazolo[3,4-d]pyrimidin-3-ylamino)-*N*-(5-methyl-isoxazol-3-yl)benzenesulfonamide (4b). Mp (^oC) 200-203; MS m/z 479 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.2 (s, 3H, CH_3), 6.0 (s, 1H, Isoxazole-H4), 6.5 (s, 2H, NH_2), 7.2-7.6 (m, 4H, aromatic ring), 7.7-8.1 (m, 5H, N-Ph), 9.6 (s, 1H, Ar-NH), 10.5 (s, 1H, SO_2-NH), 11.5 (s, 1H, OH). Anal. Calcd for $C_{21}H_{18}N_8O_4S$: C, 52.71; H, 3.79; N, 23.42. Found: C, 52.2; H, 3.2; N, 23.6.

4-(4-Amino-6-hydroxy-1-(4-methyl-6-oxo-1,6-dihydropyrimidin-2-yl)-1*H*-pyrazolo[3,4-*d*] pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (4c). Mp (^oC) 230-233;

MS m/z 509 $(M-H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.1 (s, 3H, CH_3-Pyrimidinone), 2.4 (s, 3H, CH_3), 6.0 (s, 1H, Isoxazole-H4), 6.2 (s, 1H, CH-Pyrimidinone), 6.5 (s, 2H, NH_2), 7.1-7.6 (m, 4H, aromatic ring), 9.0 (s, 1H, NH-pyrimidinone), 10.0 (s, 1H, Ar-NH), 11.1 (s, 1H, SO_2-NH), 12.5 (s, 1H, OH). Anal. Calcd for $C_{20}H_{18}N_{10}O_5S$ (H_2O): C, 45.45; H, 3.41; N, 26.52 Found: C, 46.1; H, 2.9; N, 27.2.

4-(4-Amino-6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (4d). Mp (°C) 170-172; MS m/z 419 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.1 (s, 3H, CH_3), 6.2 (s, 1H, Isoxazole-H4), 6.9 (s, 2H, NH_2), 7.2-7.6 (m, 4H, aromatic ring), 9.5 (s, 1H, Ar-NH), 10.8 (s, 1H, SO_2-NH), 11.5 (s, 1H, SH), 13 (s, 1H, pyrazole-NH or SH). Anal. Calcd for $C_{15}H_{14}N_8O_3S_2$: C, 43.05; H, 3.37; N, 26.78. Found: C, 43.9; H, 2.9; N, 25.9.

4-(4-Amino-6-mercapto-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methyl-isoxazol-3-yl)benzenesulfonamide (4e). Mp (°C) 210-213; MS m/z 493 $(M-H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.0 (s, 3H, CH_3), 5.9 (s, 1H, Isoxazole-H4), 6.8 (s, 2H, NH_2), 7.2-7.6 (m, 4H, aromatic ring), 7.5-8.0 (m, 5H, N-Ph), 10.1 (s, 1H, Ar-NH), 10.7 (s, 1H, SO_2-NH), 12.5 (s, 1H, SH). Anal. Calcd for $C_{21}H_{18}N_8O_3S_2$: C, 51.00; H, 3.67; N, 22.66. Found: C, 50.6; H, 3.2; N, 23.3.

4-(4-Amino-6-mercapto-1-(4-methyl-6-oxo-1,6-dihydropyrimidin-2-yl)-1*H*-pyrazolo[3,4-*d*] pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (4f). Mp (°C) 240-242; MS m/z 527 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.2 (s, 3H, CH_3-Pyrimidinone), 2.3 (s, 3H, CH_3), 6.1 (s, 1H, Isoxazole-H4), 6.5 (s, 1H, CH-Pyrimidinone), 6.9 (s, 2H, NH_2), 7.0-7.5 (m, 4H, aromatic ring), 9.5 (s, 1H, Ar-NH), 10.0 (s, 1H, NH-pyrimidinone), 10.8 (s, 1H, SO_2-NH), 12.1 (s, 1H, SH). Anal. Calcd for $C_{20}H_{18}N_{10}O_4S_2$: C, 45.62; H, 3.45; N, 26.60. Found: C, 46.1; H, 3.1; N, 27.1.

