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RESEARCH ARTICLE

Design and Synthesis of Novel Thiosemicarbazones as Potent Anti-breast Cancer Agents

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Abstract: ***Background*:** Thiosemicarbazones and its derivatives received a great pharmaceutical importance due to their prominent biological activities.

***Methods*:** A series of disubstituted thiosemicarbazone derivatives **(1-12)** were designed and synthesized as pure compounds in good yield. All the synthesized compounds were analyzed by spectral data. The anticancer activity of all the compounds was performed against breast cancer MCF-7 and MDA-MB-231 cell lines.

***Results*:** Most of the compounds showed activity against breast cancer MCF-7 and MDA-MB-231 cell lines with (IC_{50} = 12.25 µM – 185.35 µM) and (IC_{50} = 12.97 µM – 107.33 µM), respectively. Compound **9** presented (IC_{50} = 12.76 µM and 12.97 µM) against MCF-7 and MDA-MB-231 cell lines, respectively.

***Conclusion*:** Compound 9, was found to exhibit significant anti-breast cancer activity. This compound was further evaluated for side population percent inhibition assay on the breast cancer cell line MCF-7 at 5 and 10 µM concentration. It showed superiority to block side population by more than 80% at 5 µM concentration compared to the reference drug verapamil.

Keywords: Thiosemicarbazones, MCF-7 cell line, MDA-MB-231 cell line, breast cancer, anti-cancer activity, verapamil.

1. INTRODUCTION

Thiosemicarbazones and their derivatives received a great pharmaceutical importance due to their prominent biological activities. Thiosemicarbazones have been reported as anti-bacterial, anti-viral, anti-malarial and anti-tumor activities [1-4]. Methisazone, a drug used for the treatment of smallpox, is a classic example of thiosemicarbazone [5]. In literature, thiosemicarbazone derivatives have been reported as anticancer agents [6-9]. Examples of thiosemicarbazone derivatives reported to have significant anticancer activities are presented in Fig. (**1**). Thiosemicarbazones have been reported to inhibit the synthesis of DNA by modification in the reductive conversion of ribonucleotides to deoxyribonucleotides [10, 11]. 3-Aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP), (Triapine) displayed considerable advancements in cancer treatment and is presently in phase II clinical trials [12]. By inhibiting the ribonucleotide reductase, it suppresses the tumor growth. Therapeutic potential of 3-AP was found to be narrow due to its toxicity profile and sparingly solubility in water. The most popular cancer in women worldwide is breast cancer. Despite early detection, it remains to be the second most dominant cause of death. Breast cancer stem cells have been reported recently [13]. Prior investigations have identified adult stem cells by a side population (SP) phenotype. A side population is a small percentage, containing tumorigenic part of the total cell line. [14, 15]. Targeting both the normal cancer cells and cancer stem cells can cure cancer [16].

Therefore, there is a need in the structural modification of thiosemicarbazone derivatives to improve the potencies of existing drug candidates. In continuation of our previous research on thiosemicarbazones [17-18], we described herein, the synthesis of novel thiosemicarbazone derivatives that inhibit the growth of breast cancer cell lines especially cancer stem cell and may be useful for the treatment of breast cancer. In our earlier research on thiosemicarbazone derivatives bearing cyclohexyl moiety, the most potent compound showed activity against HER-2 expressed SKBr-3

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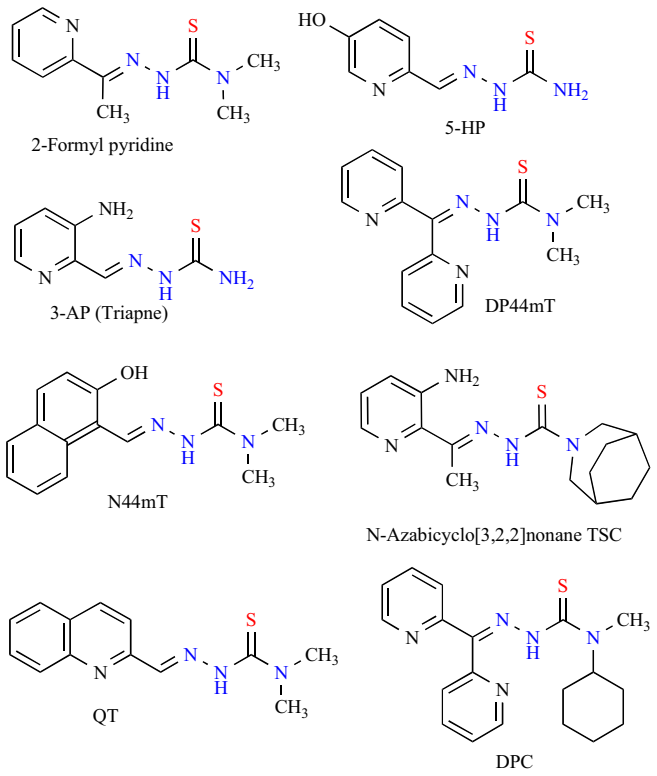


