Synthesis And Anticancer Evaluation Of Some Novel Fused Pyrazolopyrimidine Derivatives.

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Abstract: A new pyrazolopyrimidine formimidate **2b**, pyrazolopyrimidopyrimidine (**3b, 4b, 5a)**, pyrazolopyrimidine formamide **6b** and pyrazolopyridopyrimidine **7b** derivatives were designed and prepared via reaction of substituted pyrazolo[1,5-a] pyrimidine-6-carbonitrile (**1a, b**) with triethyl orthoformate, formamide, urea, formic and malononitrile in different conditions. The structures of target compounds were confirmed by elemental analyses and spectral data. Four of the newly synthesized compounds were selected by the NCI – Maryland-U.S.A. and were tested for their anticancer activity in an initial single high dose in the full NCI 60 cell line panel.

Keywords: anticancer activity, pyrazolopyrimidopyrimidine, pyrazolopyridopyrimidine.

1. Introduction

Pyrazolo[1,5-*a*]pyrimidine derivatives are considered as significant class of heterocyclic compounds with pharmacological and biological activities, such as antidepressant[1], tuberculostatic[2] , antitumor, antiviral [3-5], and antioxidant [6] activities. They act as inhibitors against KDR kinase [7,8] and COX-2[9]. Pyrazolo[1,5-a]pyrimidine derivatives are found to demonstrate antitumor effect in different cancer cell lines [10-12] and have a remarkable artificial value in the preparation of drugs with anticancer activities [13–15]. For the above reasons our target in this study was the synthesis of some new fused pyrazolopyrimidine compounds with an expected anticancer activity.

2. Results and discussion

Synthesis of compounds in scheme (1) was carried out starting by **1a,b** which produced on refluxing a mixture of (*E*)-4-((4-methoxyphenyl)diazenyl)-1H-pyrazole-3,5-diamine[16] and 2-(3,4,5-trimethoxybenzylidene) malononitrile or 2-(3,4-trimethoxybenzylidene) malononitrile, respectively in ethanol for 3h. The ^{1}HNMR spectra of **1a, b** showed two deuterium oxide exchangeable singlets at δ (5.94,7.61) ppm and δ (5.23, 6.19) ppm corresponding to NH_2-pyrazole and NH_2-pyrimidine for each start, respectively. Fusion of compound **1b** with triethyl orthoformate occurred in which compound **1b** subjected to a nucleophilic substitution reaction and inserting an ethoxy group obtaining compound **2b**. The ^{1}H NMR spectrum of compound **2b** uncovered a triplet at δ 1.21ppm and a quartet at δ 3.58ppm due to C_2H_5 protons of the ethoxymethylene moiety, also, a singlet at δ 8.35ppm for =CH- proton. Furthermore, fusion of compound **2b** with excess hydrazine hydrate produced new pyrazolo[1,5-*a*]pyrimido[4,5-*d*]pyrimidine derivative **3b**. The reaction presumably happened according to addition of a hydrazine molecule on the enamine double bond followed by elimination of an ethanol molecule and intramolecular cyclization to yield the target compound **3b**. The ^{1}H NMR spectrum of compound **3b** showed two D_2O exchangeable singlets at δ (5.87, 12.32) ppm due to NH_2 and =NH pyrimidine protons; respectively. When compound **1b** was fused with excess formamide compound **4b** was resulted. The reaction was reported to be going on condensation of the amidic carbonyl with amino group of **1b** followed by intramolecular cyclization through nucleophilic attack of the lone pair of the amide amino group on the electrophilic C≡N carbon of **1b**. The IR spectrum of **4b** show no signal for C≡N group. Fusion of **1a** with urea generated pyrazolopyrimido pyrimidinone derivative **5a** as a result of elimination of ammonia molecule from amino group of urea and amino group of **1a**, then addition of NH_2 of urea on C≡N of **1a** formed the cyclized structure. The IR spectrum of **5a** showed a broad peak at (3520-3200)cm^{-1} for OH, $2NH_2$ and NH groups, also revealed a peak at 1660 cm^{-1} is attributed to the presence of conjugated amidic C=O in pyrimidine nucleus, its ^{1}HNMR spectrum showed a deuterium exchangeable signal at δ 8.54 ppm due to NH-pyrimidinone proton, while the ^{13}CNMR spectrum revealed a signal at δ 147.3 ppm assignable to carbonyl carbon. Refluxing of compound **1b** with excess formic acid obtained pyrazolo[1,5-*a*]pyrimido formamide derivative **6b** which formed through formylation of amino group of **1b**. The cyclization due to the interaction between nucleophilic OH group and electrophilic C≡N carbon to give the iminoxazine intermediate which underwent rearrangement to form the pyrimidinone ring cannot be obtained. **IR** spectrum of **6b** showed absorption band at 3358, 3306, 3224 cm^{-1} referring to (NH_2, NH) groups, band of nitrile group (C≡N) was detected at 2218 cm^{-1}, and a sharp peak appeared at 1675 cm^{-1} for the conjugated amidic carbonyl group (C=O). The ^{1}HNMR spectrum of 9b displayed two deuterium oxide exchangeable signals corresponding to NH_2, NH protons at δ (4.31, 8.44) ppm, respectively, and a singlet signal at δ 8.15 ppm referring to proton of (CH-N) function. Condensation of **1b** with malononitrile in refluxing ethanol in presence of triethylamine gave pyrazolo[1,5-*a*] pyrido[2,3-*d*] pyrimidine derivative **7b**. Presumably, ionized malononitrile attacks the cyano group in compound **1b** before the amino group of the

