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Synthesis of new 2-substituted-10-phenylsulfonylphenothiazine conjugates and evaluation as anticancer agents by investigating their off-target mechanism

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Abstract: The approval procedure of new therapeutic drugs pass through several complex processes, starting from basic research and discovery, through preclinical and clinical phases, which take not only long time but also need enormous resources. As per statistics, only one in six molecules which enters in clinical testing eventually gets regulatory approval for marketing. Hence, repurposing of existing drugs by making use of their off-target mechanisms is becoming popular for new drug discovery. In this study, we have reported the pharmacological investigation of substituted phenothiazine, the largest class of antipsychotic drug to develop new anti-tumour agents. The structurally engineered agents (**7**) and (**10**) exhibited good antitumor activities, hence these moieties can be chosen as lead compounds for further biological and clinical studies. A constructive strategy was followed where domino synthesis is carried out to prepare the target molecules. Efficiency and ecological benefit are the added advantage of this present protocol.

Keywords: Anticancer activity, Phenylsulfonylphenothiazine-triazole conjugate, Phenylsulfonylphenothiazine-thiadiazoleconjugate, Human prostate-DU-145, Human melanoma- SK-MEL-2.

Introduction:

Almost all prostate cancers are adenocarcinomas where the cells in the prostate gland grow uncontrollably. Other types of prostate cancer include: sarcomas, small cell carcinomas, neuroendocrine tumors (other than small cell carcinomas) and transitional cell carcinomas. Only few therapeutic options are available for the treatment of prostate cancer. Out of those limited marketed drugs, abiraterone acetate is steroid based. Bicalutamide is used in combination with other drugs and its common side effect is breast enlargement. Cabazitaxel and docetaxel are semi-synthetic derivative of natural taxoid, hence total synthesis of these drugs provides extremely low yield. Degarelix is used for the hormone therapy. Enzalutamide contains diarylthio-hydantoin ring which is a complex molecule to synthesize. Thus, there is an urgency to develop new therapeutic agents for patients who have developed advanced prostate

cancer. Melanoma is another deadly form of skin cancer and strikes tens of thousands of people around the world each year. The number of cases is rising faster than any other type of solid cancer. It is usually caused by too much exposure to the Sun's ultraviolet radiation. But the link between sunshine and melanoma is still unrevealed. Very limited approved drugs are available for targeted therapies, some of them being trametinib, dabrafenib, vemurafenib, cobimetinib. Most of them are actually effective as combination therapy. Structures of these drugs substances contain various nitrogenous heterocyclic rings and also N-S linkages. It was also noticed that, most of the synthetically prepared small molecules which showed activity towards human cancer cell lines comprise nitrogen and sulfer containing heterocyclic rings *e.g.* triazole, thiadiazole moieties [1].

Over the past many years researcher made lot of efforts to explore the off-target effects of existing drugs.[2-4] Phenothiazines (PTZs) were originally developed for the treatment of schizophrenia, and bipolar disorder due to their dopaminergic activity[5] Several articles have also been published over the years where anti-tumor[6,7] and anti-cancer [8-16] activity of PTZs were revealed. In recent years, new conjugates of PTZs were prepared which showed activity as farnesyl transferase inhibitors.[17-20] Diverse biological activity of PTZs were discussed in the review article published by Mosnaim *et al.*[21] and Pluta *et al*.[22] Phenothiazine moieties were widely evaluated as anti-retroviral,[23] anti-inflammatory,[24,25] anti-tubercular,[26-28] anti-convulsant,[29] anti-HIV [28] and anti-microbial [31-33] agents.

Encouraged by the afore-mentioned versatility of PTZs and also by its similarity with the approved anticancer drugs, new PTZ conjugates have been engineered in our lab. In this study we report phenylsulfonyl phenothiazine conjugates with various nitrogen containing ring system at 2-position of the PTZ ring. We have chosen human prostate and melanoma skin cancer cell lines as the target substrates to check the antiproliferative effect of all the synthesized compounds. The synthesized compounds were evaluated for the activity against human prostate cell line DU-145 and human melanoma cell line SK-MEL-2. To our knowledge, this is the first report for such studies on these types of scaffolds.

