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Design, Synthesis and Anticancer Screening of Novel Benzothienopyarno Fused System: A Step in Discovering a Promising Anticancer Motif

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ABSTRACT

The diversity and prevalence of cancer disease is a chocking fact. Accordingly, the need for new, effective and safe anticancer agents is now mandatory. Fourteen novel tricyclic and tetracyclic benzothienopyrano and benzothienopyranopyrimidine derivatives 2, 3a-d, 4a-c, 5-8 and 9a,b were synthesized. Compounds 3b and 3d have been chosen by the NCI-USA for the 60-cell lines One-Dose screening and Five-dose assay. Both 3b and 3d exhibited very potent cytotoxic effects ($GI_{50} \leq 2.0$ μM) against almost all cell lines. 3b showed TGI values ranging from 0.463–4.75 μM while 3d showed values of 0.571–6.28 μM. The best sensitivity patterns for both 3b and 3d were against MDA-MB-435 subpanel (melanoma cancer) while they possessed a null sensitivity towards all leukemic cancer subpanels.

Keywords: Cancer, Cytotoxic effects, Cardiac diseases, Benzothienopyarno

INTRODUCTION

Cancer is a quite broad medical terminology including more than 200 different types of diseases. In reality, it is the second leading cause of death after cardiac diseases and expected to surpass this order by next few years [1]. Cancer types can be categorized into carcinoma, sarcoma, lymphoma and leukemia based on the cells from which they arise [2]. Such diseases are all possessing common features; the immortality of the cell life cycle and metastasis. In fact, the immortality of malignant cells can be considered the origin of all complications. Normal healthy cells are governed by a strict biochemical cell division mechanism. Such a mechanism leads to a well-known life span period specific for each type of cells. In reality, this mechanism is a balance between two scientific terms; cell division and apoptosis (programmed cell death) [3]. Accordingly, in cancer this controlled system is corrupted leading to the immortal, mal-functioning mass formation. The complexity of the disease and the spreadability of cancer are making it quite mandatory to discover novel chemotherapeutic agents [1]. Cancer treatment can be summarized in two main strategies. Either directly destroying the cancer tissue or indirectly killing them by depriving them of blood supply or their nutrition (Inhibiting Angiogenesis) [4]. Meanwhile, the difficulty in cancer treatment is that the aim is destroying the mal-formed cells without touching or affecting normal healthy ones. A mission which is considered impossible until now. The reason for such a difficulty is the slight differences between the tumor cell and its ancestor healthy ones. The discovery of most of cancer chemotherapeutic agents have been mostly by serendipity as an initial step. Following such a discovery and under the stress of need, the development and improvement of new members or analogues proceed after. Nitrogen mustard was discovered during the Second World War upon investigations of the lymphoid and myeloid suppression observed in soldiers accidentally exposed to the nitrogen mustard gas [5]. Accordingly, ellipticine has been first discovered as an antitumor natural alkaloid in 1959 [6,7]. Based on its tetracyclic, aromatic, planar structure, many analogues such as *retelliptine* [8,9], *pazelliptine* [10] and *datelliptium* [11] have been developed in order to achieve the virtual balance between the best therapeutic activity and the least side effects exhibited. In continuation to this aim and taking in site the tetracyclic frame of ellipcitine, we designed benzothienopyarnopyrimidine nucleus (Figure 1). The design mainly depended on maintaining the tetracyclic backbone structure by using bioisosteres rings. Rings B (pyrollo), C (benzo) and D (pyrido) in ellipticine were replaced by rings E (thieno), F (pyrano) and G (pyrimido), respectively. Accordingly, the newly synthesized compounds were submitted to NCI, USA and the selected members were screened against the antitumor activity. Compounds 3b and 3d has been chosen by the NCI-USA for the 60-cell lines One-Dose screening and Five-dose assay.

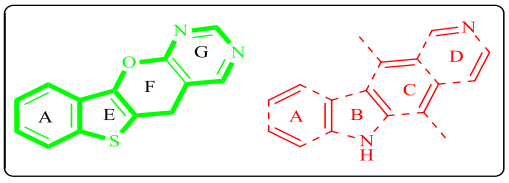


Figure 1: Benzothienopyarnopyrimidine main frame (in solid green) comparative design for ellipticine (in dashed red)

MATERIALS AND METHODS

Chemistry

Melting points were obtained on Griffin apparatus and the values given were uncorrected. IR spectra were recorded on a Shimadzu 435 spectrometer, using KBr discs. ^{1}HNMR spectra were recorded on a Mercury-300 MHz spectrometer using TMS as an internal standard. Mass spectra were recorded on a JEON JMS-AX 500 mass spectrometer. Element analysis for C, H and N were within 0.4% of the theoretical values and were performed at the regional center for Mycology and Biotechnology, Al-Azhar University. Progress of the reactions was monitored by TLC using precoated aluminum sheets silica gel MERCK 60 F 254 and was visualized by UV lamp. All chemicals were purchased from Sigma-Aldrich Company.

(*Z*)-7-chloro-2-(4-chlorobenzylidene)-1-benzothiophen-3(2*H*)-one (1)

Compound 1 can be prepared from the reaction of 7-chloro[1]benzothiophen-3(2*H*)-one with *s*-(*o*-chlorophenyl)thioglycolic acid according to the reported procedure [12].

2-amino-6-chloro-4-(4-chlorophenyl)-4*H*-benzo [4,5]thieno[3,2-*b*]pyran-3-carbonitrile (2)

The 4-chlorobenzylidenebenzothieophene 2(4 mmol) and malononitrile (5 mmol) were dissolved in ethanol (40 ml) in a 100 ml flask equipped with a condenser. Then, piperidine (1 ml) was added, and the reaction mixture was heated under reflux for 5 h. The organic phase was evaporated under reduced pressure and the residue was recrystallized from ethanol. Yield: 80%; mp 240°C; IR cm^{-1}: 3475, 3329 (NH_2), 3050 (CH aromatic), 2950 (CH aliphatic), 2196 (C≡N); ^{1}HNMR (DMSO-d_6) δ: 5.19 (s, 1H, C_4H), 7.28 (s, 2H, NH_2, D_2O exchangeable), 7.38–7.75 (3m, 7H, aromatic H); MS: m/z (% abundance) 372.2 (M^+) (27.12); Anal. Calcd. for $C_{18}H_{10}Cl_2N_2OS$: C, 57.92; H, 2.70; N, 7.51. Found: C, 58.14; H, 2.74; N, 7.64.

General procedure for preparation of series 3a-d

A solution of compound 2 (0.1 mol) in glacial acetic acid (15 ml) with the appropriate aldehyde (0.1 mol) was mixed. The reaction mixture was refluxed for 5 h. The solid formed was filtered, air dried and recrystallized from acetic acid.

7-chloro-5-(4-chlorophenyl)-2-phenyl-3*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidin-4(5*H*)-one (3a)

Yield: 80%; mp 190°C; IR cm^{-1}: 3080 (CH aromatic), 2950, 2925 (CH aliphatic), 1687 (C=O); ^{1}HNMR ($CDCl_3$) δ: 4.72 (s, 1H, C5H), 7.27–7.95 (4m, 12H, aromatic H); MS: m/z (% abundance) 476.02 (M^+) (6.34); Anal. Calcd. for $C_{25}H_{14}Cl_2N_2O_2S$: C, 62.90; H, 2.96; N, 5.87. Found: C, 63.13; H, 2.94; N, 5.99.