latter attacks one of the malononitrile cyano groups resulting in ring formation. The IR spectrum showed absorption peaks at 3399, 3292, 3187, 2194 cm^{-1} due to $3NH_2$ and C≡N groups. The ^1H NMR spectrum exhibited three singlet signals at □3.72, 3.79, 3.81 ppm corresponding to the methoxy protons $3(OCH_3)$, broad singlet signal at □5.97ppm for the amino groups $3(NH_2)$, while the multiplate signals at □6.92- 7.63 ppm were assigned for the aromatic protons.

3. Biological evaluation

3.1. NCI results.

Four compounds were selected by the National Cancer Institute (NCI, Bethesda, USA) at one dose (10^{-5}M) in the full 60 human cancer cell lines orderly into subpanels derived from nine various human cancer forms: leukemia, melanoma, lung, colon, renal, ovarian, breast, prostate and CNS. The anticancer activity of the synthesized compounds is presented in Table (1).

4. Conclusion

Compounds (**2, 3, 7**) **b** exerted mild anticancer activity against some of cell lines, while compound **1b** was found to possess mild to strong selective potent anticancer activity against certain cancer cell lines.

5. Experimental

5.1. Chemistry

Melting points were determined on Electro thermal LA 9000 SERIS. Infrared (IR) spectra were recorded on Schimadzu FT-IR Affinity-1 spectrometer .^1H ,^{13}C NMR spectra were recorded on Jeol spectrometer (500 MHz) and Bruker high performance digital FT-NMR spectrometer avance III (400 MHz) using deuterated dimethylsulfoxide (DMSO‑d6) as a solvent. The Mass spectra were carried out using a Schimadzu GC/ MS-QP-5050A mass spectrometer at 70 ev in the regional center for mycology and biotechnology, at Al-Azhar University.

General method for preparation of compounds **1a, b**

An equimolar mixture of (E)-4-((4-methoxyphenyl) diazenyl)-1H-pyrazole-3,5-diamine [16] (2.32g, 10 mmol) and 2-(3,4,5-trimethoxybenzylidene) malononitrile (2.44g, 10mmol) or 2-(3,4-trimethoxybenzylidene) malononitrile (2.14g, 10 mmol) in ethanol (30 ml) was refluxed with piperidine (1ml) for 3 h. The solid product which formed after evaporation of the solvent was collected by filtration and crystallized from ethanol to afford compounds **1a, b.**