Materials and methods:

All the key starting materials, solvents and reagents were obtained from Sigma Aldrich and Merck Specialities Pvt. Ltd. NMR spectra were recorded on a Bruker spectrometer at 400 MHz for ^{1}H and ^{13}C respectively and the chemical shifts were reported as δ value in ppm relative to TMS as internal standard. Infra red (IR) spectra were obtained using a Nicolet 6700 IR spectrometer. Mass spectra were recorded on Shimadzu mass-spectrometer. Melting points (mp) were recorded on open capillaries and are uncorrected.

Synthesis:

Synthesis of 4-[(2-aminophenyl)thio]-3-fluorobenzonitrile(3): 2-aminobenzenethiol (100 g, 798.7 mmol) was dissolved in acetone (500 mL) at 25-30 °C. Powdered potassium carbonate (145.40 g, 1052 mmol) was added to the solution and stirred for 1 hour. 3,4-difluorobenzonitrile was added in lots (178.20 g, 1281 mmol) and the mass was heated to reflux. The mixture was stirred at 55 °C for 5 hours. After completion of reaction, the mass was cooled to 25-30 °C and filtered to remove potassium carbonate. The filtrate was concentrated to dryness. Ethyl acetate (1.5 lit) and water (1.5 lit) were added to the residue and stirred for 15 min. Layers separated. Organic layer washed with water (1.5 lit), dried over sodium sulfate and concentrated. Isopropyl

ether (IPE; 300 ml) was added to the residue to get slurry, the slurry was filtered and wet cake washed with IPE. The wet cake was dried under vacuum at 50 °C (Yield: 135g, 85%): mp: 95-96 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25^{0}C, TMS) δ (ppm): 5.60 (s, 2H; NH_2), 6.62-6.66 (m, 2H), 6.87-6.89 (d, *J*=8 Hz, 1H), 7.25-7.34 (m, 2H), 7.54-7.56 (d, *J*=8 Hz, 1H), 7.84-7.87 (m, 1H); HRMS (ESI) $[M+H]^+$ 245.0547.

Synthesis of 2-cyanophenohiazine (4): Compound 3 (100 g, 378.3 mmol) was dissolved in DMF (500 mL). The solution was heated to 140 °C and stirred for 16 hours at the same temperature. After completion of reaction, the mass was cooled to 25 °C and quenched with water (2.5 lit). Product precipitation observed. The slurry was stirred for 1 hour and filtered to obtain the wet cake. The cake was dried under vacuum at 50 ^{0}C, the dried compound was coloumn purified Methanol : DCM solvent system (2 : 8) to get greenish yellow solid (Yield: 80 g, 88%): mp: 190-192^{0}C.[34]

Synthesisof10-(phenylsulfonyl)phenthiazine-2-carbonitrile (5): Benzenesulfonyl hydrazide (9.98 g, 57.9 mmol) and 2-cyanophenothiazine (12.01g, 53.5 mmol) were added in 1,4-dioxane (600 mL). tert-Butylhydroperoxide (0.28 g, 2.2 mmol, 70% solution in H_2O) followed by iodine (0.007 g, 0.11 mmol) crystals were added to the solution. The reaction mass was stirred for 30 min at 25-30^{0}C. The reaction mass was quenched with water (600 mL) and extracted with DCM (1.2 Lit). Organic layer was dried over Na_2SO_4 and concentrated. The residue was column purified using methanol : DCM (1 : 9) solvent mixture (Yield: 15.6 g, 80%): mp: 248-249 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS) δ (ppm): 6.63-6.65 (d, J=8.0; 1H), 6.77-6.90 (m, 3H), 6.98-7.05 (m, 2H), 7.09-7.11 (m,1H), 7.61-7.85 (m, 5H); For $C_{19}H_{12}N_2O_2S_2$ Calculated C 62.62, H 3.32, N 7.69, Found: C 62.62, H 3.31, N 7.69; HRMS (ESI) $[M+H]^+$ 365.0416.