Fig. (1). Thiosemicarbazone derivatives demonstrating potent anti-cancer activities.

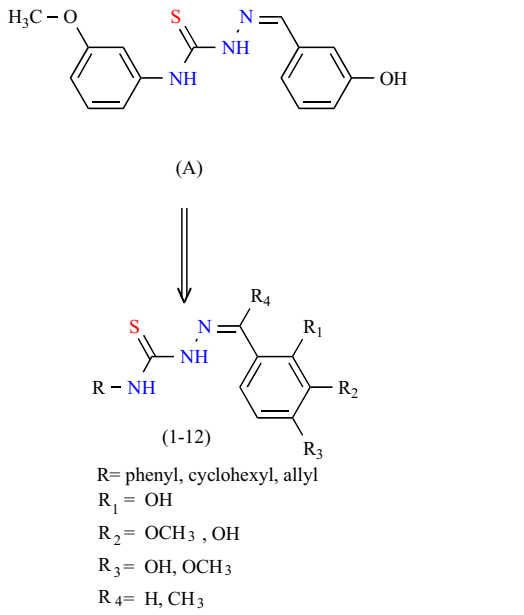


Fig. (2). Structures of lead compound **(A)**.

cells with (IC_{50}= 30.94 ± 0.19 µM) [19]. Optimization of the lead compound produced more potent compound **A**, containing 3-methoxyphenyl group targeting HER-2 overexpressed SKBr-3 cells with (IC_{50}= 17.44 ± 0.01 µM) [20]. The compound **A** was selected as a lead compound for further derivatization (Fig. **2**). Modulation of a 3-methoxyphenyl moiety was carried out with various groups, such as phenyl, cyclohexyl, and allyl. In order to search novel thiosemicarbazone derivatives with significant activity against breast cancer cell lines and cancer stem cells, thiosemicarbazone derivatives bearing phenyl, cyclohexyl, and allyl on one side and disubstituted phenyl groups at the terminal nitrogen were synthesized and their anti-cancer activities against MCF-7 and MDA-MB-231 cell lines were determined.

2. MATERIALS AND METHODS

2.1. Chemistry

In order to check the purity of the synthesized compounds, thin layer chromatography (TLC) was performed on Silica Gel 60 F_{254} coated plates (Merck). TLC spots were viewed using UV light. Spectrum BX, Perkin Elmer FT-IR spectrophotometer was used to carry out FTIR. Gallenkamp melting point apparatus was used to determine melting points. NMR Spectra were processed in DMSO-d_6 on a Bruker NMR spectrophotometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR. Mass spectroscopy was used for the measurement of molecular masses of compounds. The elemental analysis of the compounds was performed on the CHN Elementar (Analysensysteme GmbH, Germany). The elemental analysis for C, H, N, and S was within the limit of ±0.4% and ±0.3% respectively of the theoretical values.

2.1.1. Representative procedure for the synthesis of (1-12)

To a solution of phenyl/cyclohexyl/allyl thiosemicarbazide (0.0119 mol) in absolute ethanol (11 mL), water (22 mL) was added. To this solution, disubstituted aldehydes/ketones (0.0125 mol) and glacial acetic acid (0.55 mL) were added. This mixture was refluxed for an hour prior to cooling it down to room temperature. The precipitate was collected with filtration under vacuum and washed several times with cold water. The compounds were recrystallized from absolute ethanol after filtration of the precipitate in vacuum conditions and washing numerous times with cold water [21].