(E)-2,5-diamino-3-((4-methoxyphenyl)diazenyl)-7-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-a] pyrimidine-6-carbonitrile (**1a**, $C_{23}H_{22}N_8O_4$)

Yellow solid, (3.12, 66%). M.P.: 273-275 °C. IR (KBr, cm^{-1}): 3435, 3316, 3235($2NH_2$); 3080(CH-aromatic); 2945, 2843(CH-aliphatic); 2210(C≡N); 1610(C=N pyrazole, pyrimidine); 1560(C=C). ^{1}HNMR (500 MHz, DMSO-d_6): 3.71-3.86(m, 12H, $4(OCH_3)$); 5.94 (s, 2H, NH_2 pyrazole, D_2O exchangeable); 6.93-7.17 (m, 6H, Ar-H); 7.61 (s, 2H, NH_2 pyrimidine, D_2O exchangeable). Anal. Calcd for $C_{23}H_{22}N_8O_4$ (474.48); C, 58.22; H, 4.67; N, 23.62. Found: C, 58.31; H, 4.72; N, 23.69.

((E)-2,5-diamino-3-((4-methoxyphenyl)diazenyl)-7-(3,4-dimethoxyphenyl)pyrazolo[1,5-a] pyrimidine-6-carbonitrile (**1b**, $C_{22}H_{20}N_8O_3$)

Yellow solid, (2.91, 65%). M.P.: 239-241°C. IR (KBr, cm^{-1}): 3304, 3250($2NH_2$); 3080(CH-aromatic); 2936, 2834(CH-aliphatic); 2211(C≡N); 1624(C=N pyrazole, pyrimidine);1595(C=C). ^{1}HNMR (500 MHz, DMSO-d_6): 3.71-3.84(m, 9H, $3(OCH_3)$); 5.23 (s, 2H, NH_2 pyrazole, D_2O exchangeable); 6.19 (s, 2H, NH_2 pyrimidine, D_2O exchangeable); 6.74-7.79 (m, 7H, Ar-H). Anal. Calcd for $C_{22}H_{20}N_8O_3$ (444.46); C, 59.45; H, 4.54; N, 25.21. Found: C, 59.49; H, 4.65; N, 25.28.

General method for preparation of compounds (**2**, **4**) **b**

An equimolar mixture of compound **1b** (4.44g) (10 mmol) and triethoxymethane (1.48g,1.66ml,10 mmol) or formamide (0.45 g, 1.1 mL, 10 mmol) was heated until the contents melted, the reaction was maintained at temperature 200-280°C for 8 h.

*ethyl(E)-N-(2-amino-6-cyano-7-(3,4-dimethoxyphenyl)-3-((E)-(4-methoxyphenyl)diazenyl) pyrazolo[1,5-a]pyrimidin-5-yl)formimidate (**2b**, $C_{25}H_{24}N_8O_4$).*

The fused mass thus obtained was treated with ethanol, collected by filtration and crystallized from ethanol to afford compound **2b** as brown solid (2.69g, 54 %). M.p:. 129-131°C. IR (KBr, cm^{-1}): 3296, 3200 ($2NH_2$); 3040 (CH-aromatic); 2933(CH-aliphatic); 2209(C≡N); 1697 (C=N pyrazole, pyrimidine); 1590 (C=C).^{1}HNMR (500 MHz, DMSO-d_6): 1.21(t, 3H, CH_3), 3.58(q, 2H, CH_2), 3.79-3.85(m, 9H, $3(OCH_3)$), 6.10 (s, 2H, NH_2, D_2O exchangeable), 6.77-7.93 (m, 7H, Ar-H), 8.35(s, 1H, HC=N). Anal. Calcd for $C_{25}H_{24}N_8O_4$ (500.52); C, 59.99; H, 4.83; N, 22.39. Found: C, 60.02; H, 4.87; N, 22.43.

(E)-5-(3,4-dimethoxyphenyl)-9-((4-methoxyphenyl)diazenyl)pyrazolo[1,5-a]pyrimido[4,5-d]pyrimidine-4,8-diamine (**4b**, $C_{23}H_{21}N_9O_3$).