Synthesis of 10-(phenylsulfonyl) phenothiazine-2-carbothioamide (6): Compound 5 (5.02 g, 13.7 mmol) and DMF (25 ml) were added in a RB-flask. The solution was treated with NaSH (1.53g, 27.3 mmol) and $MgCl_2$ (1.23 g, 12.9 mmol). The reaction mixture was stirred for 3 hours at 25-30 ^{0}C. The mixture was poured in ice-cold water (750 mL). Product precipitated out as slurry. The slurry was filtered, wet cake washed with water and dried under vacuum at 50 °C to obtain yellow coloured solid (Yield: 4.9 g, 90%): mp: 228-232 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25^{0}C, TMS) δ (ppm): 6.66-6.68 (d, J=8.0, 1H), 7.00-7.12 (m, 3H), 7.20-7.29 (m, 2H), 7.33-7.35 (m, 1H),7.64-7.88 (m, 5H), 10.00 (s, 1H; NH), 10.38 (s, 1H, NH); For $C_{19}H_{14}N_2O_2S_3$ Calculated C 57.26, H 3.54, N 7.03, Found: C 57.25, H 3.54, N 7.03; HRMS (ESI) $[M+H]^+$ 399.02956.

Synthesis of 10-(phenylsulfonyl)-2-(1H-1,2,4-triazol-3-yl)phenothiazine (7): Compound 6 (4.01g, 10 mmol) and DMF (20 mL) were added in a RB-flask. Hydrazine hydrate (0.64 g, 20 mmol) was added drop wise to the solution. The reaction mass was stirred for 1 hour at 20 °C. Formic acid (20 mL) was added drop wise and the reaction mixture was stirred for 3 hours at 90 °C. The reaction mass was cooled to 20 °C and poured to saturated $NaHCO_3$ solution (700 mL). Product was extracted with 200 mL ethyl acetate. The organic layer was washed with 150 mL saturated brine solution, dried over Na_2SO_4 and concentrated under vacuum at 50°C. The residue was triturated with n-heptane (40 mL), filtered the slurry, washed the slurry with n-heptane and dried the wet cake under vacuum at 55 °C. The dried material was coloumn purified using methanol : DCM (1.5 : 8.5) solvent system. (Yield: 4.1 g, 74%): mp: 264-266 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS) δ (ppm): 6.65-6.67 (d, J=8.0, 1H), 6.78-6.90 (m, 3H), 6.92-7.01 (m, 2H), 7.06-7.14 (m, 1H), 7.57-7.69 (m, 5H), 8.49, (s, 1H), 14.49 (s, 1H, NH); ^{13}C NMR (400 MHz, DMSO-d6, 25

°C, TMS) δ (ppm): 157.1 (C), 141.5 (C), 140.2 (CH), 140.1 (C), 136.2 (C), 132.3 (CH), 130.2 (2CH), 129.5 (CH), 128.5 (CH), 127.7 (CH), 126.8 (CH), 126.5 (2CH), 125.1 (CH), 124.1 (CH), 123.8 (C), 122.8 (C), 118.3 (CH), 117.4 (C); For $C_{20}H_{14}N_4S_2O_2$ Calculated C 59.10, H 3.47, N 13.78, Found: C 59.75, H 3.65, N 13.55; HRMS (ESI) $[M+H]^+$ 407.0633.

Synthesis of 10-(phenylsulfonyl) phenothiazine-2-carboxylic acid (8): Compound 5 (8.01 g, 21.9 mmol) was added in 60% aqueous sulfuric acid (80mL). The solution was stirred for 3 hour at 50 °C. The reaction mass was cooled and pH was adjusted to 4.0 using aqueous alkali solution. Product precipitated out. The slurry was stirred for 1 hour at 10 °C and filtered. The wet cake was washed with water and dried under vacuum to obtain yellow coloured solid (Yield: 6.0 g, 70%): mp: 236-237 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS) δ (ppm): 6.64-6.66 (d, J=8.0, 1H), 6.98-7.10 (m, 3H), 7.18-7.27 (m, 2H), 7.32-7.34 (m,1H), 7.62-7.86 (m, 5H), 12.15 (bs, 1H, COOH); For $C_{19}H_{13}NO_4S_2$ Calculated C 59.51, H 3.42, N 3.65, Found: C 59.54, H 3.40, N 3.64; HRMS (ESI) $[M+H]^+$ 384.0363.