2.1.1.1. 2-[(4-Hydroxy-3-methoxyphenyl)methylidene]-N-phenylhydrazine-1-carbothioamide (1)

IR (KBr) cm^{-1}: 3319 (OH str.), 3142 (NH str.), 1550 (C=N str.), 1284 (NCSN str.), 1167 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 3.85 (3H, s, OCH_3), 6.82–7.59 (8H, m, Ar–H), 8.07 (1H, s, =C-H), 9.55 (1H, =NNH, D_2O exchg.), 9.99 (s,1H, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 56.3 (OCH_3), 110.4, 115.8, 123.0, 125.8, 126.5, 128.5, 128.9, 139.6, 144.0, 148.5, 149.5, 175.9 (C=S); MS (ESI) *m/z*: 301 $[M]^+$; Analysis for $C_{15}H_{15}N_3O_2S$: C (59.78) H (5.02) N (13.94) S (10.64) %; found C (60.00) H (5.04) N (13.99) S (10.61)% [22].

2.1.1.2. 2-[(3-Hydroxy-4-methoxyphenyl)methylidene]-N-phenylhydrazine-1-carbothioamide (2)

IR KBr (cm^{-1}): 333 (OH str.), 3200 (NH str.), 1550 (C=N str.), 1249 (NCSN str.), 1160 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 3.85 (3H, s, OCH_3), 6.82-7.59 (8H, m, Ar–H), 8.07 (1H, s, =C-H), 9.55 (1H, =NNH, D_2O exchg.), 9.99 (s,1H, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 56.2 (OCH_3), 125.5, 125.8, 128.4, 128.8, 129.7, 134.7, 136.7, 137.6, 139.4, 148.9, 176.0 (C=S); MS (ESI) *m/z*: 301.14 $[M]^+$; Analysis for $C_{15}H_{15}N_3O_2S$: C

(59.78) H (5.02) N (13.94) S (10.64) %; found C (59.55) H (5.03) N (13.98) S (10.60)% [22].

2.1.1.3. 2-[(2-Hydroxy-3-methoxyphenyl)methylidene]-N-phenylhydrazine-1-carbothioamide (3)

IR KBr (cm^{-1}): 3300 (OH str.), 3200 (NH str.), 1546 (C=N str.), 1206 (NCSN str.), 1150 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 3.85 (3H, s, OCH_3), 6.82-7.59 (8H, m, Ar–H), 8.07 (1H, s, =C-H), 9.55 (1H, =NNH, D_2O exchg.), 9.99 (s,1H, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 56.2 (OCH_3), 113.1, 118.5, 119.3, 121.1, 139.8, 146.3, 148.2, 178.0 (C=S); MS (ESI) *m/z*: 301.14 $[M]^+$; Analysis for $C_{15}H_{15}N_3O_2S$: C (59.78) H (5.02) N (13.94) S (10.64) %; found C (59.58) H (5.01) N (13.98)% [23].

2.1.1.4. 2-[1-(4-Hydroxy-3-methoxyphenyl)ethylidene]-N-phenylhydrazine-1-carbothioamide (4)

IR KBr (cm^{-1}): 3332 (OH str.), 3000 (NH str.), 1508 (C=N str.), 1313 (NCSN str.), 1150 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 2.35 (3H, s, CH_3), 3.85 (3H, s, -OCH_3), 6.80–7.63 (8H, m, Ar–H), 9.42 (1H, s, =NNH, D_2O exchg.), 9.98 (1H, s, NHC=S, D_2O exchg.), 10.48 (1H, s, -OH, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 14.9 (CH_3), 56.3 (OCH_3), 111.2, 115.4, 121.0, 124.1, 125.6, 125.9, 128.5, 129.1, 139.6, 147.8, 148.9, 150.2, 176.9 (C=S); MS (ESI) *m/z*: 312.14 $[M-3]^+$; Analysis for $C_{16}H_{17}N_3O_2S$: C (60.93) H (5.43) N (13.32) S (10.17) %; found C (60.70) H (5.45) N (13.37) S (10.20)%.