The reaction mixture was allowed to cool, then triturated by methanol and stirred at room temperature for 30 min. The precipitated solid was filtered, washed with cold methanol, and recrystallized from ethanol + DMF (2:1) to afford compound **4b** as black solid (2.19g, 46 %). M.p:. >360°C. IR (KBr, cm^{-1}): 3327, 3215 ($2NH_2$); 3080 (CH-aromatic); 2920 (CH-aliphatic); 1624 (4C=N pyrazole, pyrimidine); 1560 (C=C). ^{1}HNMR (400 MHz, DMSO-d_6):3.70, 3.71 (2s, 9H, $3(OCH_3)$); 5.56 (br.s, 4H, $2NH_2$, D_2O exchangeable); 7.44-7.69(m, 7H, Ar-H); 8.00 (s, 1H, =CH-pyrimidine) . MS (m/z, %): 471.99 ($M^{+\cdot}$, 28); 193.42 (100). Anal. Calcd for $C_{23}H_{21}N_9O_3$ (471.48); C, 58.59; H, 4.49; N, 26.74. Found: C, 58.65; H, 4.58; N, 26.81.

(E)-5-(3,4-dimethoxyphenyl)-4-imino-9-((4-methoxyphenyl)diazenyl)pyrazolo[1,5-a]pyrimido[4,5-d]pyrimidine-3,8(4H)-diamine (**3b**, $C_{23}H_{22}N_{10}O_3$)

A mixture of compound **2b** (5.00 g, 10 mmol) and excess hydrazine hydrate 99% (10 mL) was refluxed for 8 h. The reaction mixture was allowed to cool, and then triturated with ethanol. The separated solid was filtered and recrystallized from ethanol to afford compound **3b** as brown

solid (2.78g, 57%). M.p.: 179-181°C. IR (KBr, cm^{-1}): 3312, 3291, 3208($2NH_2$, NH); 3040(CH-aromatic); 2917(CH-aliphatic); 1624(4C=N); 1511(C=C).^{1}HNMR (500 MHz, DMSO-d_6): 3.67-3.83(m,9H,$3OCH_3$); 5.87 (br.s.,4H,$2NH_2$,D_2O exchangeable); 7.08-7.34(m,7H, Ar-H); 8.12(s,1H,CH-pyrimidine); 12.32(br.s.,1H,=NH-pyrimidine, D_2O exchangeable). MS (m/z, %): 486.26 ($M^{+\cdot}$, 10); 69.17 (100). Anal. Calcd for $C_{23}H_{22}N_{10}O_3$ (486.50); C, 56.78; H, 4.56; N, 28.79. Found: C, 56.82; H, 4.60; N, 28.82.

(E)-4,8-diamino-9-((4-methoxyphenyl)diazenyl)-5-(3,4,5-trimethoxyphenyl)pyrazolo[1,5-a] pyrimido[4,5-d]pyrimidin-2(1H)-one (**5a**, $C_{24}H_{23}N_9O_5$)

