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Research Article

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Synthesis and *in-vitro* Anticancer Activity of 3 -Cyano- 9, 12-Dimethyl -4, 14-Diimino- 2-Methylthio- Pyrimido[2,3-*b*] Pyrazolo[3,4-*e*] Pyrimido[2,3-*b*][1,3] Benzothiazole and its 2-Substituted Derivatives

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Abstract
A new heterocyclic compound 3 -Cyano- 9, 12-dimethyl -4, 14-diimino- 2-methylthio- pyrimido[2,3-*b*] pyrazolo[3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole 3 has been synthesised by the reaction of 3-amino- 4-imino- 6, 9-dimethyl- pyrazolo-[3, 4-*e*] pyrimido [2, 3-*b*] [1, 3] benzothiazole 2 with bis - methylthio methylene malononitrile in presence of anhydrous K_2CO_3 in DMF. Compound 3 has thiomethyl functionality at 2- position which was substituted by using selected nucleophiles like substituted phenols, anilines, heteryl amine and active methylene compounds to yield 4-7. All the synthesised compounds were screened for their in-vitro anticancer activity against human 60-cell lines, exhibited better activity.

Keywords Anhydrous K_2CO_3, anticancer activity, Benzothiazoles, Pyrimido, Pyrazolo.

1. Introduction

In recent years, the heterocyclic compound containing nitrogen, oxygen and Sulphur have a greater attention to achieve medicinally target molecule. The fused heterocyclic compounds like benzimidazole, benzothiazole, benzoxazole, pyrimido benzothiazoles, pyrazolo pyrimido benzoxazole contains bridged nitrogen atom are the biological interesting nucleus. Wang et al. [1] have been synthesized the benzothiazole -2- thiol derivatives and reported its antiproliferative activities on HepG2 and MCF-7 cells. Manoj N. Bhoi et al [2] synthesised novel benzothiazole containing 4*H*-pyrimido [2, 1-*b*]benzothiazoles derivatives by one pot, solvent-free microwave assisted synthesis and reported as antioxidant and antimicrobial activities. Fatemeh Chadegani et al [3] developed an efficient one pot three component method for the synthesis of pyrimido benzothiazole and its derivatives. Abdel-Mohsen HT et al [4] prepared fused pyrimido benzothiazole derivatives from catechols and 6-substituted 1, 2, 3, 4-tetrahydro-4-oxo-2-thioxo-5-pyrimidinecarbonitriles using aerial O_2 as the oxidant. Some N-bis-benzothiazole derivatives [5] were reported to as cytotoxic agent against two human monocytic cell lines (U 937, THP-1) and a mouse melanoma cell line (B16-F10). Kamal et al. [6] synthesised a series of benzothiazole linked to the pyrrolodiazepine and reported their anticancer activity, DNA thermal denaturation studies, restriction endonuclease digestion assay, and flow cytometric analysis in human melanoma cell line (A375). Mortimer et al. [7] reported in vitro antitumor properties of 2-(3, 4-dimethoxyphenyl)-5-fluorobenzothiazole. Benzothiazole containing phthalimide derivatives [8] also exhibited *in vitro* cytotoxicity on human cancer cell lines. N D Amnerkar et al [9] reported a series benzothiazole derivatives and evaluated for neurotoxicity and hepatotoxicity and behavioural study.

Vijay N. Bhosale et al [10] reported antibacterial activity of Aryl / Heteryl fused pyrazolo [3¢,4¢: 4,5] pyrimido[2,1-*b*][1,3]benzothiazoles and In view of the reported biological activities and application of benzothiazoles moiety, we thought it worthwhile to design and synthesis of fused pyrazolo pyrimido benzothiazoles 2, pyrimido pyrazolo pyrimido bnezothiazoles 3 and its substituted derivatives by using selected nucleophiles like substituted phenols, anilines, heteryl amines and active methylene compounds to offered compounds 4-7, further evaluated their in-vitro anticancer activity at National Cancer Institute, Maryland, USA.