7-chloro-2,5-*bis*(4-chlorophenyl)-3*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidin-4(5*H*)-one (3b)

mp 195°C; IR cm^{-1}: 3090, 3080 (CH aromatic), 2918, 2848 (CH aliphatic), 1683 (C=O); ^{1}HNMR ($CDCl_3$) δ: 4.71 (s, 1H, C5H), 7.27–7.94 (4m, 11H, aromatic H); MS: m/z (% abundance) 509.98 (M^+) (0.13); Anal. Calcd. for $C_{25}H_{13}Cl_3N_2O_2S$: C, 58.67; H, 2.56; N, 5.47. Found: C, 58.70; H, 2.52; N, 5.51.

7-chloro-5-(4-chlorophenyl)-2-(4-fluorophenyl)-3*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidin-4(5H)-one(3c)

mp 204°C; IR cm^{-1}: 3080 (CH aromatic), 2910-2850 (CH aliphatic), 1687 (C=O); ^{1}HNMR ($CDCl_3$) δ: 4.71 (s, 1H, C5H), 7.27–7.94 (4m, 11H, aromatic H); MS: m/z (% abundance) 494.01 (M^+) (0.13); Anal. Calcd. for $C_{25}H_{13}Cl_2FN_2O_2S$: C, 60.62; H, 2.65; N, 5.66. Found: C, 60.65; H, 2.70; N, 5.64.

7-chloro-5-(4-chlorophenyl)-2-(4-methoxyphenyl)-3*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidin-4(5*H*)-one (3d)

mp 182°C; IR cm^{-1}: 3068, 3051 (CH aromatic), 2920, 2849 (CH aliphatic), 1681 (C=O); ^{1}HNMR ($CDCl_3$) δ: 3.90 (s, 3H, OCH_3), 4.70 (s, 1H, C5H), 7.02–7.99 (4m, 11H, aromatic H); ^{13}CNMR (75 MHz, $CDCl_3$) δ: 22.21, 22.67, 24.34, 25.59, 31.19, 124.26, 132.80, 133.02, 139.79, 154.33; MS: m/z (% abundance) 506.03 (M^+) (0.13); Anal. Calcd. for $C_{26}H_{16}Cl_2N_2O_3S$ C, 61.55; H, 3.18; N, 5.52. Found: C, 61.49; H, 3.20; N, 5.50.

General procedure for preparation of series 4a-c

A solution of compound 2 (0.1 mol) in tetrahydrofuran (THF) (25 ml) with the appropriate aldehyde (0.1 mol) was mixed together. The reaction mixture was refluxed for 4 h. The solid formed was filtered, air dried and recrystallized from acetone.

(*E*)-2-(benzylideneamino)-6-chloro-4-(4-chlorophenyl)-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile (4a)

Yield: 43%; mp 100°C; IR cm^{-1}: 3062 (CH aromatic), 2950 (CH aliphatic), 2196 (C≡N), 1660 (C=N); ^{1}HNMR ($CDCl_3$) δ: 4.80, 4.98 (s, 1H, C4H), 7.23–7.63 (m, 12H, H aromatic), 7.65-7.66 (m, 1H, CH=N); MS: m/z (% abundance) 461.00 (M^+) (2.89); Anal. Calcd. for $C_{25}H_{14}Cl_2N_2OS$: C, 65.08; H, 3.06; N, 6.07. Found: C, 65.10; H, 3.09; N, 6.04.

(*E*)-6-chloro-2-((4-chlorobenzylidene)amino)-4-(4-chlorophenyl)-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile (4b)

Yield: 50%; mp 199°C; IR cm^{-1}: 3050 (CH aromatic), 2980 (CH aliphatic), 2196 (C≡N), 1658 (C=N); ^{1}HNMR ($CDCl_3$) δ: 4.81, 4.98 (2s, 1H, C4H), 7.23–7.40 (m, 11H, H aromatic), 7.64-7.67 (m, 1H, CH=N); MS: m/z (% abundance) 494.00 (M^+) (0.33); Anal. Calcd. for $C_{25}H_{13}Cl_3N_2OS$: C, 60.56; H, 2.64; N, 5.65. Found: C, 60.82; H, 2.68; N, 5.72.

(*E*)-6-chloro-4-(4-chlorophenyl)-2-((4-fluorobenzylidene)amino)-4*H*-benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile (4c)

Yield: 50%; mp 241°C; IR cm^{-1}: 3010 (CH aromatic), 2924 (CH aliphatic), 2193 (C≡N), 1658 (C=N); ^{1}HNMR ($CDCl_3$) δ: 4.81, 4.99 (2s, 1H, C4H), 7.23–7.40 (m, 11H, H aromatic), 7.64-7.70 (m, 1H, CH=N); MS: m/z (% abundance) 479.00 (M^+) (31.58); Anal. Calcd. for $C_{25}H_{13}Cl_2FN_2OS$: C, 62.64; H, 2.73; N, 5.84. Found: C, 62.70; H, 2.76; N, 5.80.

7-chloro-5-(4-chlorophenyl)-2-methyl-3*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidin-4(5*H*)-one (5)

Compound 2 (0.1 mol) was dispersed in acetic anhydride (10 ml). The reaction was heated under reflux for 10 h. The reaction mixture was then cooled and poured on ice. The separated solid was filtered, washed with water, air dried and recrystallized from acetonitrile to yield 5. Yield: 58%; mp>300°C; IR cm^{-1}: 3421 (NH), 2926, 3050 (CH aromatic), 2999, 2924 (CH aliphatic), 1651 (C=O); 1602 (C=N); ^{1}HNMR (DMSO-d_6) δ: 2.33 (s, 3H, CH_3), 5.43 (s, 1H, C5H), 7.33–7.83 (3m, 8H, H aromatic, 1H, NH); MS: m/z (% abundance) 414.00 (M^+) (20.88); Anal. Calcd. for $C_{20}H_{12}Cl_2N_2O_2S$: C, 57.84; H, 2.91; N, 6.75. Found: C, 58.09; H, 2.97; N, 6.93.

4,7-dichloro-5-(4-chlorophenyl)-2-methyl-5*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidine (6)

In an ice-jacketed round-bottomed flask, compound 5 (0.1 mmol) was dispersed in phosphorous oxychloride (4 ml). Dry pyridine (0.4 ml) was then added drop wise with continuous stirring and under efficient cooling. The reaction was heated under reflux for 2 h. The reaction mixture was then cooled and poured on ice. The separated solid was filtered, washed with water, air dried and recrystallized from methanol. Yield: 60%; mp >212°C; IR cm^{-1}: 3095 (CH aromatic), 2950, 2900 (CH–aliphatic), 1609 (C=N); ^{1}HNMR (DMSO-d_6) δ: 2.51 (s, 3H, CH_3), 6.01 (s, 1H, C5H), 7.40–8.01 (3m,-7H, H aromatic); MS: m/z (% abundance) 432.00 (M^+) (13.78); Anal. Calcd. for $C_{20}H_{11}Cl_3N_2OS$: C, 55.38; H, 2.56; N, 6.46. Found: C, 55.35; H, 2.60; N, 6.40.