Synthesis of 10-(phenylsulfonyl)-N'-(pyrazin-2-yl)phenothiazine-2-carbohydrazide (9): Compound 8 (1.01 g, 2.6 mmol) was added in DCM (10 mL). 2-hydrazinopyrazine (2-HP, 0.34 g, 3.1 mmol) was added to the solution and cooled to 5 °C. Propylphosphonic anhydride (1.98 g, ~50 wt. % in ethylactate, 3.1 mmol) and N,N-diisopropylethylamine (0.40 g, 3.1 mmol) were added and the reaction mixture was stirred for 30 min at 0-5 °C. The reaction mass was poured in water and extracted with (2x 10 mL) DCM. The combined organic layer was washed with saturated brine solution, dried over sodium sulfate and concentrated. The residue was coloumn purified using methanol : DCM (1.5 : 8.5) solvent system (Yield: 0.74 g, 60 %): mp: 285-287 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25^{0}C, TMS) δ (ppm): 6.64-6.66 (d, J=8.0, 1H), 6.77-6.92 (m,3H), 7.00-7.14 (m, 3H), 7.57-7.69 (m, 5H), 7.94-8.28 (m, 2H), 8.29 (s, 1H), 9.55 (s, 1H), 10.28 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d6, 25 °C, TMS) δ (ppm): 165.1 (C), 154.2 (C), 149.1 (CH), 141.5 (C), 140.2 (C), 135.5 (C), 134.5 (C), 133.8 (CH), 132.7 (CH), 131.9 (CH), 129.5 (2CH), 128.8 (CH), 127.5 (CH), 127.1 (CH), 126.4 (2CH), 126.0 (CH), 123.5 (CH), 122.8 (C), 121.9 (C), 117.2 (CH), 116.4 (CH); For $C_{23}H_{17}N_5O_3S_2$ Calculated C 58.09, H 3.60, N 14.73, Found: C 58.21, H 3.74, N 14.77; HRMS (ESI) $[M+H]^+$ 476.0849.

Synthesis of 10-(phenylsulfonyl)-2-(1,3,4-thiadiazol-2-yl)phenothiazine (10): Compound 8 (4.02 g, 10.4 mmol) and 2-methyl THF (40 mL) were added in a RB-flask. Formic hydrazide (0.67 g, 10.9 mmol) was charged under stirring at 25°C. Clear solution obtained. The reaction mass was cooled to -5 °C. Charged *N, N*- diisopropylethylamine (4.0 mL, 22.96 mmol) and stirred for 10 min at -10 °C. Propylphosphonic anhydride (9.54g, ~50 wt. % in ethylactate, 15.0 mmol) solution was added over a period of 30 min and stirred for 3 hours at 5°C. Reaction mass was quenched with water (100 mL) and diluted with ethyl acetate (100 mL). Layers separated and organic layer washed with water (100 mL). Organic layer concentrated under vacuum. THF (50 mL) was added to the residue, stirred for 15 min. Charged P_2S_5 (3.33 g,15.0mmol) and heated the mass to reflux. The reaction mass was stirred for 2 hours at reflux condition. Cooled the reaction mass to 25 °C and quenched with 5% $NaHCO_3$ solution. Extracted with ethyl acetate (100 mL). Washed organic layer with water (100 mL) and concentrated under vacuum. The residue was coloumn purified using methanol : DCM (1 : 9) solvent system (Yield: 3.4 g, 78 %): mp: 260-261°C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS) δ (ppm): 6.64-6.66 (d, J=8.0, 1H), 6.78-6.92 (m, 3H), 7.00-7.14 (m, 3H), 7.57-7.70 (m, 5H), 9.79 (s, 1H); ^{13}C NMR (400 MHz,

DMSO-d6, 25 °C, TMS) δ (ppm): 164.8 (C), 143.6 (CH), 141.5 (C), 140.6 (C), 136.2 (C), 132.0 (C), 132.5 (CH), 130.2 (2CH), 129.5 (CH), 128.8 (CH), 128.2 (2CH), 127.8 (2CH), 126.8 (CH), 123.8 (C), 124.1 (CH), 118.3 (CH), 117.4 (CH); For $C_{20}H_{13}N_3O_2S_3$ Calculated C 56.72, H 3.09,N 9.92, Found: C 56.32, H 3.18, N 9.85; HRMS (ESI) $[M+H]^+$ 424.0244.