2.1.1.5. N-cyclohexyl-2-[(4-hydroxy-3-methoxyphenyl)methylidene]hydrazine-1-carbothioamide (5)

IR KBr (cm^{-1}): 3335 (OH str.), 2928 (NH str.), 1510 (C=N str.), 1281 (NCSN str.), 1117 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 1.12–1.91 (10 H, m, cyclohexyl), 4.20 (1H, s, cyclohexyl), 6.80–7.32 (3H, m, Ar–H), 7.80 (1H, s, =CH), 7.97 (1H, s, =NNH, D_2O exchg.), 11.28 (1H, s, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 21.5 (CH_2), 25.3 (CH_2), 25.5 (CH_2), 32.3 ($2CH_2$), 52.9 (CH), 56.2 (OCH_3), 110.9, 115.9, 122.0, 125.8, 143.2, 148.3, 149.2, 175.7 (C=S); MS (ESI) *m/z*: 308.38 $[M+1]^+$; Analysis for $C_{15}H_{21}N_3O_2S$: C (58.61) H (6.89) N (13.67) S (10.43) %; found C (58.38) H (6.87) N (13.72) S (10.40)%.

2.1.1.6. N-cyclohexyl-2-[(3-hydroxy-4-methoxyphenyl)methylidene]hydrazine-1-carbothioamide (6)

IR KBr (cm^{-1}): 3304 (OH str.), 3001 (NH str.), 1548 (C=N str.), 1280 (NCSN str.), 1125 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 1.12–1.91 (10 H, m, cyclohexyl), 4.20 (1H, s, cyclohexyl), 6.93–7.29 (3H, m, Ar–H), 7.86 (1H, s, =CH), 7.93 (1H, s, =NNH, D_2O exchg.), 9.17 (1H, s, NHC=S, D_2O exchg.), 11.25 (1H, s, -OH, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 21.5 (CH_2), 25.3 (CH_2), 25.5 (CH_2), 32.3 ($2CH_2$), 52.8 (CH), 56.0 (OCH_3), 112.0, 113.2, 120.9, 127.3, 143.1, 147.1, 150.0, 175.7 (C=S); MS (ESI) *m/z*: 306.14 $[M-1]^+$; Analysis for $C_{15}H_{21}N_3O_2S$: C (58.61) H (6.89) N (13.67) S (10.43) %; found C (58.39) H (6.88) N (13.70) S (10.41)%.

2.1.1.7. N-cyclohexyl-2-[(2-hydroxy-3-methoxyphenyl)methylidene]hydrazine-1-carbothioamide (7)

IR KBr (cm^{-1}): 3348 (OH str.), 3200 (NH str.), 1550 (C=N str.), 1263 (NCSN str.), 1121 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 1.12–2.0 (10 H, m, cyclohexyl), 4.20 (1H, s, cyclohexyl), 6.70–7.50 (3H, m, Ar–H), 7.92 (1H, s, =CH), 8.41 (1H, s, =NNH, D_2O exchg.), 11.38 (1H, s, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 25.3 ($2CH_2$), 25.5 (CH_2), 32.2 ($2CH_2$), 53.0 (CH), 56.2 (OCH_3), 113.1, 118.4, 119.4, 121.0, 139.7, 146.4, 148.3, 175.9 (C=S); MS (ESI) *m/z*: 307.40 $[M]^+$; Analysis for $C_{15}H_{21}N_3O_2S$: C (58.61) H (6.89) N (13.67) S (10.43) %; found C (58.40) H (6.90) N (13.69) S (10.44)%.

2.1.1.8. N-cyclohexyl-2-[1-(4-hydroxy-3-methoxyphenyl)ethylidene]hydrazine-1-carbothioamide (8)

IR KBr (cm^{-1}): 3335 (OH str.), 2936 (NH str.), 1550 (C=N str.), 1201 (NCSN str.), 1117 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 1.35-1.91 (10 H, m, cyclohexyl), 2.27 (3H, s, CH_3), 4.20 (1H, s, cyclohexyl), 6.80–7.42 (3H, m, Ar–H), 7.94 (1H, s, =CH), 7.80 (1H, s, =NNH, D_2O exchg.), 10.09 (1H, s, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 14.6 (CH_3), 18.9 (CH_2), 25.0 (CH_2), 25.5 (CH_2), 32.1 (CH_2), 32.2 (CH_2), 52.6 (CH), 56.5 (OCH_3), 110.5, 115.5, 120.5, 129.3, 147.8, 148.7, 148.8, 176.9 (C=S); MS (ESI) *m/z*: 321.68 $[M]^+$; Analysis for $C_{16}H_{23}N_3O_2S$: C (59.78) H (7.21) N (13.7) S (9.98) %; found C (59.55) H (7.24) N (13.74) S (9.95) %.