7-chloro-5-(4-chlorophenyl)-2-methyl-4-(4-methylpiperazin-1-yl)-5*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidine (7)

Compound 6 (0.1 mol) was dissolved in absolute ethanol (10 ml). *N*-methylpiperazine (0.1 mol) was then added and the reaction was heated under reflux for 2 h. The separated solid after cooling was filtered, air dried and recrystallized from ethanol to yield **7**. Yield: 55%; mp 84°C; IR cm^{-1}: 3057, 3022 (CH aromatic), 2976, 2850 (CH aliphatic), 1645 (C=N); ^{1}HNMR (DMSO-d_6) δ:2.95 (s, 3H, CH_3), 2.95 (s, 4H, $2CH_2$), 3.07 (s, 4H, $2CH_2$), 3.09 (s, 3H, NCH_3), 4.24 (s, 1H, C5H), 6.77–7.23 (3m, 7H, H aromatic); MS: m/z (% abundance) 496.00(M^+) (0.21); Anal. Calcd. for-$C_{25}H_{22}Cl_2N_4OS$: C, 60.36; H, 4.46; N, 11.26. Found: C, 60.40; H, 4.43; N, 11.29

7-chloro-5-(4-chlorophenyl)-5H-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-d]pyrimidin-4-amine (8)

Compound 2 (2.7 mmol) was mixed with formamide (10 ml). The reaction was heated under reflux for 5 h. The separated solid after cooling was filtered, air dried and recrystallized from methanol. Yield: 65%; mp>195°C; IR cm^{-1}: 3410, 3211 (NH_2), 3064, 3034 (CH aromatic), 2920-(CH aliphatic), 1647 (C=N); ^{1}HNMR (DMSO-d_6) δ: 4.17 (s, 1H, C5H), 7.31–8.40 (2m, 9H, H aromatic, NH_2); MS: m/z (% abundance)-399.00 (M^+) (3.10); Anal. Calcd. for $C_{19}H_{11}Cl_2N_3OS$: C, 57.01; H, 2.77; N, 10.50. Found: C, 57.18; H, 2.73; N, 10.67.

General procedure for preparation of 9a,b

Compound 8 (0.1 mol) was dissolved in dry dioxane (10 ml). The appropriate aldehyde was added to the solution and the reaction was heated under reflux for 6 h. The separated solid was filtered, washed with water, air dried and recrystallized from acetonitrile to afford the appropriate 9a,b.

(*Z*)-7-chloro-*N*-(4-chlorobenzylidene)-5-(4-chlorophenyl)-5*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidin-4-amine (9a)

Yield: 68%; mp>300°C; IR cm^{-1}: 3066, 3034 (CH aromatic), 2974, 2908 (CH aliphatic), 1658 (C=N);^{1}HNMR ($CDCl_3$) δ: 4.14–4.24 (1m, 1H, C5H), 7.08–8.13 (2m, 11H, H aromatic), 8.28–8.49 (m, 1H, N=CH), 8.52–8.53 (m, 1H, C2H); MS: m/z (% abundance) 521.00 (M^+) (0.27); Anal. Calcd. for $C_{26}H_{14}Cl_3N_3OS$: C, 59.73; H, 2.70; N, 8.04. Found: C, 59.80; H, 2.76; N, 8.08.

(*Z*)-7-chloro-5-(4-chlorophenyl)-*N*-(4-methoxybenzylidene)-5*H*-benzo[4',5']thieno[2',3':5,6]pyrano[2,3-*d*]pyrimidin-4-amine (9b)

Yield: 75%; mp 75°C; IR cm^{-1}:-3067, 3032 (CH aromatic), 2972, 2910, 2856 (CH aliphatic), 1640 (C=N); ^{1}HNMR (DMSO-d_6) δ: 3.34 (s, 3H, OCH_3), 4.14, 4.28 (2s, 1H, C5H), 7.34–7.65 (2m, 11H, H aromatic), 7.93–8.00 (m, 1H, N=CH), 8.37–8.41 (m, 1H, C2H); MS: m/z (% abundance) 517.00 (M^+) (0.61); Anal. Calcd. for $C_{27}H_{17}Cl_2N_3O_2S$: C, 62.55; H, 3.31; N, 8.11. Found: C, 62.50; H, 3.27; N, 8.14.

***In vitro* anticancer screening**

NCI 60 cell one-dose screen

Anticancer activity screening of the newly synthesized compounds was measured *in vitro* utilizing 60 different human tumor cell lines provided by US National Cancer Institute according to previously reported standard procedure [13-15] as follows:

Cells are inoculated into 96 well microtitre plates in 100 µL. After cell inoculation, the microtiter plates are incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line are fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addiction (Tz). Experimental drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 µg/ml gentamicin. Additional four, 10-fold or ½ log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100 µL of these different drug dilutions are added to the appropriate microtiter wells already containing 100 µL of medium, resulting in the required final drug concentrations. Following drug addition, the plates are incubated for an additional 48 h at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed *in situ* by the gentle addition of 50 µL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µL) at 0.4% (w/v) in 1% acetic acid is added to each well, and plates are incubated for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm.

For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 µL of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

$$[(Ti-Tz)/(C-Tz)] \times 100 \text{ for concentrations for which } Ti \geq Tz$$

$$[(Ti-Tz)/Tz] \times 100 \text{ for concentrations for which } Ti<Tz$$

NCI 60 cell five-dose screen

Compounds which exhibit significant growth inhibition in the One-Dose Screen are evaluated against the 60 cell panel at five concentration levels.

The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated into 96 well microtiter plates in 100 µL at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs. After 24 h, two plates of each cell line are fixed *in situ* with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz). Experimental drugs are solubilized in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 µg/ml gentamicin. Additional four, 10-fold or ½ log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100 µl of these different drug dilutions are added to the appropriate microtiter wells already containing 100 µl of medium, resulting in the required final drug concentrations. Following drug addition, the plates are incubated for an additional 48 h at 37°C, 5% CO_2, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed *in situ* by the gentle addition of 50 µl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4 % (w/v) in 1% acetic acid is added to each well, and plates are incubated for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 µl of 80 % TCA (final concentration, 16 % TCA). Using the seven absorbance measurements [time zero, (Tz), control growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations levels. Percentage growth inhibition is calculated as:

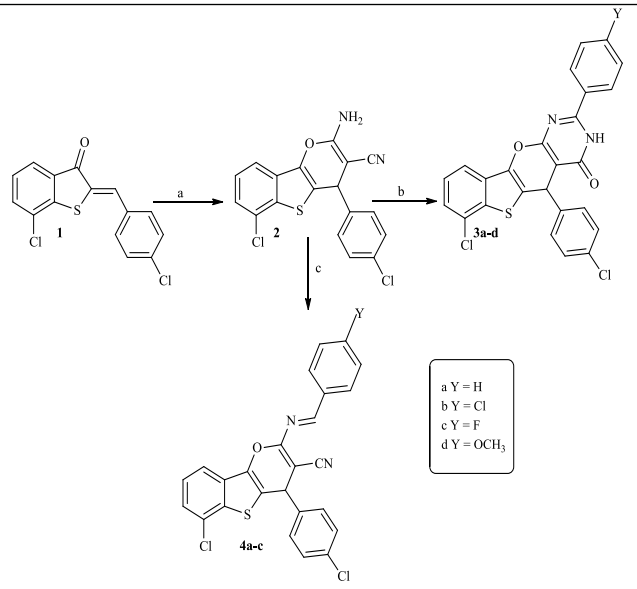
$$[(Ti-Tz)/(C-Tz)] \times 100 \text{ for concentrations for which } Ti \geq Tz$$

$$[(Ti-Tz)/Tz] \times 100 \text{ for concentrations for which } Ti<Tz$$

Three dose response parameters are calculated for each experimental agent. Growth inhibition of 50% (GI50) is calculated from [(Ti-Tz)/(C-Tz)] × 100=50, which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) is calculated from Ti=Tz. The LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from [(Ti-Tz)/Tz] × 100=-50. Values are calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