Synthesis of 10H-phenothiazine-2-carboxylic acid (11): 2-cyanophenothiazine (4.01 g, 17.8 mmol) was added in 60% aqueous sulfuric acid (16mL). The solution was stirred for 3 hours at 50 °C. The reaction mass was cooled and pH was adjusted to 4.0 using aqueous alkali solution to obtain slurry. The slurry was stirred for 1 hour at 10 °C and filtered. The wet cake was washed with water and dried under vacuum to obtain yellow coloured solid (Yield: 3.1 g, 72 %): mp: 219-222 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS) δ (ppm): 6.64-6.66 (d, J=8.0, 1H), 6.98-7.10 (m, 3H), 7.18-7.27 (m, 2H), 7.32-7.34 (m,1H), 8.72 (s, 1H; NH), 12.12 (bs, 1H; COOH); For $C_{13}H_9NO_2S$ Calculated C 64.18, H 3.73, N 5.76, Found: C 64.25, H 3.72, N 5.73; HRMS (ESI) $[M+H]^+$ 244.0433.

Synthesis of N'-(pyrazin-2-yl)-10H-phenothiazine-2-carbohydrazide (12): Compound 11 (1.95g, 8.0 mmol) was added in DCM. 2-hydrazinopyrazine (1.02g, 9.2 mmol) was added to the solution and cooled to 5 °C. Propylphosphonic anhydride (~50 wt. % ethyl acetate solution, 5.91g, 9.3 mmol) and N,N-diisopropylethylamine (1.2g, 9.3 mmol) was added and the reaction mixture was stirred for 30 min at 0-5 °C. The reaction mass was poured in water and extracted with (2x 10 mL) DCM. The combined organic layer was washed with saturated brine solution, dried over sodium sulfate and concentrated. The residue was coloumn purified using methanol : MDC (9 : 1) solvent system (Yield: 0.74 g, 65 %): mp: 271-272 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS) δ (ppm): 6.65-6.66 (d, J=6.8; 1H), 6.77-6.92 (m, 3H), 7.00-7.14 (m, 3H), 7.94 (s, 1H), 7.95-8.28 (m, 3H), 8.30 (s, 1H), 9.79 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d6, 25 °C, TMS), δ (ppm): 165.1 (C), 154.5 (C), 149.2 (CH), 142.5 (C), 142.0 (C), 134.5 (CH), 134.0 (C), 131.9 (CH), 128.9 (CH), 128.2 (CH), 128.0 (CH), 125.7 (CH), 123.1 (C), 123.3 (CH), 117.5 (C), 116.3 (CH), 114.1 (CH); For $C_{17}H_{13}N_5OS$ Calculated C 60.88, H 3.91,N 20.88, Found: C 61.02, H 3.77, N 20.79; HRMS (ESI) $[M+H]^+$ 336.0918.

Synthesis of 10H-phenothiazine-2-carbothioamide (13): 2-cyanophenothiazine (4.01g, 17.8 mmol) and DMF (25 mL) were charged in a RB-flask. The solution was treated with NaSH (1.96 g, 35.0 mmol) and $MgCl_2$ (3.33 g, 35.0 mmol). The reaction mixture was stirred at 25 °C for 3 hours. The mixture was poured in ice-cold water (600 mL). Product precipitated out as slurry. The slurry was filtered, wet cake washed with water and dried under vacuum at 50 °C to obtain yellow coloured solid (Yield: 3.9 g, 86%): mp: 212-217 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS), δ (ppm): 6.66-6.68 (d, J=8.0, 1H), 7.00-7.12 (m, 3H), 7.20-7.29 (m, 2H),7.33-7.35 (m,1H), 8.75 (s, 1H; NH), 10.00 (s, 1H; CSNH), 10.40 (s, 1H; CSNH); For $C_{13}H_{10}N_2S_2$ Calculated C 60.43, H 3.90, N 10.84, Found: C 60.42, H 3.89, N 10.83; HRMS (ESI) $[M+H]^+$ 259.0362.