2. Results and Discussion

In continuation to our previous work [11], we have reported the novel heterocyclic compound containing diimino fused pyrimido pyrazolo pyramido benzothiazole with five cycles. 3-amino- 4-imino- 6, 9-dimethyl- pyrazolo-[3, 4-*e*] pyrimido [2, 3-*b*] [1, 3] benzothiazole 2 was prepared by the reaction of 3-Cyano -6, 9-dimethyl -2-methylthio -4-imino -4*H*-pyrimido [2, 1-*b*] [1, 3] benzothiazole 1 and 80% Hydrazine hydrate in presence of anhydrous K_2CO_3. Moreover compound 2 on being subjected to cyclization in presence of anhydrous K_2CO_3 in DMF with bis -methylthio methylene malononitrile to yield new fused heterocycles 3 -Cyano- 9, 12-dimethyl -4, 14-diimino- 2-methylthio- pyrimido[2,3-*b*] pyrazolo[3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole 3. Anhydrous K_2CO_3 play an important role to maintain basic condition which favour to the cyclization.

Compound 3 has thiomethyl functionality at 2- position, which was get substituted by using selected nucleophiles like substituted phenols, anilines, heteryl amine and active methylene compounds to yield 2-substituted derivatives (4-7) of 3 -Cyano- 9, 12-dimethyl -4, 14-diimino- 2-methylthio- pyrimido[2,3-*b*] pyrazolo[3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole.