DISCUSSION

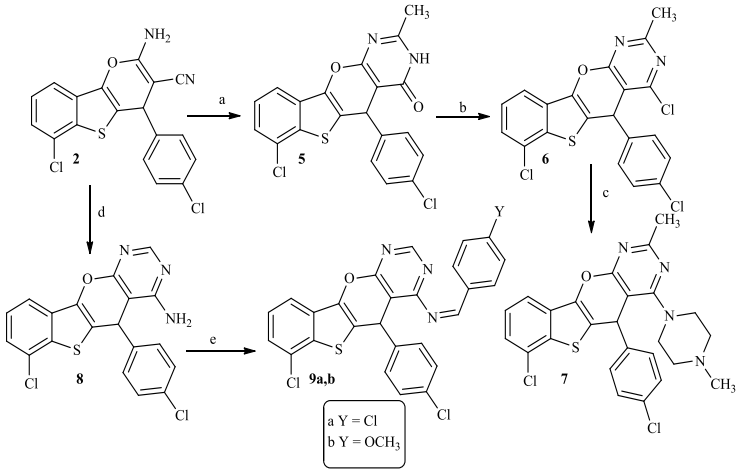
This piece of work is sketched out in two schemes. Scheme 1 outlines the synthesis of compounds 3a-d and 4a-c, while the preparation of compounds 5-9a,b are illustrated in Scheme 2. Accordingly, 7-chloro-2-(4-chlorobenzylidene)-1-benzothiophen-3-one (thioaurone) 1 was prepared from the previously reported precursors [13-16]. It is worth mentioning that thioaurones are quite well studied structures from the synthetic chemistry point of view [17,18]. The 2-amino-3-cyanopyrane ring cyclization has been previously reported [19]. In accordance, the reaction of 1 with malononitrile in the presence of piperidine afforded the benzo[4,5]thieno[3,2-*b*]pyran-3-carbonitrile derivative 2. Compound 2 is considered as the corner stone for the preparation of the rest of tricyclic and tetracyclic derivatives 3a-d-9a,b. In Scheme 1, the reaction of the appropriate aromatic aldehydes with the 2-aminobenzothienopyrano-3-carbonitrile derivative 2 in acetic acid afforded series 3. The formation of the tetracyclic series 3a-d was confirmed by different spectroscopic methods [20]. The disappearance of the very sharp cyano peak in the IR. Spectrum at 2196 cm^{-1} and the appearance of the cyclic amidic carbonyl peaks at 1687 and 1683 cm^{-1} was a preliminary evidence for the success of cyclization. The ^{1}HNMR and MS spectra confirmed the proposed structure. On the other hand, upon only switching the solvent to dry tetrahydrofuran (THF) and refluxing the reactants for only 4 h the corresponding imines 4a-c were formed. Once more, the cyano peak in the IR. Spectra were a leading mark to the success of the reaction. The appearance of the extra aromatic protons at the range of δ 7.23-7.63 and the multiple peak corresponding to 1 proton for the imine (CH=N) at the range of δ 7.65-7.66 confirmed the imine bond formation.



Reagents and conditions :a) $CH_2(CN)_2$, reflux 5 h, C_2H_5OH, Piperidine; b) ArCHO, reflux 5 h, acetic acid; c) ArCHO, refux 4 h, THF.

Scheme 1: Preparation of compounds 2-4a-c

In Scheme 2, refluxing of the 2-amino-4-(4-chlorophenyl)benzothienopyran-3-nitrile derivative 2 in acetic anhydride afforded the 2-methylpyrimid-4-one tetracyclic structure 5 in a similar manner procedure [21]. Refluxing of compound 5 in phosphorous oxychloride for 2 h afforded the corresponding chloro derivative 6. The reaction of *N*-methylpiperazine with 2 in absolute ethanol yielded 7. On the other hand, refluxing of 2 with formamide for 5 h afforded the 4-aminopyrimidino tetracyclic derivative 8. Condensation of 8 with the appropriate aromatic aldehydes (4-chlorobenzaldehyde and 4-methoxybenzaldehyde) in dry dioxane yielded compounds 9a and 9b respectively.



Reagents and conditions : a) $(CH_3CO)_2O$, reflux 10 h ; b) $POCl_3$, pyridine, reflux 2 h; c) *N*-Methylpiperazine, C_2H_5OH, reflux 2 h; d) $HCONH_2$, reflux 5 h ; e) ArCHO, dioxane, reflux 6 h.

Scheme 2: Preparation of compounds 5-9a,b

Anticancer screening results

The National Cancer Institute (NCI) at the NIH, Bethesda, Maryland, USA has chosen two compounds out of the thirteen newly synthesized compounds for anticancer evaluation under the drug discovery program of the NCI. The denoted compounds were 3b and 3d. The chosen compounds were evaluated at a single dose (10^{-5} M) utilizing 60 different human tumor cell lines representing nine main different types of cancers: leukemia, lung, colon, melanoma, renal, CNS, breast, prostate and ovarian cancer panels. The results obtained from the single dose test of the selected compounds are shown in Tables 1-4. Both 3b and 3d have shown promising results. Thus, they were subjected to Five-dose screening assay at concentrations 0.01, 0.1, 1.0, 10.0 and 100.0 μM. The graphs of the Five-dose screening assay results are provided in the supplementary data section. Compounds 3b and 3d have been chosen for the *in vitro* disease-oriented anticancer screening system. This screening system covers a panel of 60 human cancer cell lines [14]. Namely, leukemia, Non-small Cell Lung Cancer (NSCLC), colon cancer, Central Nervous System (CNS), prostate cancer, melanoma, ovarian cancer, renal and breast ones. The above-mentioned cell lines were adapted to a single growth medium, with reproducible growth and drug sensitivity profiles. Furthermore, 3b and 3d have been tested at Five-dose concentrations; 10^{-4}, 10^{5}, 10^{-6}, 10^{-7} and 10^{-8} M. The percentage growth was calculated according to the previously reported Optical Density (OD) method [22]. The dose response curves of 3b and 3d against each cell line from the nine human cancer subpanels were created by plotting the Percentage Growth (PG) against the $\log_{10}$ of the corresponding drug concentration for each cell line by subpanel group (Figures 2 and 3). Accordingly, the +50, 0 and -50 horizontal lines are drawn, respectively.

Table 1: The Sixty human tumor cell line anticancer screening data at a single dose assay (10^{-5} M) as a percent cell growth for compounds 3b and 3d (Leukemia and Non-Small Cell Lung Cancer cell lines)

Panel	Cell name	3b	3d
Leukemia	CCRF-CEM	-4.4659	-3.9405
Leukemia	HL-60(TB)	-15.368	-13.976
Leukemia	K-562	-0.1845	2.4819
Leukemia	RPMI-8226	0.98373	2.21918
Leukemia	SR	-14.94	-11.653
Non-Small Cell Lung Cancer	A549/ATCC	-63.685	-54.957
Non-Small Cell Lung Cancer	EKVX	32.9079	24.5802
Non-Small Cell Lung Cancer	HOP-62	7.02529	-5.719
Non-Small Cell Lung Cancer	HOP-92	1.47134	-15.699
Non-Small Cell Lung Cancer	NCI-H226	9.20049	15.8003
Non-Small Cell Lung Cancer	NCI-H23	-0.8589	3.66636
Non-Small Cell Lung Cancer	NCI-H322M	16.701	-5.6624
Non-Small Cell Lung Cancer	NCI-H460	-30.375	-10.751
Non-Small Cell Lung Cancer	NCI-H522	-32.906	-45.616

Table 2: The Sixty human tumor cell line anticancer screening data at a single dose assay (10^{-5} M) as a percent cell growth for compounds 3b and 3d (Colon and CNS Cancer cell lines)