2.1.1.9. 2-[(4-Hydroxy-3-methoxyphenyl)methylidene]-N-(prop-2-en-1-yl)hydrazine-1-carbothioamide (9)

IR KBr (cm^{-1}): 3318 (OH str.), 3200 (NH str.), 1550 (C=N str.), 1278 (NCSN str.), 1121 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 3.80 (3H, s, -OCH_3), 4.22 (2H, d, CH_2), 5.10 (2H, m, =CH_2), 5.88 (1H, m, =CH), 6.77–7.37 (3H, m, Ar–H), 7.95 (1H, s, =CH), 8.52 (1H, s, =NNH, D_2O exchg.), 11.35 (1H, s, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 21.6 (CH_2), 46.1 (=CH_2), 56.3 (OCH_3), 110.5, 115.8, 112.4, 125.9, 135.6, 143.3, 148.4, 149.2, 177.2 (C=S); MS (ESI) *m/z*: 264.14 $[M-1]^+$; Analysis for $C_{12}H_{15}N_3O_2S$: C (54.32) H (5.70) N (15.84) S (12.08) %; found C (54.11) H (5.71) N (15.90) S (12.05)%.

2.1.1.10. 2-[(3-Hydroxy-4-methoxyphenyl)methylidene]-N-(prop-2-en-1-yl)hydrazine-1-carbothioamide (10)

IR KBr (cm^{-1}): 3149 (OH str.), 2929 (NH str.), 1542 (C=N str.), 1272 (NCSN str.), 1121 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 3.80 (3H, s, -OCH_3), 4.22 (2H, d, CH_2), 5.10 (2H, m, =CH_2), 5.89 (1H, m, =CH), 6.77–7.37 (3H, m, Ar–H), 7.95 (1H, s, =CH), 8.52 (1H, s, =NNH, D_2O exchg.), 11.35 (1H, s, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 22.7 (CH_2), 46.1 (=CH_2), 56.0 (OCH_3), 112.0, 113.4, 115.9, 120.8, 127.5, 135.6, 143.0, 147.1, 150.1, 177.3 (C=S); MS (ESI) *m/z*: 265.13 $[M]^+$; Analysis for $C_{12}H_{15}N_3O_2S$: C (54.32) H (5.70) N (15.84) S (12.08) %; found C (54.50) H (5.69) N (15.87) S (12.07)%.

2.1.1.11. 2-[(2-Hydroxy-3-methoxyphenyl)methylidene]-N-(prop-2-en-1-yl)hydrazine-1-carbothioamide (11)

IR KBr (cm^{-1}): 3308 (OH str.), 3000 (NH str.), 1578 (C=N str.), 1281 (NCSN str.), 1208 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 3.83 (3H, s, -OCH_3), 4.22 (2H, t, CH_2), 5.10 (2H, m, =CH_2), 5.91 (1H, m, =CH), 6.78–7.57 (3H, m, Ar–H), 8.44 (1H, s, =CH), 8.59 (1H, s, =NNH, D_2O exchg.), 11.51 (1H, s, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 11.7 (CH_2), 46.2 (=CH_2), 56.3 (OCH_3), 113.1, 115.9, 118.5, 119.4, 121.2, 135.5, 139.7, 146.4, 148.3, 177.4 (C=S); MS (ESI) *m/z*: 265.30 $[M]^+$; Analysis for $C_{12}H_{15}N_3O_2S$: C (54.32) H (5.70) N (15.84) S (12.08) %; found C (54.52) H (5.72) N (15.90) S (12.04)%.