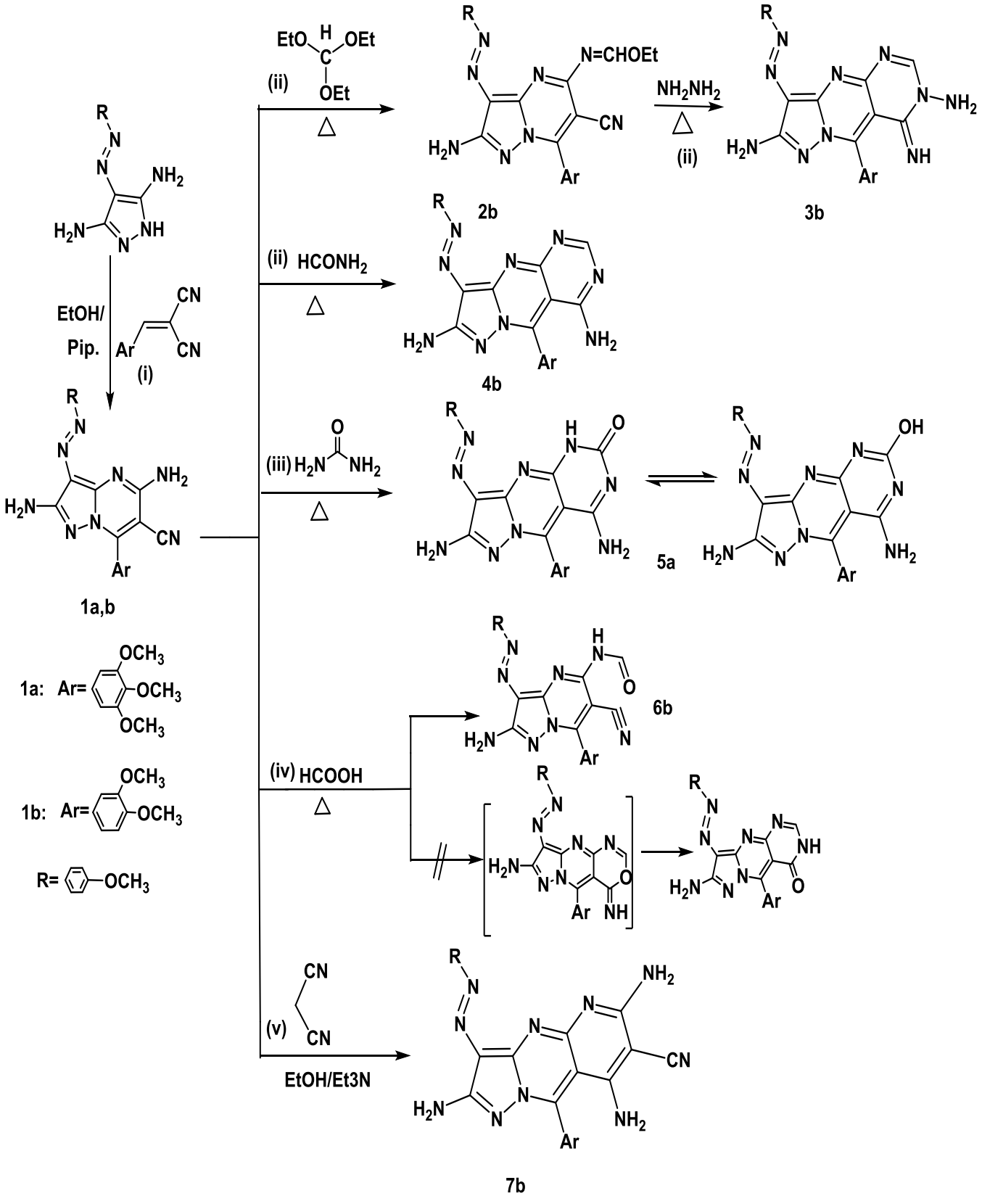
10 mmol of **1a** (4.74g) was heated with 20 mmol of urea (1.20g) at 180–200 °C for 6 h. Cooled solid was then dissolved in 2 N NaOH and the solution was boiled with charcoal for 10 min and filtered. The boiling filtrate was acidified with glacial acetic acid. This acidified solution was filtered hot to yield the product that was purified by dissolving small sample in boiling dilute NaOH and precipitating it from hot solution of acetic acid. The solid product which formed was collected by filtration and washed by ethanol, methanol, ethyl acetate, acetone, benzene, hexane, petroleum ether and DMF to afford compound **5a** as brown solid (2.61g, 50 %). M.p.: >360°C. IR (KBr, cm^{-1}):3520-3200(broad, OH, $2NH_2$, NH); 3003(CH-aromatic); 2836 (CH-aliphatic); 1660 (conjugated amidic C=O); 1635 (3C=N pyrazole, pyrimidine); 1580 (C=C). ^{1}HNMR (400 MHz, DMSO-d_6): 3.80-3.81 (m, 12H, $4(OCH_3)$); 6.34 (s,2H,NH_2- pyrazole,D_2O exchangeable); 6.97(s, 2H, NH_2-pyrimidinone, D_2O exchangeable); 6.99-7.84 (m, 6H, Ar-H); 11.23 (br.s,2H,NH-pyrimidinone, OH(tautomer),D_2O exchangeable).^{13}CNMR (400 MHz, DMSO-d_6): 55.7, 55.8(4(OCH_3); 86.6(C_2 pyrazole); 100.0, 114.5, 122.2, 127.4, 130.1, 139.3, 153.7, 160.9(phenyl $C_{12,\ 20\text{-}22,\ 25\text{-}32}$); 109.2($C_6$ fused pyrimidopyrimidinone); 147.3(C=O); 149.1(C_3 fused pyrazolopyrimidine); 152.3(C_{15}=N pyrazole); 157.4(C_9=N pyrimidinone); 165.5(C_7 pyrimidine); 166.7(C_{11}=N fused pyrimidopyrimidinone). MS (m/z, %): 517.24 ($M^{+\cdot}$, 9); 52.62 (100). Anal. Calcd for $C_{24}H_{23}N_9O_5$ (517.51); C, 55.70; H, 4.48; N, 24.36. Found: C, 55.79; H, 4.55; N, 24.43.