Panel	Cell name	3b	3d
Colon Cancer	COLO 205	-55.932	-62.288
Colon Cancer	HCC-2998	-45.38	-34.511
Colon Cancer	HCT-116	-45.932	-58.644
Colon Cancer	HCT-15	-37.398	-29.065
Colon Cancer	HT29	-52.417	-49.364
Colon Cancer	KM12	-17.359	-8.1602
Colon Cancer	SW-620	-11.093	-17.524
CNS Cancer	SF-268	-0.0873	-9.9476
CNS Cancer	SF-295	-20.044	1.1225
CNS Cancer	SF-539	-63.229	-51.804
CNS Cancer	SNB-19	14.1707	12.7334
CNS Cancer	SNB-75	-22.049	-12.695
CNS Cancer	U251	-37.7	-34.265

Table 3: The Sixty human tumor cell line anticancer screening data at a single dose assay (10^{-5} M) as a percent cell growth for compounds 3b and 3d (Melanoma and Ovarian Cancer cell lines)

Panel	Cell name	3b	3d
Melanoma	LOX IMVI	-33.757	-49.609
Melanoma	MALME-3M	51.7598	39.7891
Melanoma	M14	-39.311	-36.342
Melanoma	MDA-MB-435	-19.552	0.07198
Melanoma	SK-MEL-2	7.32489	-11.452
Melanoma	SK-MEL-28	17.9814	23.3913
Melanoma	SK-MEL-5	-17.711	-23.274
Melanoma	UACC-257	21.342	12.2076
Melanoma	UACC-62	3.42183	5.56845
Ovarian Cancer	IGROV1	13.1796	12.5306
Ovarian Cancer	OVCAR-3	-50.361	-71.446
Ovarian Cancer	OVCAR-4	17.2744	24.4075
Ovarian Cancer	OVCAR-5	-3.9548	-13.842
Ovarian Cancer	OVCAR-8	-31.471	-22.998
Ovarian Cancer	NCI/ADR-RES	-17.241	-4.3966
Ovarian Cancer	SK-OV-3	-2.5287	-22.586

Table 4: The Sixty human tumor cell line anticancer screening data at a single dose assay (10^{-5} M) as a percent cell growth for compounds 3b and 3d (Renal, Prostate and Breast Cancer cell lines)

Panel	Cell name	3b	3d
Renal Cancer	786-0	-50.146	-50.146
Renal Cancer	A498	3.7943	-2.7909
Renal Cancer	ACHN	23.3701	8.97994
Renal Cancer	CAKI-1	7.82483	-6.3954
Renal Cancer	RXF 393	-25.421	-27.388
Renal Cancer	SN12C	-0.0627	-35.088
Renal Cancer	TK-10	-7.4318	-35.592
Renal Cancer	UO-31	15.5944	6.39734
Prostate Cancer	PC-3	4.5421	1.23965
Prostate Cancer	DU-145	2.09416	-5.6723
Breast Cancer	MCF7	5.58569	4.608
Breast Cancer	MDA-MB-231/ATCC	-28.842	-41.159
Breast Cancer	HS 578T	-9.2252	-15.575
Breast Cancer	BT-549	-7.9171	-26.862
Breast Cancer	T-47D	-2.5117	11.9421
Breast Cancer	MDA-MB-468	5.06104	5.7721

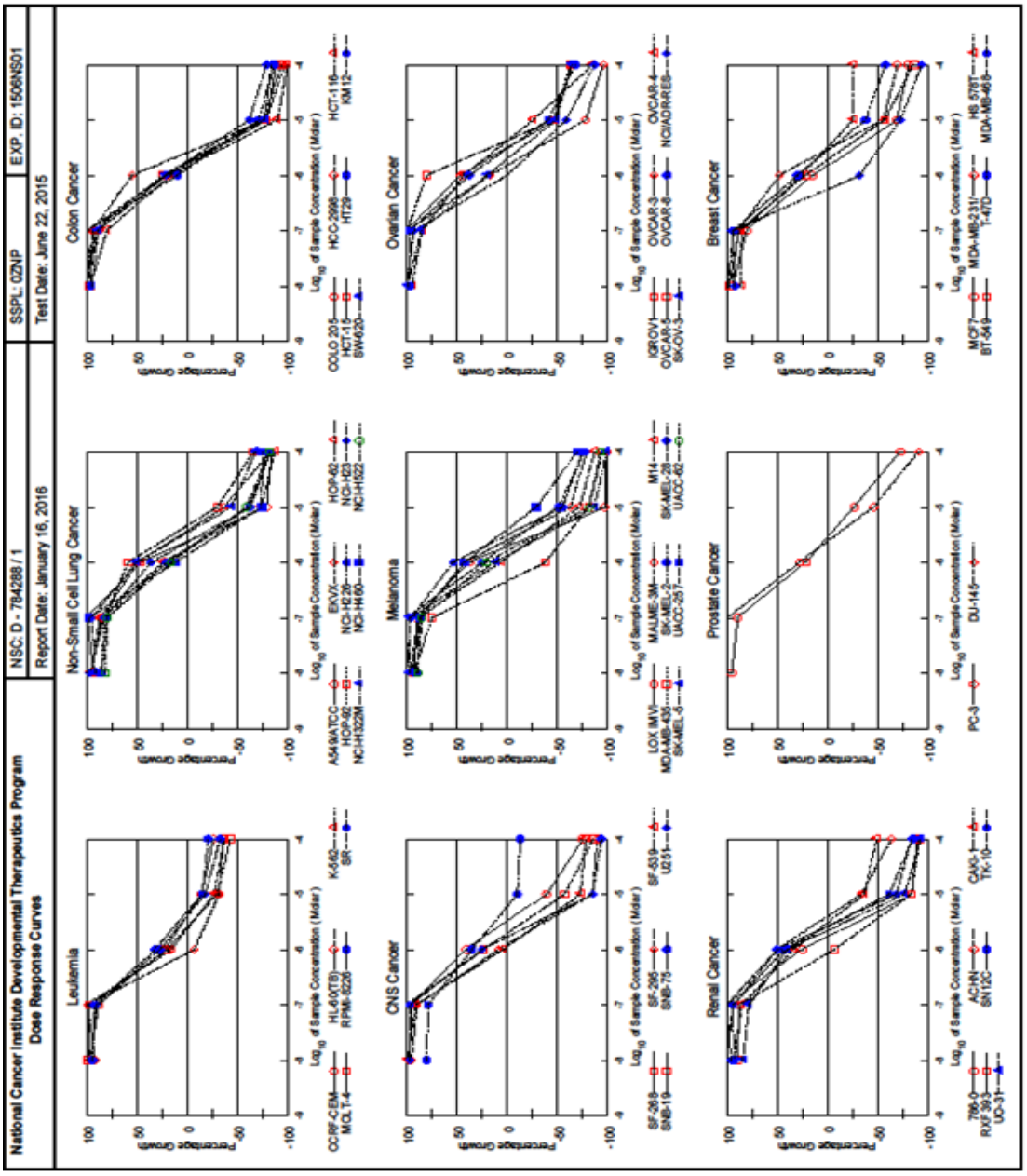


Figure 2: The dose-response curves of 3b against the NCI's 60 human cancer cell lines

