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Article

Lead Optimization of 2-Cyclohexyl-*N*-[(*Z*)-(3-methoxyphenyl/3-hydroxyphenyl) methylidene]hydrazinecarbothioamides for Targeting the HER-2 Overexpressed Breast Cancer Cell Line SKBr-3

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Abstract: Lead derivatives of 2-cyclohexyl-*N*-[(*Z*)-(3-methoxyphenyl/3-hydroxyphenyl) methylidene]hydrazinecarbothioamides **1**–**18** were synthesized, characterized and evaluated *in vitro* against HER-2 overexpressed breast cancer cell line SKBr-3. All the compounds showed activity against HER-2 overexpressed SKBr-3 cells with IC_{50} = 17.44 ± 0.01 μM to 53.29 ± 0.33 μM. (2*Z*)-2-(3-Hydroxybenzylidene)-*N*-(3-methoxyphenyl)hydrazinecarbothioamide (**12**, IC_{50} = 17.44 ± 0.01 μM) was found to be most potent compound of this series targeting HER-2 overexpressed breast cancer cells compared to the standard drug 5-fluorouracil

(5-FU) (IC_{50} = 38.58 ± 0.04 μM). Compound **12** inhibited the cellular proliferation via DNA degradation.

Keywords: thiosemicarbazones; HER-2; SKBr-3 cells; BT-474 cells; cancer stem cells

1. Introduction

Thiosemicarbazones constitute an important class of pharmacophore that has been explored by medicinal chemists owing to its wide range of biological activities, which include antibacterial, antimalarial, antiviral and antitumor activities [1–3]. Methisazone which was used for the treatment of smallpox, is an example of a thiosemicarbazone drug [4]. Thiosemicarbazone derivatives having potent anticancer activities have been reported in the literature [5–8]. Triapine, a potent anti-proliferative is effective against several cancer types. It obstructs tumor growth by inhibiting ribonucleotide reductase. Triapine (3-AP, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone) has shown significant usefulness in the treatment of cancer and is currently in phase II clinical trials [9]. The therapeutic potential of 3-AP is however limited because of its poor water solubility and toxicity profile.

Worldwide the most common cancer and the second most leading cause of mortality in women is breast cancer, despite improvements in early detection. Recently the existence of breast cancer stem cells has been reported. Because of their resistance to conventional treatment, small populations of cells that are relatively resistant to therapy and able to repopulate *in vivo*, called cancer stem cells (CSCs), are believed to be responsible for treatment failures. CSCs have fundamental implications for the early detection, prevention and treatment of cancer. Drug discovery programs for cancer usually select compounds which have the property of inducing cytotoxic effects in cancer cell lines [10]. Unfortunately, the cytotoxic effects *in vitro* and inhibition of tumor growth *in vivo* is not the end story for curing cancer in preclinical models because of the existence of CSCs, as tumors are maintained by a self-renewing CSC population [11]. There are now various research findings which have confirmed cancer stem cells in leukemia [12], breast [13], brain [14], lung [15], colon [16]. To cause relapse, CSCs must have survived primary treatment [17]. Studies suggested that aldehyde dehydrogenase 1 (ALDH-1) is a more potent marker of breast CSCs [18–20]. ALDH-1-positive cells are resistant to conventional chemotherapy with paclitaxel and epirubicin [21]. ALDH-1-positive breast cancer CSCs can induce tumor formation with a few as 500 cells. Breast cancer cells that expressed ALDH-1 were more likely to be estrogen receptor (ER) negative, progesterone receptor (PR) negative and human-epidermal growth factor receptor-2 (HER-2) positive. Several reports demonstrated that HER-2 regulate CSCs. Cells displaying stem cell properties such as sphere formation or increased aldehyde dehydrogenase expression also have increased HER-2 expression compared with bulk cell population [22]. Traditionally breast cancer can be classified into three main subtypes: luminal, basal like and human epidermal growth factor receptor-2 positive $(HER\text{-}2)^{+}$. Clinical and laboratory evidences have indicated that overexpression of HER-2 may render tumor cells resistant to several anticancer drugs [23]. Thus, there remains an urgent need for new pharmaceutical compounds and compositions to effectively eradicate and target cancer stem cells. We need to target both proliferating cells as well as cancer stem cells in order to cure cancer [24].