2.1.1.12. 2-[1-(4-Hydroxy-3-methoxyphenyl)ethylidene]-N-(prop-2-en-1-yl)hydrazine-1-carbothioamide (12)

IR KBr (cm^{-1}): 3348 (OH str.), 3100 (NH str.), 1550 (C=N str.), 1263 (NCSN str.), 1121 (C=S str.); ^{1}H NMR (DMSO–d_6, 500 MHz) δ: 3.83 (3H, s, -OCH_3), 4.25 (2H, m, CH_2), 5.14 (2H, m, =CH_2), 5.92 (1H, m, =CH), 7.32–7.45 (3H, m, Ar–H), 8.50 (1H, s, =CH), 9.37 (1H, s, =NNH, D_2O exchg.), 10.16 (1H, s, NHC=S, D_2O exchg.); ^{13}C NMR (DMSO–d_6, 125 MHz) δ: 14.6 (CH_2), 46.2 (=CH_2), 56.3 (OCH_3), 111.1, 115.5, 115.7, 120.7, 129.3, 135.6, 147.8, 148.7, 149.1, 178.3; MS (ESI) *m/z*: 279.08 $[M]^+$; Analysis for $C_{13}H_{17}N_3O_2S$: C (55.89) H (6.13) N (15.04) S (11.48) %; found C (55.70) H (6.15) N (15.10) S (11.45)%.

2.2. Cell lines

MCF-7 cells were grown in McCoy's 5A (GIBCO, 8717, Grovement Cir, Gaithersberg, MD, USA), and MDA-MB-231 cells were grown in DMEM (Sigma, 82024 Taufkirchen, Germany). MCF-7 and MDA-MB-231 breast cancer cell lines were procured from the American Type Culture Collection (ATCC) (0801 University Boulevard, Manassas, VA, USA). Hemocytometer was used to calculate the number of cells.

2.3. Measurement of IC_{50}

IC_{50} was mathematically calculated as IC_{50} = fixed dose (20) × 50/ (formazan quantity of treated cells/formazan quantity of untreated cells) × 100 [24].

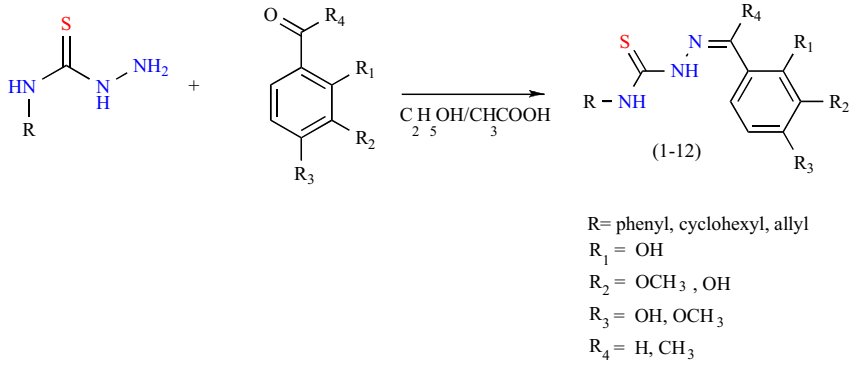
2.4. Side Population Staining by DYECYCLE Violet Stain

Functionally, to gate only side population cells, verapamil 50 µM was used. All analyses were performed on a FACS LSRII (BD Biosciences, San Jose, CA, USA) [25].

3. RESULTS AND DISCUSSIONS

The synthesis of thiosemicarbazone derivatives **(1-12)** was carried out as shown in Scheme **1**. The phenyl/cyclohexyl/allyl thiosemicarbazides were reacted with disubstituted aldehydes/ketones in the presence of absolute ethanol and glacial acetic acid to yield final thiosemicarbazones **(1-12)**. Elemental analysis verified the purities of compounds. The compounds were confirmed and characterized by spectroscopic methods. The spectra of all thiosemicarbazones showed D_2O exchangeable singlet at δ 7.80–9.55 ppm corresponding to NH protons and NHC=S protons, respectively. The presence of all carbon atoms for compounds was confirmed by ^{13}C NMR spectra. Carbon signal of the C=S group of thiosemicarbazone appeared in the 175.6–178.0 ppm region. The experimental part contains the detailed spectral results of ^{1}H NMR, ^{13}C NMR spectra, and mass spectra of all the synthesized compounds. The physicochemical data of all the synthesized compounds are given in Table **1**.