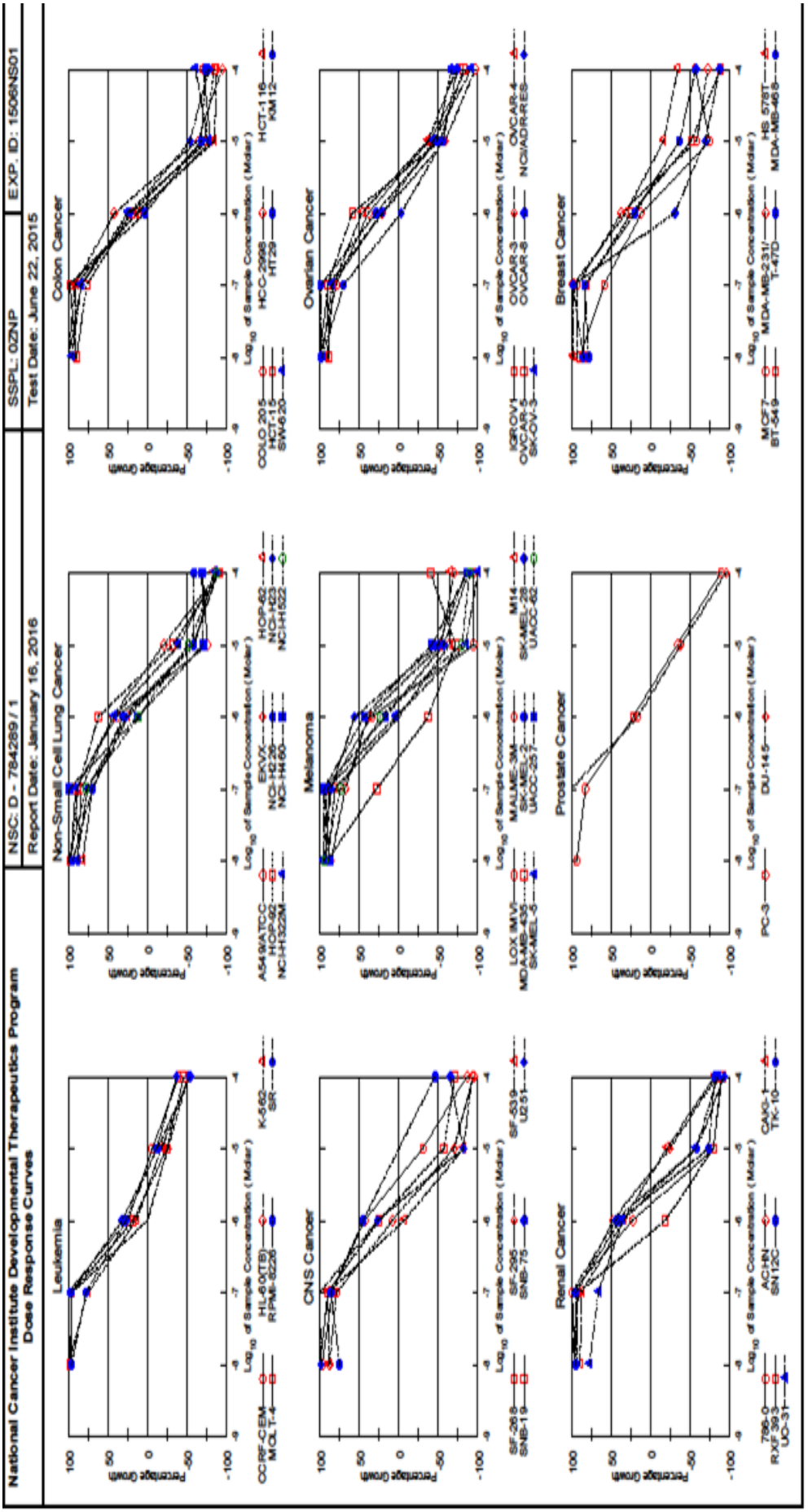


Figure 3: The dose-response curves of 3d against the NCI's 60 human cancer cell lines

Table 5: The cytotoxicity spectrum for compounds 3b and 3d against the NCI's 60 human cancer cell lines

	3b			**3d**		
Panel/Cell line	**GI_{50} (*m*M)**	**TGI (*m*M)**	**LC_{50} (*m*M)**	**GI_{50} (*m*M)**	**TGI (*m*M)**	**LC_{50} (*m*M)**
Leukemia						
CCRF-CEM	0.383	4.12	>100	0.462	6.28	>100
HL-60(TB)	0.289	0.853	>100	0.3	0.982	81.1
K-562	0.405	2.32	>100	0.26	2.7	81.4
MOLT-4	0.409	2.54	>100	0.386	2.64	>100
RPMI-8226	0.504	4.75	>100	0.529	5.1	79.2
SR	0.436	3.95	>100	0.354	5.05	>100
Non-small Cell Lung Cancer						
A549/ATCC	0.836	2.32	5.86	0.625	2.2	5.98
EKVX	0.926	3.71	19.4	0.71	4.74	26.1
HOP-62	0.374	1.76	5.7	0.275	1.96	7.68
HOP-92	1.27	4.61	36.3	1.33	4.58	20.2
NCI-H226	0.323	1.78	7.18	0.329	2.24	8.15
NCI-H23	0.513	2.32	7.36	0.343	2.09	8.08
NCI-H322M	1.03	3.44	17.5	0.717	3.31	1.65
NCI-H460	0.365	1.39	5.31	0.365	1.38	5.58
NCI-H522	0.296	1.63	7.54	0.259	1.48	9.15
Colon Cancer						
COLO 205	0.364	1.57	5.05	0.355	1.32	4.71
HCC-2998	1.09	2.57	6.07	0.68	2.48	7.39
HCT-116	0.301	1.44	4.23	0.341	1.38	4.42
HCT-15	0.418	1.74	5.59	0.301	1.66	5.79
HT29	0.368	1.33	5.46	0.325	1.09	5.58
KM12	0.432	1.78	7.15	0.349	1.91	8.86
SW-620	0.388	1.68	5.32	0.376	1.75	5.39
CNS Cancer						
SF-268	0.684	3.24	19.7	0.68	3.82	21.7
SF-295	0.31	1.24	4.25	0.256	1.27	5.54
SF-539	0.296	1.12	4.97	0.259	0.84	3.75
SNB-19	0.469	1.98	8.24	0.413	2.01	8.12
SNB-75	0.454	5.88	>100	0.738	9.98	>100
U251	0.446	1.68	4.79	0.483	1.76	5.06
Melanoma						
LOX IMVI	0.59	1.91	4.5	0.504	1.84	4.49
MALME-3M	0.669	2.5	7.46	0.409	2.62	9.09
M14	0.3	1.24	5.34	0.239	0.973	5.92
MDA-MB-435	0.167	0.463	1.92	0.0451	0.274	NA
SK-MEL-2	0.482	2.15	9.78	0.374	1.69	8.11
SK-MEL-28	1.1	3.15	9.05	1.15	3.39	10.1
SK-MEL-5	0.354	1.32	4.24	0.297	1.11	4.05
UACC-257	0.68	3.9	32.5	0.776	3.29	1.54
UACC-62	0.351	1.6	4.89	0.298	1.7	5.16
Ovarian Cancer						
IGROV1	0.848	3.11	14	0.512	3.12	17.4
OVCAR-3	0.402	1.5	5.07	0.383	1.85	7.73
OVCAR-4	0.695	4.25	25.5	0.846	3.66	23.2
OVCAR-5	1.72	4.31	17.7	1.2	3.4	9.6
OVCAR-8	0.626	3.02	21.3	0.5	2.37	12.8
NCI/ADR-RES	0.292	1.01	6.95	0.187	0.919	7.59
SK-OV-3	0.352	1.95	11.2	0.349	2.09	13.5
Renal Cancer						
786-0	0.48	1.7	4.9	0.43	1.63	5.03
ACHN	1	3.91	33.2	0.897	4.51	25.8
CAKI-1	0.488	3.03	>100	0.49	4.16	30.2
RXF 393	0.251	0.85	3.63	0.235	0.675	3.22
SN12C	0.715	2.56	7.74	0.64	2.23	6.17
TK-10	0.987	2.62	6.89	0.846	2.67	8.15
UO-31	0.551	2.2	5.87	0.329	2.39	8.13
Prostate Cancer						
PC-3	0.37	2.69	31.4	0.337	2.39	18.7
DU-145	0.499	2.38	12.4	0.394	1.96	15.7
Breast Cancer						
MCF7	0.282	1.47	5.84	0.151	1.37	5.13
MDA-MB-231/ATCC	0.887	2.89	8.77	0.589	2.43	8.06
HS 578T	0.396	3.23	>100	0.521	4.44	>100
BT-549	0.373	1.89	8.1	0.355	2.07	9.41
T-47D	0.48	2.78	40.4	0.326	2.2	41
MDA-MB-468	0.225	0.56	2.73	0.234	0.571	3.01