4-(4,6-Dimercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)-benzenesulfonamide (5). A mixture of 5-amino-4-cyano-1-substituted pyrazole derivatives (0.01mol) and carbon disulfide (4 ml) in pyridine (20 ml) was refluxed for 8 hrs, then allowed to cool to room temperature. The solid precipitated was collected by filtration and recrystallized from dimethyl formamide. Mp (°C) 260-263; MS m/z 435 $(M)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.5 (s, 3H, CH_3), 6.1 (s, 1H, Isoxazole-H4), 7.2-7.6 (m, 4H, aromatic ring), 9.5 (s, 1H, Ar-NH), 11.3 (s, 1H, SO_2-NH), 12.0 (s, 1H, SH), 12.5 (s, 1H, pyrazole-NH), 13.5 (s, 1H, SH). Anal. Calcd for $C_{15}H_{13}N_7O_3S_3$: C, 41.37; H, 3.01; N, 22.51. Found: C, 40.8; H, 3.7; N, 22.9.

4-(4-Hydrazinyl-6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)-benzenesulfonamide (6). General procedure

To a mixture of hydrazine hydrate (5.0g, 99.9 mmol) and ethanol (5 ml), 4,6-dimercapto-1H-pyrazolo[3,4-d]pyrimidine derivative (2.6g, 5.95 mmol) was added and the mixture was heated under reflux for 15 minutes. After the reaction was completed, the precipitated crystals were collected by filtration and washed with water and ethanol to afford the hydrazinyl derivative (6). Mp (°C) 255-158; MS m/z 451 $(M+H_2O)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.0(s, 3H, CH_3), 5.0 (d, 2H, NH_2-hydrazine), 6.0 (s, 1H, Isoxazole-H4), 7.0-7.5 (m, 4H, aromatic ring), 9.9 (s, 1H, Ar-

NH), 11.1 (s, 1H, SO_2-NH), 11.7 (s, 1H, SH), 13.2 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{15}H_{15}N_9O_3S_2$: C, 41.56; H, 3.49; N, 29.08. Found: C, 42.2; H, 4.1; N, 29.9.

4-(4-(2-(Arylidene)hydrazinyl)-6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (7). A mixture of the hydrazinylderivative (1.4g, 3.33 mmol) and the appropriate aldehyde (9.99 mmol) in dimethyl formamide was stirred at room temperature for 12 hrs. After the reaction was completed, the solution was evaporated under reduced pressure and the residue was triturated with ethanol to give crystals, which were collected by filtration and recrystallized from a mixture of ethanol and dimethyl formamide.

4-(4-(2-(2,4-Dihydroxybenzylidene)hydrazinyl)-6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (7a).

Mp (^{o}C) 185-188; MS m/z 554 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.3(s, 3H, CH_3), 6.1 (s, 1H, Isoxazole-H4), 6.5 (d, 1H, H5-Phenyl), 7.0-7.4 (m, 4H, aromatic ring), 7.5 (s, 1H, H3-Phenyl), 7.7 (d 1H, H6-Phenyl), 8.2 (s, 1H, N=CH), 9.5 (s, 1H, Ar-NH), 9.8 (s, 1H, OH), 11.0 (s, 1H, SO_2-NH), 11.5 (s, 1H, SH), 11.7 (s, 1H, OH), 12.1 (s, 1H, NH-Hyrazone), 13.2 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{22}H_{19}N_9O_5S_2$: C, 47.73; H, 3.46; N, 22.77. Found: C, 47.2; H, 4.1; N, 22.9.

4-(4-(2-Benzylidenehydrazinyl)-6-mercapto-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (7b). Mp (^{o}C) 222-225; MS m/z 522 $(M)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.0 (s, 3H, CH_3), 6.0 (s, 1H, Isoxazole-H4), 7.1-7.7 (m, 9H, aromatic ring), 8.5 (s, 1H, N=CH), 9.2 (s, 1H, Ar-NH), 11.2 (s, 1H, SO_2-NH), 10.7 (s, 1H, NH-Hyrazone), 11.1 (s, 1H, SH), 13.0 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{22}H_{19}N_9O_3S_2$ (H_2O): C, 48.98; H, 3.53; N, 23.38. Found: C, 49.4; H, 4.1; N, 23.9.

4-(5-Mercapto-3-substituted-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-9-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (8a,b). A mixture of the hydrazinyl derivative (1.4g, 3.33 mmol) and triethyl orthoformate or triethyl orthoacetate (10.8mmol) in dimethyl formamide (30ml) was heated at 100^0 for 1 h. After the reaction was completed, the solution was evaporated under reduced pressure and the residue was triturated with ethyl acetate to give the titled compound (8a,b).