Cell growth inhibition assay was used for the *in vitro* antiproliferative activity. WST-1 was used according to the protocol for the calculation of IC_{50} for each compound (Table **2**). The compounds showed activity against luminal MCF-7 cells with IC_{50} ranging between (12.2 ± 0.59 µM) to (185 ± 0.35 µM). The compounds also showed activity against basal cell line MDA-MB-231 with IC_{50} ranging between (12.9 ± 0.76 µM) to (107 ± 0.33 µM). Compound **12** was found to be inactive up to 300 µM concentration. Compound **9** was found to be the most potent compound of the series with IC_{50} = (12.7 ± 0.64 µM) and (12.9 ± 0.76 µM) against luminal MCF-7 and basal cell line MDA-MB-231 respectively compared to the standard drug 5-fluorouracil (5-FU) IC_{50} = (15.23 ± 0.80 µM) and (29.38 ± 1.24 µM) against MCF-7 and MDA-MB-231, respectively. This compound was then further assessed for side population percent inhibition assay on the MCF-7 cell line at 5 µM and 10 µM concentration (Fig. **3**). The reference drug verapamil was used to



Scheme 1. Synthetic protocol of compounds **(1-12)**.

Table 1. Physical data of the synthesized compounds (1-12).

Compounds	R	R_1	R_2	R_3	R_4	Molecular Weight	Yield (%)	M.p. (°C)
1	Phenyl	H	OCH_3	OH	H	$C_{15}H_{15}N_3O_2S$	80	165-167
2	Phenyl	H	OH	OCH_3	H	$C_{15}H_{15}N_3O_2S$	85	170-172
3	Phenyl	OH	CH_3	H	H	$C_{15}H_{15}N_3O_2S$	90	195-197
4	Phenyl	H	OCH_3	OH	CH_3	$C_{16}H_{17}N_3O_2S$	75	198-200
5	Cyclohexyl	H	OCH_3	OH	H	$C_{15}H_{21}N_3O_2S$	75	108-110
6	Cyclohexyl	H	OH	OCH_3	H	$C_{15}H_{21}N_3O_2S$	70	168-170
7	Cyclohexyl	OH	OCH_3	H	H	$C_{15}H_{21}N_3O_2S$	85	≥230
8	Cyclohexyl	H	OCH_3	OH	CH_3	$C_{16}H_{23}N_3O_2S$	75	178-180
9	Allyl	H	OCH_3	OH	H	$C_{12}H_{15}N_3O_2S$	70	125-127
10	Allyl	H	OH	OCH_3	H	$C_{12}H_{15}N_3O_2S$	75	176-178
11	Allyl	OH	OCH_3	H	H	$C_{12}H_{15}N_3O_2S$	70	218-220
12	Allyl	H	OCH_3	OH	CH_3	$C_{13}H_{17}N_3O_2S$	65	128-130

block side population cells at 50 μM concentration. The untreated MCF-7 cells showed a 4.8% side population. When the MCF-7 cells were treated with compound **9**, 0.8% and 0.7% side population were obtained at 5 μM and 10 μM concentrations, respectively as compared to verapamil (50 μM) with 0.6% of side population. Compound **9** showed superiority to block side population by more than 80% at lower concentration compared with the reference drug verapamil.

The design of new compounds was based on compound (**A**) and the structural modifications were done not only to obtain derivatives with higher anti-proliferative activity but also to collect data regarding structure-activity relationship (SAR). We showed that the presence of pharmacophore (NHC=SNHN) is essential for the activity. The anti-proliferative activity of the compounds was affected by replacing 3-methoxyphenyl with phenyl, cyclohexyl and allyl groups. Phenyl group at the proximal end decreased the activity whereas the anti-proliferative activity was increased by replacing with cyclohexyl and allyl groups. The compounds containing allyl group were found to be most active in the series. The disubstituted phenyl moiety at the terminal nitrogen had a minimum effect on the activity. Methyl group at R^4 increased the anti-proliferative effect. These results indicate that substitution at the proximal nitrogen of the thiosemicarbazone has a more significant role in the anti-proliferative activity than the substitution at the terminal nitrogen of the thiosemicarbazone.