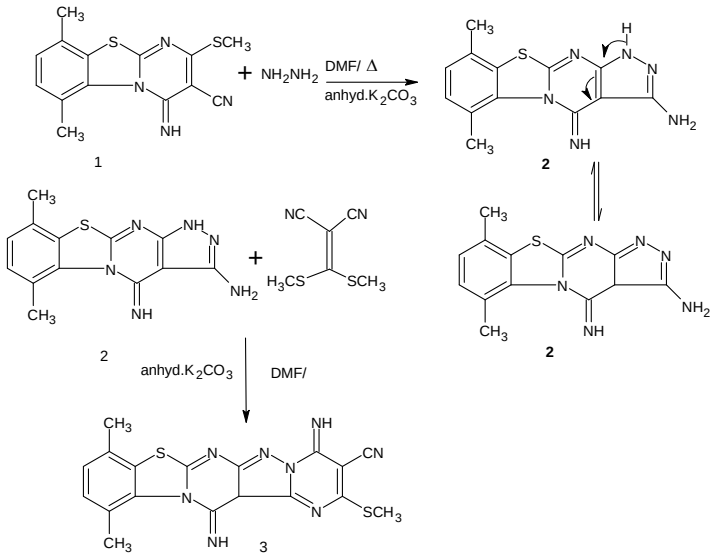

Figure 1: Reaction Scheme-I

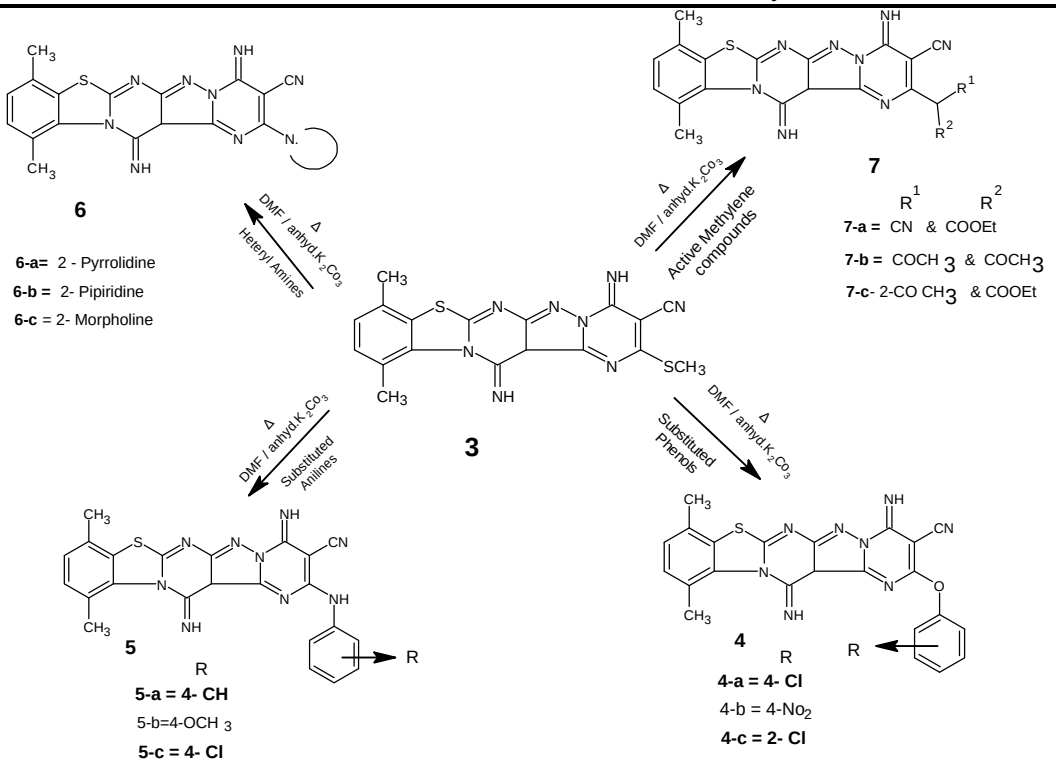

Figure 2: Reaction Scheme-II

2.1. Experimental Section

The melting points of all compounds were determined in open capillary tubes and are uncorrected. Infrared (IR) spectra were recorded in KBr on SHIMADZ-FTIR Spectrophotometer in cm^{-1}. ^1H Nuclear Magnetic Resonance (NMR) spectra were recorded on FT Gemini 300MHz Spectrometer using DMSO-d_6/$CDCl_3$ and TMS as internal reference. Chemical shift values are expressed in δ (ppm). Mass spectra were recorded on SCHIMADZU- GCMS Spectrometer using EI technique.

General Method:

Synthesis of 3-amino- 4-imino- 6, 9-dimethyl- pyrazolo-[3, 4-*e*] pyrimido [2, 3-*b*] [1, 3] benzothiazole (2): A mixture of 3-Cyano -6, 9-dimethyl -2-methylthio -4-imino -4*H*-pyrimido [2, 1-*b*] [1, 3] benzothiazole **1** (0.01mole) and Hydrazine hydrate 5 ml, was refluxed in the presence of 25 ml of dimethyl formamide and a pinch of anhydrous potassium carbonate (0.5gm) for five hours. The reaction mixture was monitored by TLC. The reaction mixture was cooled at room temperature and poured in ice cold water, the separated solid product was filtered washed with water and recrystallized from DMF-ethanol to offered solid compound **2**. Yield: 68%, M.p: 255^0 C **,** IR: (KBr / cm^{-1}): 3406 cm^1 (=NH), 3329,3215 cm^1, 1624 cm^1 (C=N). ^{1}HNMR: (60 MHz, DMSO): δ2.4(s 3H Ar-CH_3), δ 2.5 (s 