4-(5-Mercapto-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-9-ylamino)-*N*-(5-methyl-isoxazol-3-yl)benzenesulfonamide (8a). Mp (^{o}C) 262-265; MS m/z 444 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 1.9 (s, 3H, CH_3), 6.1 (s, 1H, Isoxazole-H4), 7.1-7.6 (m, 4H, aromatic ring), 8.6 (s, 1H, CH-Triazole), 9.5 (s, 1H, Ar-NH), 11.0 (s, 1H, SO_2-NH), 12.1 (s, 1H, SH), 13.2 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{16}H_{13}N_9O_3S_2$: C, 43.33; H, 2.95; N, 28.43. Found: C, 43.9; H, 3.1; N, 28.9.

4-(5-Mercapto-3-methyl-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo-[4,3-*c*]pyrimidin-9-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (8b). Mp (^{o}C) 243-245; MS m/z 458 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.1 (s, 3H, CH_3-Isoxazole), 2.3 (s, 3H, CH_3-Triazole) 6.4 (s, 1H, Isoxazole-H4), 7.0-7.5 (m, 4H, aromatic ring), 10.1 (s, 1H, Ar-NH), 11.3 (s, 1H, SO_2-NH), 12.6

(s, 1H, SH), 13.3 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{17}H_{15}N_9O_3S_2$: 44.63; H, 3.30; N, 27.55. Found: C, 44.9; H, 3.9; N, 27.9.

4-(3,5-Dimercapto-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-9-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (8c). A mixture of hydrazinylpyrazolo[3,4-d]pyrimidine derivative (1.4g, 3.33 mmol) and carbon disulfide (5 ml) in pyridine (15 ml) was refluxed for 10 hrs and then allowed to cool. The reaction mixture was triturated with water and acidified with dilute hydrochloric acid. The solid product was filtered, washed with water and air dried to give compound (8c). Mp (^{o}C) 280-283; MS m/z 477 $(M+2H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.0 (s, 3H, CH_3), 6.2 (s, 1H, Isoxazole-H4), 7.2-7.6 (m, 4H, aromatic ring), 9.5 (s, 1H, Ar-NH), 10.7 (s, 1H, SO_2-NH), 12.1 (s, 1H, SH-Triazole), 12.3 (s, 1H, SH), 13.1 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{16}H_{13}N_9O_3S_3$: C, 40.41; H, 2.76; N, 26.51.55. Found: C, 42.1; H, 3.3; N, 27.3.

4-(5-Mercapto-3-aryl-7*H*-pyrazolo[4,3-e][1,2,4]triazolo[4,3-*c*]pyrimidin-9-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (8d,e).

A mixture of 4-arylmethylidene hydrazinopyrazolo[3,4-d]pyrimidine derivatives (2.0 mmol) with 70% nitric acid (0.22 ml, 2.4 mmol) in dimethyl formamide (40 ml) was heated at 100^0 for 6 hrs. After the reaction was completed, the reaction mixture was evaporated under reduced pressure to its half volume and allowed to cool then the resulting crystals were collected by filtration to give the desired compound.

4-(5-Mercapto-3-phenyl-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-9-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (8d). Mp (^{o}C) 238-240; MS m/z 520 $(M+H)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.2 (s, 3H, CH_3), 6.1 (s, 1H, Isoxazole-H4), 7.2-8.0 (m, 9H, aromatic ring), 9.2 (s, 1H, Ar-NH), 10.9 (s, 1H, SO_2-NH), 12.1 (s, 1H, SH), 13.2 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{22}H_{17}N_9O_3S_2$: C, 50.86; H, 3.30; N, 24.26. Found: C, 50.1; H, 3.3; N, 23.5.

4-(3-(2,4-Dihydroxyphenyl)-5-mercapto-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-9-ylamino)-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide (8e). Mp (^{o}C) 228-231; MS m/z 551 $(M)^+$; ^{1}HNMR (DMSO-d_6) (δ ppm) 2.1 (s, 3H, CH_3), 6.2 (s, 1H, Isoxazole-H4), 6.2 (d, 1H, H3-Phenyl), 6.4 (s, 1H, H5-Phenyl), 7.1-7.6 (m, 5H, aromatic ring-H6-Phenyl), 9.4 (s, 1H, Ar-NH), 9.8 (s, 1H, OH), 10.2 (s, 1H, OH), 10.8 (s, 1H, SO_2-NH), 11.9 (s, 1H, SH), 13.0 (s, 1H, pyrazole-NH). Anal. Calcd for $C_{22}H_{17}N_9O_5S_2$ (H_2O): C, 46.40; H, 2.99; N, 22.14. Found: C, 47.2; H, 3.3; N, 22.9.