CONCLUSION

In conclusion, we synthesized novel thiosemicarbazone derivatives **(1-12)** from the lead compound and were confirmed by spectral data. The synthesized compounds **(1-12)** were evaluated *in vitro* against breast cancer cell lines MCF-7 and MDA-MB-231. All the compounds except compound **12** were active against both the cell lines. Compound **9** was having the most potent anti-proliferative activity against both the tested cell lines. It presented more than 80% inhibition of side population in comparison to the standard drug verapamil using the MCF-7 cell line. It can act a lead for targeting breast cancer cell line MCF-7 and MDA-MB-231. There is wide scope for the further development of this compound in pharmacokinetic and pharmacodynamic studies.

Table 2. *In vitro* cytotoxicity of compounds against breast cancer cell lines MCF-7 and MDA-MB-231.

Compounds	IC_{50}[a] (μM) MCF-7 (Mean ±SD)*	IC_{50} (μM) MDA-MB-231 (Mean ±SD)*
1	185±0.35	107±0.33
2	30±0.64	25.6±0.81
3	20±0.72	24.5±0.13
4	16±0.91	18.6±0.43
5	14.7±0.86	14.5±0.62
6	13.8±0.30	13.4±0.56
7	17.3±0.84	20.9±0.81
8	13±0.10	13.3±0.56
9	12.7±0.64	12.9±0.76
10	12.2±0.59	13.8±0.79
11	12.5±0.33	14.2±0.91
12	In[b]	In
5-FU[c]	15.23±0.80	29.38 ± 1.24

[a] IC_{50}: concentration of the compound (μM) producing 50% cell growth inhibition after 48 h of compound exposure. [b] Inactive within 300 (μM) concentration range. [c] 5-FU: 5-fluorouracil. *Mean ±SD of three independent experiments.

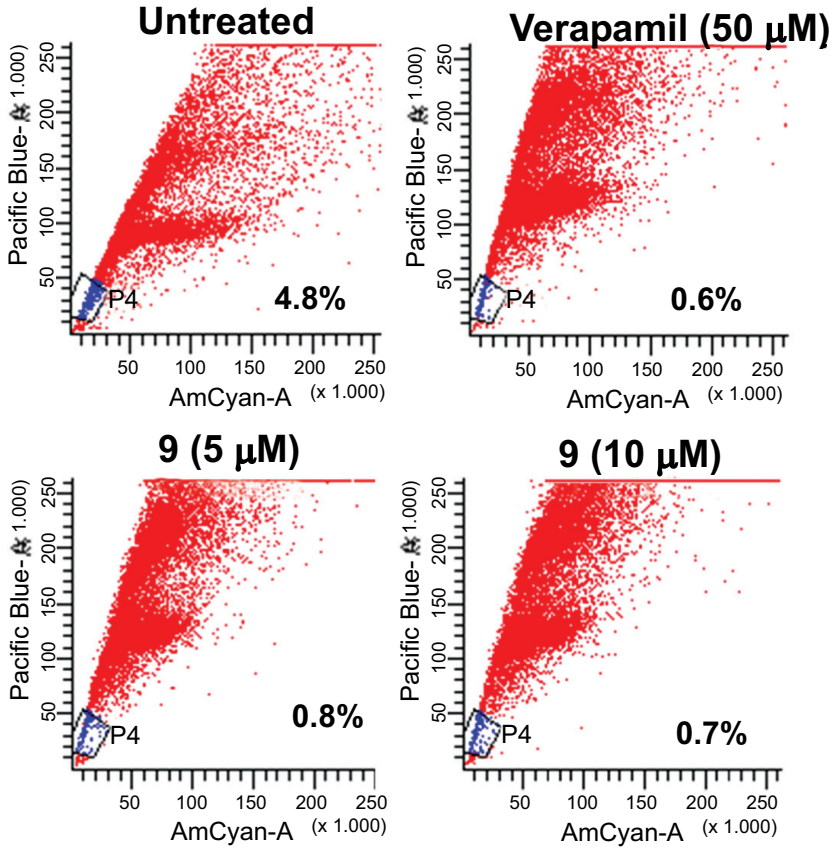


Fig. (3). The side population percent inhibition on breast cancer cell line MCF-7 at (5 µM) and (10 µM) concentration of compound **9** and reference drug verapamil (50 µM).

AUTHOR'S CONTRIBUTIONS

Compound synthesis were performed by M.A.B.; Al-T.M. performed the anti-proliferative activity, M.A.A. and A.M.N. helped in the analysis and preparation of the manuscript. A. Al-D performed the flow cytometry.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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