Therefore there is high potential in structural modification of thiosemicarbazone (TSC) derivatives to improve the existing drug candidates. In our previous research on TSC derivatives bearing a cyclohexyl moiety, the synthesized compounds showed activity against HER-2 expressed SKBr-3 cells with IC_{50} = 25.6 ± 0.07 μM − 61.6 ± 0.4 μM. The two compounds (2-cyclohexyl-*N*-[(*Z*)-(3-methoxyphenyl) methylidene]hydrazinecarbothioamide (**C2**) and (2-cyclohexyl-*N*-[(*Z*)-(3-hydroxyphenyl)-methylidene]hydrazinecarbothioamide (**C10**) (Figure 1) showed inhibitory effects on $ALDH^+$ population more effectively than the reference drug, salinomycin. Compound **C2** was the most active, showing a 50% inhibitory effect [25]. These two compounds **C2** and **C10** were selected as lead compounds for further derivatization. Modulation of the cyclohexyl moiety was performed using different groups like phenyl, substituted phenyl, benzyl, alkyl, alkenyl, etc. In order to discover novel TSC derivatives with significant activity against CSCs. TCS derivatives bearing 3-hydroxy/3-methoxy phenyl on one side and different groups at the terminal nitrogen were synthesized and their antitumor activities on SKBr-3 and BT-474 cell line were determined.

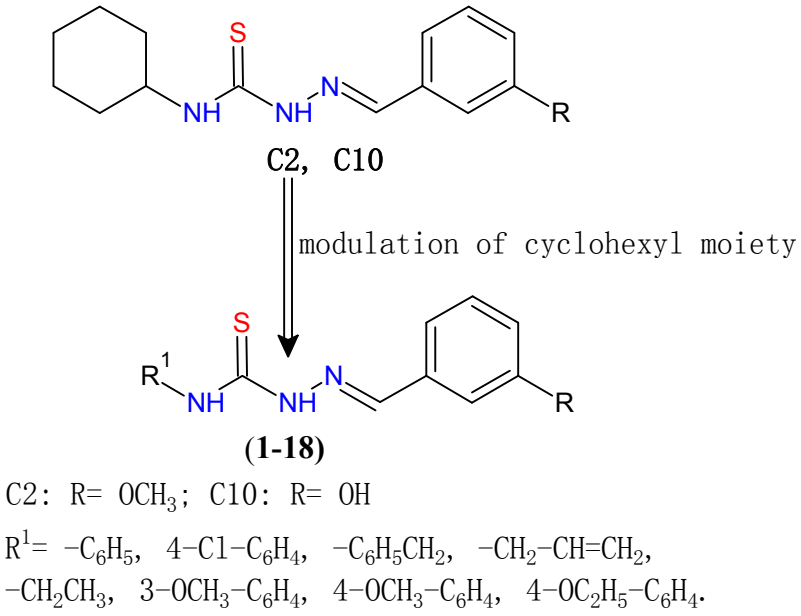


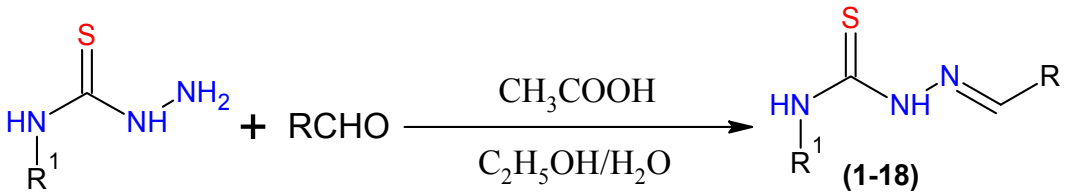
Figure 1. Structures of lead compound **C2**, **C10** and newly synthesized compounds **1**–**18**.