References

1. Sherr, C. J. *Science* **1996**, 274, 1672.
2. Senderowicz, A. M.: Sausville, E. A. *J. Natl. Cancer Inst.* **2000**, *92*, 376.
3. Shapiro, G. I.; Harper, J. W. *J. Clin. Invest.* **1999**; 04, 1645.
4. Harbour, J. W.; Luo, R. X.; Dei Santi, A.; Postigo, A. A. Dean, D. C. *Cell* **1999**; 98, 859
5. Krek, W.; Xu, G.; Livingston, D. M. *Cell* **1995**, 83, 1149.
6. Chen, Y-N. P.; Sharma, S. K.; Ramsey, T. M. et al. *Proc.Natl. Acad. Sci.* U S A **1999**, *96*, 4325.
7. Hinds, P. W. *Cancer Cell* **2003**, *3*, 305.
8. Tetsu, O.; McCormick, F. *Cancer Cell* **2003**, *3*, 233.
9. Pevarello, P.; Villa, M. *Expert Opin. Ther. Patents* **2005**, *15*, 675.
10. Jagabandhu, D.; Robert, V. M.; Sidney, P.; Rosemary, Z.; Ding, R. S.; Kim, W. M.; Kathleen, G.; Arthur, M. D.; John, S. S.; Hongjian, Z.; Susan, E. K.; Kevin, K.; Murray, M.; Joel, C. B.; John, H. D.; Gary, L. S.; Katerina, L. *Bioor. & Med. Chem. Lett.* **2008,** 18, 2652.
11. Schenone, S.; Ranise, A.; Bondavalli, F.; Brullo, C.; Fossa, P.; Mosti, L.; Menozzi, G.; Carraro, F.; Naldini, A.; Bernini, C.; Manetti, F.; Botta, M. *Bioorg. & Med. Chem. Lett.* **2004**, *14*, 2511.
12. Dong, C. K.; Yeo, R. L.; Beom-Seok, Y.; Kye, J. S.; Dong, J. K.; Bong, Y. C.; Kyung, H. Y. *Eur. J. Med. Chem*. **2003**, *38*, 525.
13. Ibrahim D. A. *Europ. J. Med. Chem*. **2009**, doi:10.1016/j.ejmech. 2009.01.003
14. Spartan'06 Wavefunction, Inc. Irvine, CA
15. Molegro Virtual Docker, www.molegro.com
16. Yoshinori Tominaga; Yasumasa Honkawa; Mayumi Hara; Akira Hosomi *J. Heterocyclic Chem***. 1990**, 27, 775.
17. (a) Hamdouchi, C.; Keyser, H.; Collins, E.; Jaramillo, C.; de Diego, E.; Spencer, C.; Dempsey, J.; Anderson, B.; Leggett, T.; Stamm, N.; Schultz, R.; Watkins, S.; Cocke, K.; Lemke, S.; Burke, T.; Beckmann, R.; Dixon, J.; Gurganus, T.; Rankl, N.; Houck, K. A.; Zhang, F.; Vieth, M.; Espinosa, J.; Timm, D.; Campbell, R.; Patel, B.; Brooks, H.B. *Mol. Cancer Ther*. **2004**, *3*, 1. (b) Jaramillo, C.; de Diego, E.; Hamdouchi, C.; Collins, E.; Keyser, H.; Sanchez-Martinez, C.; del Prado, M.; Norman, B.; Brooks, H.; Watkins, S.; Spencer, C.; Dempsey, J.; Anderson, B.; Leggett, T.; Schultz, R.; Watkins, S.; Lemke, S.; Burke, T.; Beckmann, R.; Dixon, J.; Gurganus, T.; Rankl, N.; Houck, K. A.; Zhang, F.; Vieth, M.; Espinosa, J.; Timm, D.; Campbell, R.; Patel, B. *Bioorg. Med. Chem. Lett*. **2004**, *14*, 6095.