Synthesis of 2-(1H-1,2,4-triazol-3-yl)-10H-phenothiazine: (14): Compound 13 (3.04 g, 117 mmol) and DMF (15 mL) were charged in a RB-flask. Hydrazine hydrate (0.73 g, 22.8 mmol) was added drop wise to the solution. The reaction mass was stirred for 1 hour at 20 °C. Formic acid (15 mL) was added drop wise and the reaction mixture was stirred for 3 hrs at 90 °C. The reaction mass was cooled to room temperature (RT) and poured to saturated $NaHCO_3$ (525 mL) solution. Product was extracted with (2x 100 mL) Ethyl acetate. The combined organic layer was washed with

(3x 50 mL) saturated brine solution, dried over Na_2SO_4 and concentrated under vacuum at 50 °C. The residue was triturated with n-heptane (30 mL), filtered and dried. The dried product was coloumn purified using methanol : MDC (9 : 1) solvent system (Yield: 2.2 g, 72%): mp: 255-258 °C; ^{1}H NMR (400 MHz, DMSO-d6, 25 °C, TMS), δ (ppm): 6.65-6.67 (d, J=8.0, 1H), 6.78-6.92 (m, 3H), 7.00-7.14 (m, 3H), 8.50 (s, 1H), 8.81 (s, 1H), 14.50 (s, 1H); ^{13}C NMR (400 MHz, DMSO-d6, 25 °C, TMS), δ (ppm): 158.9 (C), 143 (C), 142.8 (C), 141.5 (CH), 130 (CH), 129 (CH), 128 (CH), 127.8 (C), 126.8 (CH), 124.1 (C), 123.1 (CH), 117.5 (C), 117.4 (CH), 114.2 (CH); For $C_{20}H_{13}N_3O_2S_3$ Calculated C 63.14, H 3.78, N 21.04, Found: C 63.44, H 3.54, N 21.36; HRMS (ESI) $[M+H]^+$ 267.0703.

SRB assay:

The biological activity testing has been conducted at Advance centre for treatment, Research & Education in Cancer (ACTREC), Navi Mumbai by SRB assay. [35, 36] The cell lines were grown in RPMI 1640 medium containing 10% foetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental compounds.

Synthesized compounds were initially solubilized in dimethyl sulfoxide at 100 mg/mL and diluted to 1mg/mL using water and stored frozen prior to use. At the time of compound addition, an aliquot of frozen concentrate (1mg/mL) was thawed and diluted to 100 µg/mL, 200 µg/mL, 400 µg/mL and 800 µg/mL with complete medium containing test article. Aliquots of 10 µl of these different sample dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final sample concentrations i.e.10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL.

After compound addition, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells x 100.

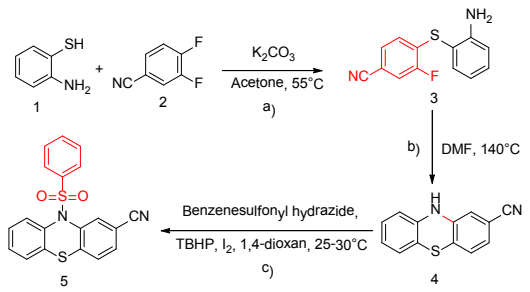
Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of sample at the four concentration levels (Ti), the percentage growth was calculated at each of the sample concentration levels. Percentage growth inhibition was calculated as: [Ti/C] x 100 %.

Results and discussion:

Chemistry:

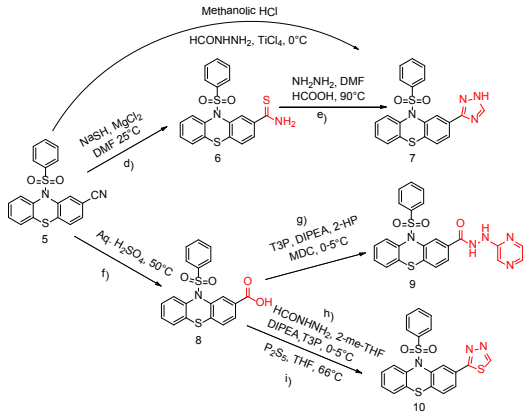
The condensation of 2-aminobenzenethiol

(**1**) and 3,4-difluorobenzonitrile (**2**) produced compound (**3**). Desired PTZ ring system (**4**) was prepared by Smiles rearrangement of compound (**3**). Compound (**4**) was further reacted with benzenesulfonyl hydrazide to produce 2-cyano-10-phenylsulfonylphenothiazine (**5**). Sulfonylation at nitrogen of phenothiazine was a challenging task. Employing previous reports, use of various organic bases, *e.g.* TEA, disiopropylehtylamine, DBU, lithium hexamethyldisilazen etc. in combination with benzenesulfonyl chloride or benzenesulfonyl anhydride resulted only 0-4 % yield. The present protocol of sulfonylation using tert-Butylhydroperoxide (TBHP) and catalytic iodine provided 80 % Compound (**5**) which was used as a starting material to prepare various 2-substituted phenylsulfonylphenothiazine compounds as outlined in **scheme 2**.