3H Ar-CH_3), δ 2.6 (s 1H CH), δ 4.4 (broad 2H NH_2), δ 6.8 (d 1H Ar-H), δ 7.6 (d 1H Ar-H), δ 8.2 (s 1H =NH). Mass : (m/z): = 284 (30%). M.F: $C_{13}H_{12}N_6S$, Found 284, Calculate (%): C 54.91, H 4.25, N 29.56, and S11.28.

Synthesis of 3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-methylthio- pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido [2,3-*b*][1,3] benzothiazole (3) : A mixture of 3-amino- 4-imino- 6, 9-dimethyl- pyrazolo-[3, 4-*e*] pyrimido [2, 3-*b*] [1, 3] benzothiazole **2 (** 0.01mole) and bis-methylthio methylene malononitrile (0.01mole) was refluxed in the presence of 25 ml of DMF and a pinch of anhydrous potassium carbonate for 6-7 hours. The reaction mixture was monitored by TLC, further it was cooled at room temperature and poured in ice cold water, the separated solid product was filtered washed with water and recrystallized from DMF -ethanol to yield compound **3**. Yield: 80 %, M.p: 310 ^{0} C, IR: (KBr / cm^{-1}): 3311, 3209 cm^1 (N-H two =NH stretch), 2910, 2860 (C-H), 2208 (-CN), 1629

(C=N).cm[1]. [1]H-NMR: (DMSO-d[6]): δ 2.3 (s 3H Ar-CH_3), δ 2.4 (s 3H Ar-CH_3), δ 2.6 (s 3H -SCH_3) δ 3.5 (s 1H CH), δ 7.2 (d 1H Ar-H), δ 7.6 (d 1H Ar-H), δ 8.2 (s 1H =NH). Mass : (m/z:): 429 (M+Na) (M‡ 10 %), [13]CNMR(DMSO-d[6]): δ 18.5 , 20 ,39 ,78, 121 ,124, 125 ,126 ,127,129 ,130 , 131 , 136, 138 ,151, 165. M.F: $C_{18}H_{14}N_8S_2$. Found 406 + Na. Calculuted(%) : C 53.19, H 3.47, N 27.57, S 15.78.