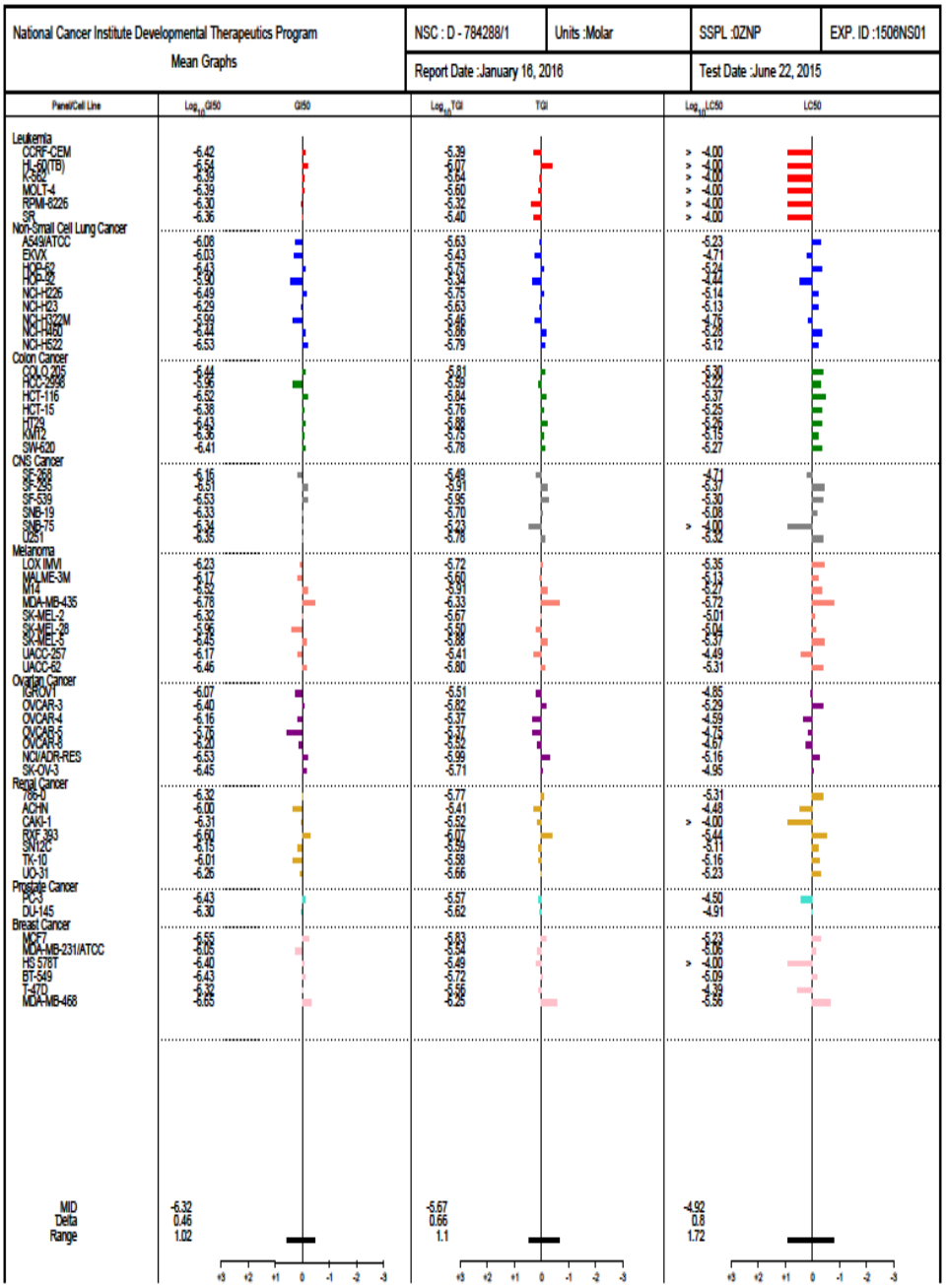


Figure 4: The mean graphs of 3b against the NCI's 60 human cancer cell lines

Thus, the molar drug concentrations corresponding to points where the curves cross these lines represent the values to cause 50% growth inhibition (GI_{50}), total growth inhibition (TGI) and 50% cell killing (LC_{50}), respectively. Interestingly, whenever these response parameters cannot be obtained by the interpolation, the value given is the highest concentration tested proceeded by the sign '>'. In accordance, both 3b and 3d exhibited excellent GI and TGI values with values 100 < μM. Both 3b and 3d showed a sort of sensitivity against melanoma cancer cell lines, MDA-MB-435 subpanel. Compound 3b showed activity towards all leukemic cell lines, SNB-75 subpanel (CNS cancer), CAK-1 (renal cancer) and finally HS578T (breast cancer). Regarding 3d, the sensitive subpanels were CCRF-CEM, MOLT-4, and SR (leukemic cancer), SNB-75 (CNS cancer), HS578T (breast cancer). The values of GI_{50} TGI and LC_{50} are summarized in Table 5. Both 3b and 3d exhibited very potent cytotoxic effects ($GI_{50} \leq 2.0$ μM) against almost all cell lines. Regarding TGI, 3b and 3d show values ranging from 0.463–4.75 μM and 0.571–6.28 μM, respectively. Figures 4 and 5 represent the NCI's mean graph plot for the response parameters in Table 5.