(E)-N-(2-amino-6-cyano-7-(3,4-dimethoxyphenyl)-3-((4-methoxyphenyl)diazenyl)pyrazolo[1,5-a]pyrimidin-5-yl)formamide (**6b**, $C_{23}H_{20}N_8O_4$)

Compound **1b** (4.44g, 10 mmol) was refluxed in excess formic acid (10 mL) for 16 h. The reaction mixture was allowed to cool, and then triturated by ethanol. The separated solid was filtered and recrystallized from ethanol to afford compound **6b** as brown solid (2.11g, 44 %). M.p:. 264-266°C. IR (KBr, cm^{-1}): 3358, 3306, 3224 (NH_2, NH); 3080 (CH-aromatic); 2954, 2937 (CH-aliphatic); 2218 (C≡N); 1675 (conjugated amidic C=O); 1632, 1601 (2C=N pyrazole, pyrimidine); 1513 (C=C). ^{1}HNMR (400 MHz, DMSO-d_6): 3.69, 3.78, 3.83(3s, 9H, $3(OCH_3)$); 4.31 (s, 2H, NH_2, D_2O exchangeable); 6.30-8.06(m, 7H, Ar-H); 8.15(s, 1H, CH-formamide); 8.40, 8.44(2s, 1H, NH, D_2O exchangeable).

(E)-2,6,8-triamino-9-(3,4-dimethoxyphenyl)-3-((4-methoxyphenyl)diazenyl)pyrazolo[1,5-a]pyrido[2,3-d]pyrimidine-7-carbonitrile (**7b**, $C_{25}H_{22}N_{10}O_3$).

An equimolar mixture of compound **1b** (4.44g, 10 mmol) and malononitrile (0.66 g, 10 mmol) were refluxed in absolute ethanol (30 mL) containing 5 drops of triethylamine for 9 h. The reaction mixture was allowed to cool and the obtained solid was filtered off, washed with ethanol and recrystallized from ethanol to afford compound **7b** as brown solid (2.99g, 58 %). M.p:. 179-181°C. IR (KBr, cm^{-1}): 3399, 3292, 3187 ($3NH_2$); 3080 (CH-aromatic); 2956, 2835 (CH-aliphatic); 2194(C≡N); 1640, 1610 (3C=N pyrazole, pyrimidine); 1563 (C=C). ^{1}HNMR (400 MHz, DMSO-d_6): 3.72, 3.79, 3.81(3s, 9H, $3(OCH_3)$); 5.97 (br.s, 6H, $3NH_2$, D_2O exchangeable); 6.92-7.63(m, 7H, Ar-H). MS (m/z, %): 510.31 ($M^{+\cdot}$, 34); 78.08 (100). Anal. Calcd for $C_{25}H_{22}N_{10}O_3$ (510.52); C, 58.82; H, 4.34; N, 27.44. Found: C, 58.89; H, 4.40; N, 27.52.