General method for the synthesis of compounds (4-7):

Compound **3** (0.01 mole) was refluxed with selected nucleophiles like substituted phenols, Anilines, Heteryl amines and active methylene compounds independently in presence of a pinch of anhydrous K_2CO_3 in DMF for 5-6 hours. The reaction mixtures were monitored by TLC cooled and kept for overnight. Reaction mixture was poured in ice cold water, solid get separated and recrystlized from ethanol to yield compound **4-7.**

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-(*p*-chloro phenoxy)-pyrimido[2,3 *b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (4-a): Yield: 51 %, M.p: 300[0]C, IR: (KBr / cm^{-1}): 3300 cm^{-1} broad (=NH, -NH), 2920 cm^{-1}(C-H); 2196 cm^{-1}(C≡N); 1543 cm^{-1} (C=N), 1259 cm^{-1} and 1070 cm^{-1} (C-O-C) asymmetric & symmetric stretching**, Mass :** (m/z:): 488 (M+2, 10 %),486 (M‡ 30 %), M. F: $C_{23}H_{15}N_8OSCl$, Found: 486 , Calculated (%) : C 56.73, H 3.10, N 23.01, S 6.59.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-(*p*-nitro phenoxy)-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (4-b) : Yield: 60 %, M.p:280 [0]C , IR: (KBr / cm^{-1}) :3398 cm^{-1} broad (NH); 2920 cm^{-1}(C-H); 2198 cm^{-1}(C≡N); 1550 cm^{-1} (C=N), 1454 cm^{-1} , 1380 cm^{-1} (-NO_2), 1026 cm^{-1} ,1159 cm^{-1}(C-O-C **),** [1]H-NMR: (60 MHz,DMSO): δ 2.21 (s 3H Ar-CH_3), δ 2.3 (s 3H Ar-CH_3), δ 3.58 (s 1H CH), δ 6.8-7.4 (m 6H Ar-H), δ 7.6 (s 1H =NH) δ 7.98(s 1H =NH), Mass : (m/z:):496 (M-1, 10 %), M. F: $C_{23}H_{15}N_9O_3S$, Found : 497, Calculated (%): C 55.53, H 3.04, , N 25.34, S 6.45.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-(*o*-chloro phenoxy)-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (4c): Yield: 45 %, M.p: 280[0]C, IR: (KBr / cm^{-1}): 3446, 3333 cm^{-1}(=NH); 2918 cm^{-1}(C-H); 2196cm^{-1}(C≡N); 1600 cm^{-1} (C=N), 1257 cm^{-1} and 1024cm^{-1} (C-O-C) stretching , M.F: $C_{23}H_{15}N_8OSCl$, Found: 486, Calculated (%): C 56.73, H 3.10, N 23.01, S 6.59.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2- *p*-(toludino)-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (5-a): Yield: 62 %, M.p: 320[0]C**,** IR: (KBr / cm^{-1}): 3335 (=NH), 3228 (N-H broad), 2926 (C-H), 2210 (-CN), 1645 (C=N).cm[1], [1]H-NMR: (60 MHz,$CDCl_3$): δ 2.25 (s 3H Ar-CH_3), δ 2.4 (s 3H Ar-CH_3), δ 2.6 (s 3H Ar CH_3), δ 3.5 (s 1H -CH), δ 4.6 (broad, 1H -NH), δ 6.8-7.4 (m 6H Ar-H), δ 8.4 (broad 2H =NH), Mass: (m/z:): 465 (M‡ 10 %), M.F: $C_{24}H_{19}N_9S$, Found: 465, Calculated (%) : C 61.92, H 4.11, N 27.08, S 6.89.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2- *p*-(anisidino)-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (5-b): Yield: 58 %, M. p: 325[0]C**,** IR: (KBr / cm^{-1}): 3408(=NH), 3315 cm^{-1} (NH); 2916, 2856 cm^{-1}(C-H); 2200cm^{-1}(C≡N) 1612 cm^{-1} (C=N), 1112, 1024 cm^{-1} (C-O-C) stretching, Mass : (m/z): 481, (M‡ 25 %), M.F: $C_{24}H_{19}N_9OS$, Found : 481, Calculated (%): C 58.86, H 3.98, N 26.18, S 6.66.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2- (*p*-chloro aniline) -pyrimido[2,3-*b*] pyrazolo[3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (5-c) : Yield: 50%, M.P: 295[0]C, IR: (KBr / cm^{-1}): 3410(=NH) ,3313 cm^{-1}(NH); 2912cm^{-1}(C-H); 2189 cm^{-1}(C≡N); 1626 cm^{-1} (C=N; Mass : (m/z, RA%): 487(M+2; 15%), 485(M^+ ,45%), M.F: $C_{23}H_{16}N_9S$ Cl, Found : 485, Calculated (%) : C 56.85, H 3.32, N 25.94, S 6.60.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-pyrrolidino-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (6-a): Yield: 67%, M.p: 320^{0}C, : IR: (KBr / cm^{-1}): 3408,3309 cm^{-1}(=NH); 2912 cm^{-1}(C-H); 2214 cm^{-1}(C≡N); 1629cm^{-1} (C=N), Mass : (m/z): 430 (M+1, 25%). M.F: $C_{21}H_{19}N_9S$, Found : 429, Calculated (%) C 58.73, H 4.46, N 29.35, S 7.47.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-piperidino-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (6-b): Yield: 50%, M.p: 270^{0}C, IR: (KBr / cm^{-1}): 3502 cm^{-1}(broad=NH); 2924, 2854 cm^{-1}(C-H); 2202cm^{-1}(C≡N); 1639 cm^{-1} (C=N), Mass (m/z,): 443(M‡25%), M.F: $C_{22}H_{21}N_9S$, Found: : 443, Calculated (%): C 59.58, H 4.77, N 28.42, S 7.23.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-morpholino-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (6-c) : Yield: 57%, M.p: 295^{0}C, IR: (KBr / cm^{-1}): 3414, 3327, cm^{-1}(NH); 2912, 2860 cm^{-1}(C-H); 2185cm^{-1}(C≡N); 1631 cm^{-1} (C=N), 1261, 1024 cm^{-1}(C-O-C); ^{1}HNMR (DMSO): δ 1.8 (s 3H Ar-CH_3), δ 2.0 (s 3H Ar-CH_3), δ 2.4 (t 2H -NCH_2), δ 2.85 (t 2H OCH_2), δ 3.4(s1H -CH), δ 7.6 (d 1H Ar-H), δ 7.9 (d 1H Ar-H) δ 8.8 (s 2H =NH) , M.F: $C_{21}H_{19}N_9O$, Found: 445, Calculated (%): C 56.62, H 4.30, N 28.30, S 7.20.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2--(*α*-ethyl cyano acetyl)-pyrimido[2,3-*b*] pyrazolo[3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (7-a): Yield: 55%, M.p: 310^{0}C, IR: (KBr / cm^{-1}): 3308, 3211 cm^{-1}(NH); 2966 cm^{-1}(C-H); 2216 cm^{-1}(C≡N); 1645 cm^{-1} (C=O), Mass: (m/z): 471 (M‡ 10 %), M.F: $C_{22}H_{17}N_9O_2S$, Found : 471, Calculated (%): C 56.04, H 3.63, N 26.74, S 6.80.