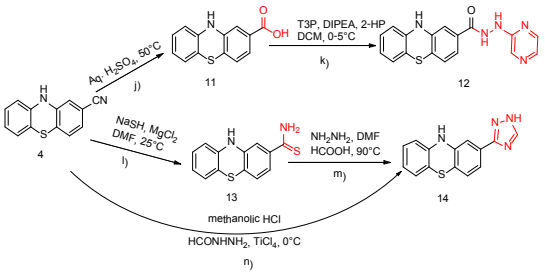


Scheme 1: Synthesis of 2-cyano-10-phenylsulfonylphenothiazine

In step d), compound (**6**) was produced when the cyano-group at 2-position of PTZ ring converted to thioamide-group. In step-e) triazole ring was constructed to afford compound (**7**)**.** [37] In another series (step f) nitrile functional group of compound (**5**) was transformed to the corresponding acid to obtain compound (**8**) which was further reacted with 2-hydrazino pyrazine (step g) to afford compound (**9**). In step h) and i), compound (**5**) was converted to its corresponding thiadiazole derivative (**10**). In order to understand the importance of phenylsulfonyl group on antiproliferative activity of 2-substitutedphenothiazine derivatives, another set of compounds were prepared following the synthetic route as shown in **scheme 3)** where phenothiazine derivatives do not have the phenylsulfonyl moiety. In step j) 2-cyanophenothiazine was converted to its corresponding acid derivative which was finally reacted with 2-hydrazinopyrazine (step k) to produce compound (**12**). In step l) 2-cyanophenothiazine was converted to its analogous thioamide moiety (**13**). The construction of triazole ring in compound 13 was then carried out to get compound (**14**). Synthesis of compound (**14**) from substrate (**4**) was another crucial development. Poor yield (61%) obtained when reported methodology was employed via carbothioamide intermediate (**13**). Moreover, the process was not environment friendly since it involved use of mercapto compound and high temperature. Hence, it was required to develop a milder and efficient protolcol for synthesis of triazole. The newly developed protocol to synthesize compound (14) is a domino reaction. In a single pot (step n), compound (**4**) was stirred in methonilic HCl at 0 °C for 6 hours to produce pinner salt which was further treated with formic hydrazide and drop wise addition of titanium chloride to provide compound (**14**) in 91% yield. Similarly, compound (**7**) was also synthesized from compound (**5**) using this current protocol, which has furnished 94% yield.



Scheme 2: Synthesis of 2-substituted-10-phenylsulfonylphenothiazine conjugates



Scheme 3: Synthesis of 2-substituted phenothiazine conjugates

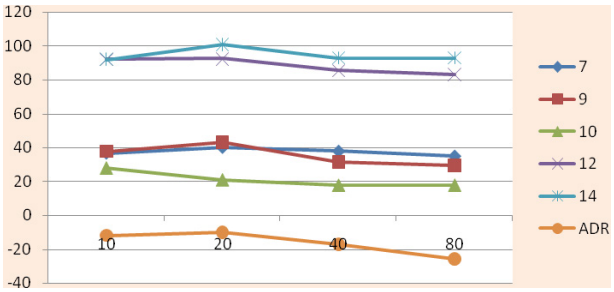


Figure 1: Growth Curve of human prostate cancer cell line DU-145

Antiproliferative activity:

The antiproliferative activity of the synthesized compounds (**7**, **9**, **10**, **12** and **14**) was evaluated on human prostate cancer cell line DU-145 and human melanoma cell line SK-MEL-2 by SRB assay after 48 hours of test sample treatment.

Structure activity relationship (SAR): The tested compounds showed moderate to good activities against both the human cancer cell lines. Among these conjugates, (**7**), (**9**) and (**10**) exhibited the highest antiproliferative potency (**Figure 1**) with GI50 of <10 for DU-145 cell lines (**Table 1**). The melanoma cell line (SK-MEL-2) was also found to be very sensitive for few compounds amongst which (**7**) and (**10**) exhibited the highest antiproliferative potency (**Figure 2**) with GI50 of <10µM (**Table 2**).