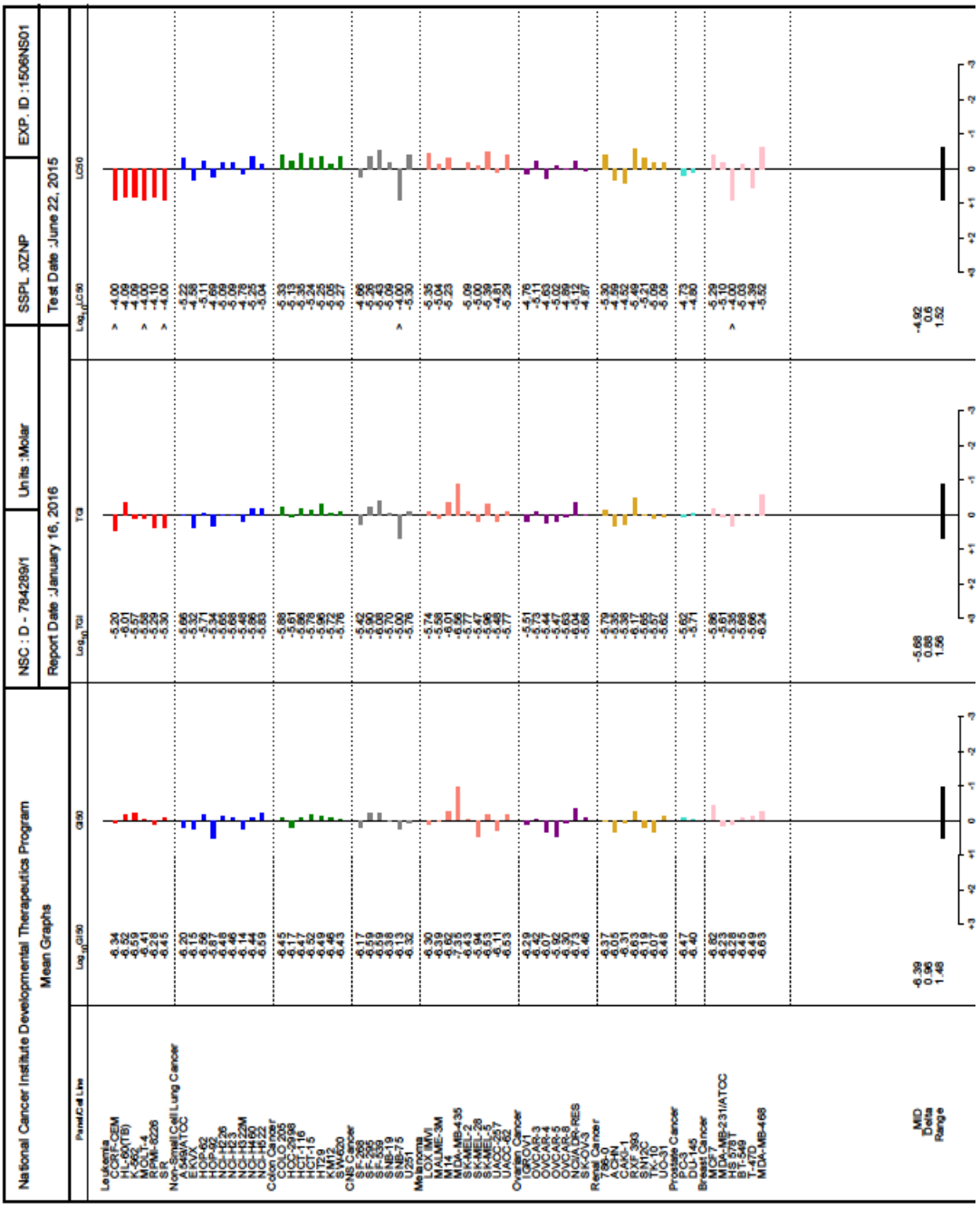


Figure 5: The mean graphs of 3d against the NCI's 60 human cancer cell lines

Such a graph plot easily enables the visual scanning of the produced data for potential patterns of selectivity for specific cell lines or particular subpanels with respect to a selected response parameter [23]. The mean graph plot shows the pattern at the three main parameters, GI_{50}, TGI, and LC_{50}. The vertical line for each parameter implies the mean value of all the test data against the cell lines. Accordingly, bars extending to the left side mean that the sensitivity of the cell lines is less than the average while ones extending to the right imply the sensitivity of the cell line exceeding the average. Those values exceeding the 100 μM, it is not applicable to determine the desired parameter (e.g. LC_{50} parameter against all leukemic cancer subpanels for both 3b and 3d). Accordingly, the best sensitivity patterns for both 3b and 3d were against MDA-MB-435 subpanel (melanoma cancer).

CONCLUSION

New thienopyrimidine fused system has been designed, synthesized and screened against their anticancer activity. Compounds 3b and 3d were selected and evaluated by National Cancer Institute (USA) at single dose (10^{-5} M). The tested compounds showed promising activities against most of tumor cell lines. Accordingly, they were subjected to Five-dose screening assay at concentrations 0.01, 0.1, 1.0, 10.0 and 100.0 μM. Both 3b and 3d exhibited very potent cytotoxic effects ($GI_{50} \leq 2.0$ μM) against almost all cell lines. 3b and 3d showed TGI values ranging from 0.463–4.75 μM and 0.571–6.28 μM, respectively. The best sensitivity patterns for both 3b and 3d were against MDA-MB-435 subpanel (melanoma cancer).

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

REFERENCES

[1] R.L. Siegel, K.D. Miller, A. Jemal, *Cancer. J. Clin*., **2015**, 65, 5.
[2] T.R. Golub, D.K. Slonim, P. Tamayo, C. Huard, M. Gaasenbeek, J.P. Mesirov, H.M. Coller, L. Loh, J.R. Downing, M.A. Caligiuri, C.D. Bloomfield, E.S. Lander, *Sci*., **1999**, 286, 531.
[3] T.L. Lemke, D.A. Williams, Lippincott Williams & Wilkins, Philadephia, **2008**.
[4] Y. Lu, R.I. Mahato, Springer Science & Business Media, **2009**.
[5] B.A. Chabner, T.G. Roberts, *Nat. Rev. Cancer*., **2005**, 5, 65.
[6] H.J. Knölker, K.R. Reddy, **2008**, 65, 181.
[7] M. Ohashi, T. Oki, *Expert Opin. Ther. Pat*., **2008**, 6, 1285.
[8] C. Ducrocq, F. Wendling, J.C. Chermann, M. Tourbez-Perrin, C. Rivalle, P. Tambourin, F. Pochon, E. Bisagni, *J. Med. Chem*., **1980**, 23, 1212.
[9] J. Kattan, M. Durand, J.P. Droz, M. Mahjoubi, J.P. Marino, M. Azab, *Am. J. Clin. Oncol*., **1994**, 17, 242.
[10] M.P. Fontaine-Aupart, E. Renault, C. Videlot, F. Tfibel, R. Pansu, M. Charlier, P. Pernot, *Photochem. Photobiol*., **1999**, 70, 829.
[11] E.M. Acton, V.L. Narayanan, P.A. Risbood, R.H, Shoemaker, D.T. Vistica, M.R. Boyd, *J. Med. Chem*., **1994**, 37, 2185.
[12] C.E. Dalgliesh, F.G. Mann, *J. Chem. Soc*., **1945**, 893.
[13] M.C. Alley, D.A. Scudiero, A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, M. R. Boyd, *Cancer. Res*., **1988**, 48, 589.
[14] B.A.C.M.R. Grever, S.A. Schepartz, *Semin. Oncol*., **1992**, 19, 622.
[15] M.R. Boyd, K.D. Paull, *Drug Dev. Res*., **1995**, 34, 91.
[16] L.K.A. Rahman, *J. Chem. Soc. Perkin. Trans. I*., **1984**, 385.
[17] K. Sukanta, C. Mukherjee, T.K. Pradhan, A. De, *Arkivoc*, **2006**, 45.
[18] M.T. Konieczny, W. Konieczny, *Heterocycles*, **2005**, 451.
[19] M. Bakhouch, G. Al Houari, M. Daoudi, A. Kerbal, M. El yazidi, *Mediterr. J. Chem*., **2015**, 4, 9.
[20] D.L. Pavia, G.M. Lampman, G.S. Kriz, J.A. Vyvyan, *Cengage Learning*, **2008**.
[21] A.M. El-agrody, M.S. Abd El-latif, A.H. Fakery, A.H. Bedair, *J. Chem. Res.*, **2000**, 26.
[22] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, *J. Natl. Cancer Inst.*, **1990**, 82, 1107.
[23] K.D. Paull, R.H. Shoemaker, L. Hodes, A. Monks, D.A. Scudiero, L. Rubinstein, J. Plowman, M.R. Boyd, *J. Natl. Cancer. Inst*., **1989**, 81, 1088.