3-Cyano- 9, 12-dimethyl -4, 14-diimino- 2-(-acetyl acetonyl) -pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (7-b): Yield: 55 %,M.p: 320^{0}C, IR: (KBr / cm^{-1}): 3280 cm^{-1}(=NH); 2980 cm^{-1}(C-H); 2210cm^{-1}(C≡N); 1712cm^{-1} (C=O). Mass (m/z): 458 (M‡ 40 %), M F: $C_{22}H_{18}N_8O_2S$, Found: 458, Calculated (%): C 57.63, H 3.96, N 24.44, S 6.99.

3-Cyano- 9, 12-dimethyl- 4,14-diimino- 2--(*α*-Ethyl acetoacetyl)-pyrimido[2,3-*b*] pyrazolo [3,4-*e*] pyrimido[2,3-*b*][1,3] benzothiazole (7-c): Yield: 60%,M.p: 300 ^{0}C, IR: (KBr / cm^{-1}): 3446,3331, cm^{-1}(=NH); 2854 cm^{-1}(C-H); 2114cm^{-1}(C≡N); 1693 cm^{-1} (C=O) ester, 1259 cm^{-1} (C-O-C) stretch, ^{1}HNMR (DMSO): δ 1.2 (t 3H CH_3) δ 2.1 (s 3H Ar-CH_3), δ 2.3 (s 3H Ar-CH_3), δ 2.5 (q 2H CH_2), δ 3.2 (s 3H $COCH_3$), δ 4.2 (broad 2H, 2CH) δ 7.3 (d 1H Ar-H), δ 7.7 (d 1H Ar-H) δ 7,9 (broad 2H =NH). **Mass**: (m/z): 490 (M+2, 05%). M.F: $C_{23}H_{20}N_8O_3S$, Found: 488, Calculated (%): C 56.55, H 4.13, N 22.94, S 6.56

2.2. Anticancer Activity

All the synthesized compounds were screened for their in-vitro anticancer activity at National Cancer Institute of Maryland. USA. Compound 2 was selected by NCI for in-vitro anticancer activity by DTP processes. The in-vitro anticancer activities were screened against 60 human cell lines at a single dose of 10 mg against different types of cancer like lung, Renal, Leukaemia, Prostate Breast cancer, CNS, Colon and Melanoma cancer (Table 1). Results of activity were reported in mean graph. The mean value for human 60 cell line is 104.08; only selected cell lines are shown in activity table. Negative values project towards the right of the vertical line and it represents cellular sensitivities to the test agent that expected the mean. Positive value project towards the left of the vertical line it represents cell lines are sensitivities to the test agent that are less than the average values. The compounds with cell lines appearing on the negative side in the mean graph exhibit growth of inhibition (GI) of cancer cell to that of particular cancer.

In present work, we reported compounds 2-7 along with its derivatives. Only compound 2 exhibited the anticancer activity against human 60 cell lines. Compound 2 has amino functionality at 3-position in fused pyrazolo pyrimido benzothiazole. The anticancer activity of compound 2 exhibited against different types of selected cancer lines: Leukemia (RPMI-8226), Lung (NCI-H522), Colon (HCT-116), CNS (SNB-75), Melanoma (SK-MEL-2), Ovrian (OVCAR-8), Renal (UO-31), Prostate (PC-3), Breast (T-47D). Compound 2 exhibited maximum anticancer activity

against Prostate (PC-3) and Renal (UO-31) cancer, but rest of the compounds were not selected by DTP process to screening the anticancer activity, due to five rings in their structure.

Table 1: Anticancer activity

Cancer Type	Selected Cell Line↓	Compound – 2 NSC Code-765947
Leukemia	RPMI-8226	-10.93
Lung	NCI-H522	-16.69
Colon	HCT-116	-2.86
CNS	SNB-75	-15.61
Melanoma	SK-MEL-2	-7.97
Ovrian	OVCAR-8	-6.13
Reanal	UO-31	-18.32
Prostate	PC-3	-18.39
Breast	T-47D	-10.01
Mean from Graph		**104.08**

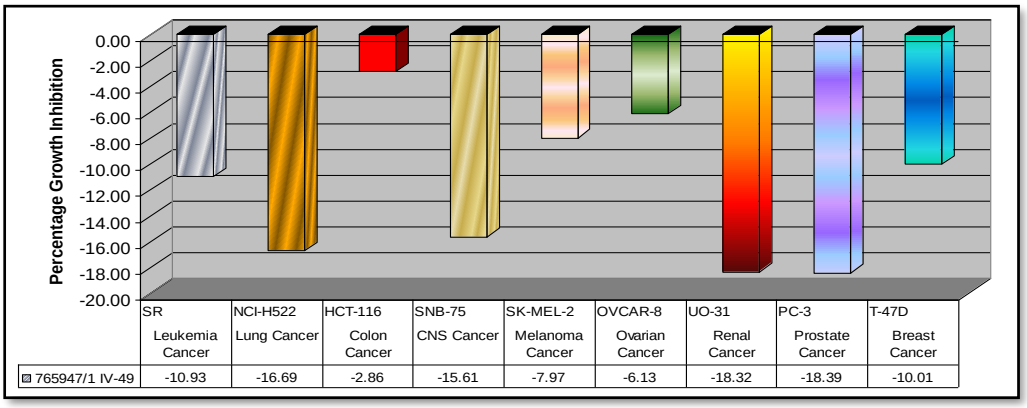

Figure 3: Graphical presentation of anticancer activity of compound 2. (NSC Code-765947)

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