Table 1: % cell growth of human prostate cancer cell line DU-145

Compound	Human prostate cancer cell line DU-145 (% Control Growth)				GI50 (µM)
	Sample concentrations (µg/ml)				
	10	20	40	80	
7	36.8	40.2	37.9	35.0	<10
9	37.7	43.2	31.4	29.3	<10
10	28.0	21.1	17.9	18.4	<10
12	92.1	92.5	85.4	83.1	>80
14	91.9	100.8	92.7	92.7	>80

Adriamycin (ADR) was used as the positive control. TGI values of ADR were <10 for both DU-145 and SK-MEL-2 cell lines. SAR analysis indicated that, antiproliferative potencies of the molecules in which benzenesulfonyl moity was not present, e.g., compounds **(12)** and **(14)** were substantially low as compared to (**7**), (**9**) and (**10**). This fact leads to the very important conclusion that, the phenylsulfonyl moiety is essential for maintaining the antiproliferative potency of the compounds.

It has been observed that, amongst the three most active compounds (**7**) and (**9**) showed similar activity on DU-145, whereas (**10**) showed much higher activity. This suggests that the moiety present at 2-position of phenylsulfonyl phenothiazine ring also played an integral role in maintaining the antiproliferative activity. Presence of thiadiazole ring at 2-position of phenylsulfonylphenothiazine derivative has increased the activity of the compound (**10**) as compared to the presence of triazole ring in (**7**) or 2-hydrazinopyrazine ring in (**9**).

This type of variation in antitumor activity was also noted for another cell line SK-MEL-2, where (**7**) and (**10**) exhibited more activity than (**9**), (**12**) and (**14**). This again conforms to our previous conclusion, i.e. the presence of phenylsulfonyl moiety at the 10-position of the PTZ group is the major crucial factor for

maintaining the antiproliferative potency of the compounds.

Table 2: % cell growth of Human melanoma cell line SK-MEL-2

Compound	Human melanoma cell line SK-MEL-2 (% Control Growth)				GI50
	Sample concentrations (µg/ml)				
	10	20	40	80	
7	2.7	18.0	14.6	8.8	<10
9	35.8	48.0	51.8	51.1	<10
10	2.2	12.9	12.2	10.9	<10
12	39.0	44.5	49.3	52.6	<10
14	40.9	97.5	133.8	74.5	<10
ADR	-12.5	-9.1	-21.6	-4.1	<10

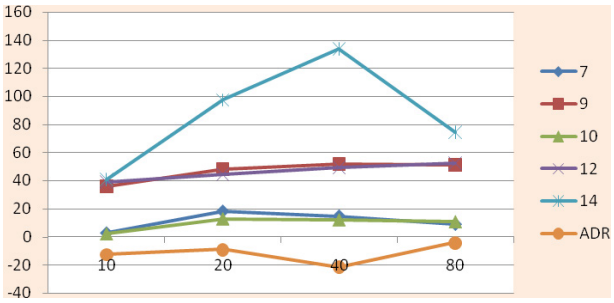


Figure 2: Growth Curve of human melanoma cell line SK-MEL-2

We further rationalized that in case of melanoma cell line compound (**7**) and (**10**) showed almost same activity unlike prostate cell line. This variation is attributable to the change in functionality at 2-position of the phenothiazine ring.

Conclusion:

In the present study, five new 2-substituted-10-phenylsulfonylphenothiazine conjugates were synthesized. All synthesized compounds (**7, 9, 10, 12** and **14**) were evaluated for their activity against human prostate DU-145 and human melanoma SK-MEL-2 cell lines. Among these compounds, compound (**10**) can be considered as the most promising anticancer agent against DU-145 prostate cell line. Compounds (**7**) and (**10**) also showed significant inhibitory effect on SK-MEL-2 melanoma cell line. Substantial loss in antiproliferative potencies have been observed for the compounds (**12**) and (**14**), which do not have phenylsulfonyl group moiety. Among the synthesized compounds, (**7**) and (**10**) were identified as potential antiproliferative agents and selected as lead compounds for further studies.

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