Conditions:(i) reflux, 3h;(ii) fusion, 8h; (iii) reflux, 6h; (iv) reflux, 16h; (v)reflux, 9h.

Scheme (1)

5.2. NCI methodology

All compounds submitted to the NCI 60 Cell screen are tested at a single high dose (10^{-5} M) as previously reported [17].

Acknowledgments

The authors wish to express all thanks to Naional Institute of Health (NIH), Bethesda, MD, USA, for the screening studies of anticancer activity reported in the paper.

Table (1): *Evaluation of anticancer activity (Percentage of Growth inhibition) of the selected compounds for most sensitive cell lines.*

Comp. No.	NCI-No.	Mean Growth%	Range of Growth %	Panel Subpanel cell line (Growth inhibition percent)
1b	812492	73.78	66.30 36.94 to 103.24	Leukemia: CCRF-CEM (47.32), HL-60(TB) (30.76), K-562 (29.10), MOLT-4 (59.79), RPMI-8226 (56.46), SR (22.41). Non-Small Cell Lung Cancer: A549/ATCC (29.13), EKVX (20.88), HOP-62 (29.66),HOP-92 (50.48), NCI-H23 (14.14), NCI-H322M (17.73), NCI-H460 (27.40), NCI-H522 (33.84). Colon Cancer: COLO 205 (16.33), HCT-116 (26.81), HCT-15 (25.73), HT29 (14.30), KM12 (25.30). CNS Cancer: SF-295 (23.82), SF-539 (24.90), SNB-75 (44.08), U251 (15.83). Melanoma: M14 (20.87), MDA-MB-435 (21.85), SK-MEL-5 (21.88). Ovarian Cancer: IGROV1 (42.65), OVCAR-4 (25.03), OVCAR-5 (15.11), OVCAR-8 (38.43), SK-OV-3 (30.30). Renal Cancer: A498 (28.56), ACHN (23.58), CAKI-1 (32.47), RXF 393 (34.01), SN12C (23.95), TK-10 (26.87), UO-31 (38.29). Prostate Cancer: PC-3 (33.15), DU-145 (31.73). Breast Cancer: MCF7 (60.20), MDA-MB-231/ATCC(38.46), HS 578T (31.59), BT-549 (28.56), T-47D (63.06), MDA-MB-468 (53.63).
2b	812500	90.33	61.86 55.42 to 117.28	Leukemia: CCRF-CEM (37.03), HL-60(TB) (21.71), MOLT-4 (39.28), RPMI-8226 (26.58), SR (30.05). Non-Small Cell Lung Cancer: A549/ATCC (13.51), HOP-62 (19.41), HOP-92 (33.15), NCI-H522 (19.55). Colon Cancer: COLO 205 (28.13), HCT-116 (22.02), KM12 (13.90). CNS Cancer: SF-539 (20.37). Ovarian Cancer: OVCAR-8 (24.67), SK-OV-3 (17.94). Renal Cancer: CAKI-1 (18.37), UO-31 (26.47). Breast Cancer: MDA-MB-231/ATCC(22.53), BT-549 (16.78),T-47D (44.58).
3b	812501	99.47	47.21 72.51 to 119.72	Leukemia: CCRF-CEM (24.01), Non-Small Cell Lung Cancer: HOP-92 (19.91), NCI-H226 (15.62), NCI-H522 (26.26). Renal Cancer: A498 (19.27), Breast Cancer: MDA-MB-231/ATCC(27.49), T-47D (13.31).
7b	812494	94.14	77.03 60.92 to 137.95	Non-Small Cell Lung Cancer: EKVX (28.89), NCI-H522 (15.69). CNS Cancer: SNB-75 (31.96). Ovarian Cancer: IGROV1 (39.08). Renal Cancer: CAKI-1 (25.25), UO-31 (27.48).Breast Cancer: MCF7 (32.87), MDA-MB-231/ATCC(22.72), T-47D (28.80).

Range: Variation in value between the most sensitive cell lines and the most inefficient one.

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