2. Results and Discussion

2.1. Chemistry

The synthesis of thiosemicarbazone derivatives **1**–**18** was carried out in single step as shown in Scheme 1. Thiosemicarbazides were reacted with 3-methoxybenzaldehyde/3-hydroxybenzaldehyde in the presence of acetic acid to yield 2-cyclohexyl-*N*-[(*Z*)-(3-methoxyphenyl/3-hydroxyphenyl) methylidene]hydrazinecarbothioamide derivatives **1**–**18**. The purity of the compounds was checked by TLC and elemental analysis. The compounds were characterized and confirmed by spectral data. In the ^{1}H-NMR spectra, the signals of the respective protons of the derivatives were verified on the basis of chemical shifts, multiplicity and coupling constants. The spectra of all synthesized compounds showed a D_2O exchangeable singlet at 6.3–8.3 ppm and 11.1–11.7 ppm corresponding to NH protons and

NHC=S protons. The analytical and spectral data of the compounds was in good agreement with the composition of the synthesized compounds. The physiochemical property data of the of all compounds is given in Table 1.



R = 3-OCH$_3$-C$_6$H$_4$; 3-OH-C$_6$H$_4$

R^1= -C$_6$H$_5$, 4-Cl-C$_6$H$_4$, -C$_6$H$_5$CH$_2$, -CH$_2$-CH=CH$_2$, -CH$_2$CH$_3$, 3-OCH$_3$-C$_6$H$_4$, 4-OCH$_3$-C$_6$H$_4$, 4-OC$_2$H$_5$-C$_6$H$_4$.

Scheme 1. Synthetic protocol of compounds **1**–**18**.

Table 1. Physical data of the synthesized compounds **1**–**18**.

Compounds	R	R^1	Molecular Formula	Yield %	Mp (°C)	*C*Log P
1	3-Methoxyphenyl	Phenyl	$C_{15}H_{15}N_3OS$	70	161–162	4.42
2	3-Methoxyphenyl	4-Chlorophenyl	$C_{15}H_{14}ClN_3OS$	75	197–198	5.41
3	3-Methoxyphenyl	Prop-2-en	$C_{12}H_{15}N_3OS$	72	115–116	2.70
4	3-Methoxyphenyl	Benzyl	$C_{16}H_{17}N_3OS$	68	127–129	3.65
5	3-Methoxyphenyl	Ethyl	$C_{11}H_{15}N_3OS$	72	122–123	2.40
6	3-Methoxyphenyl	3-Methoxyphenyl	$C_{16}H_{17}N_3O_2S$	65	148–150	4.68
7	3-Methoxyphenyl	4-Methoxyphenyl	$C_{16}H_{17}N_3O_2S$	70	156–157	4.37
8	3-Methoxyphenyl	4-Ethoxyphenyl	$C_{17}H_{19}N_3O_2S$	68	160–161	4.91
9	3-Methoxyphenyl	3-Chlorophenyl	$C_{15}H_{14}ClN_3OS$	70	138–140	5.45
10	3-Hydroxyphenyl	Phenyl	$C_{14}H_{13}N_3OS$	78	212–213	3.95
11	3-Hydroxyphenyl	4-Chlorophenyl	$C_{14}H_{12}ClN_3OS$	65	222–224	4.94
12	3-Hydroxyphenyl	3-Methoxyphenyl	$C_{15}H_{15}N_3O_2S$	70	185–186	4.21
13	3-Hydroxyphenyl	Prop-2-en	$C_{11}H_{13}N_3OS$	60	94–97	2.22
14	3-Hydroxyphenyl	Benzyl	$C_{15}H_{15}N_3OS$	65	130–132	3.17
15	3-Hydroxyphenyl	4-Methoxyphenyl	$C_{15}H_{15}N_3O_2S$	72	182–183	3.90
16	3-Hydroxyphenyl	4-Ethoxyphenyl	$C_{16}H_{17}N_3O_2S$	70	200–202	4.43
17	3-Hydroxyphenyl	3-Chlorophenyl	$C_{14}H_{12}ClN_3OS$	75	198–200	4.98
18	3-Hydroxyphenyl	Ethyl	$C_{10}H_{13}N_3OS$	60	152–154	1.93

2.2. Anti-Proliferative in Vitro *Activity*

In vitro anti-proliferative activity was measured by the cell growth inhibition assay. For the determination of IC_{50} for each compound, WST-1 reagent was used according to the protocol (Table 2). From our previous experience, TCS derivatives showed selectivity against HER-2 overexpressed cancer cells over luminal and basal subtypes. All the compounds showed activity against HER-2 overexpressed SKBr-3 cell with IC_{50} values ranging between 17.44 ± 0.01 μM to 53.29 ± 0.33 μM. Compound **12** (IC_{50} = 17.44 ± 0.01 μM) was found to be most potent compound of this series targeting HER-2 overexpressed breast cancer cells compared to the standard drug 5-fluorouracil (5-FU) (IC_{50} = 38.58 ±

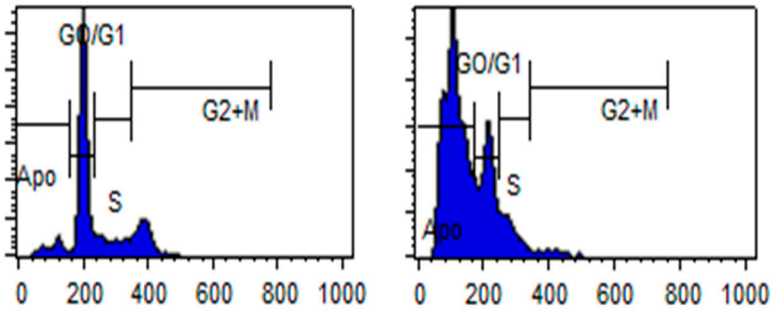
0.04 μM). To gain insight into the anti-proliferation mechanism, the effect on cell cycle distribution was investigated by fluorescence-activated cell sorting (FACS) analysis. SKBr-3 cells were exposed to 10 μM of compound **12** for 48 h and the result was the accumulation of the cells on DNA degradation phase, which is a strong indication that the treatment induced apoptosis by breakdown of the cells' DNA. This was also accompanied by a compensatory decrease in G_1, S and M phase cells. Histograms show the number of cells per channel (vertical axis) *vs.* DNA content (horizontal axis). The values indicate the percentage of cells in the relevant phases of the cell cycle. The analysis shows increase in apoptosis of cells (DNA degradation) by 8 folds compared with untreated cells (Figure 2).

Table 2. *In vitro* cytotoxic activity of compounds against breast cancer cell line SKBr-3.

Compounds	[a] IC_{50} (μM)
	SKBr-3
1	45.67 ± 0.18
2	23.69 ± 0.06
3	17.89 ± 0.03
4	34.52 ± 0.18
5	23.00 ± 0.16
6	24.55 ± 0.02
7	25.00 ± 0.03
8	23.76 ± 0.01
9	27.00 ± 0.03
10	22.32 ± 0.02
11	26.49 ± 0.02
12	17.44 ± 0.01
13	29.26 ± 0.05
14	27.74 ± 0.03
15	53.29 ± 0.33
16	24.75 ± 0.08
17	18.28 ± 0.05
18	22.33 ± 0.03
5-FU	38.58 ± 0.04

[a] IC_{50}: Concentration of the compound (μM) producing 50% cell growth inhibition after 48 h of compound exposure.

These results suggested that compound **12** inhibited the cellular proliferation via DNA degradation. The apoptotic effect of compound **12** was evaluated using annexin-V staining. Cells were harvested after treatment by compound **12** (10 μM) for 48 h and incubated with annexin V-FITC and PI as described in the Experimental Section. Ten thousand cells were analyzed per determination. Dot plots show annexin V-FITC binding on the X axis and PI staining on the Y axis. Dots represent cells as follows: lower left quadrant, normal cells ($FITC^-/PI^-$); lower right quadrant, apoptotic cells ($FITC^+/PI^-$); upper left quadrant, necrotic cells ($FITC^-/PI^+$), upper right quadrant late apoptotic ($FITC^+/PI^+$) (Figure 3).



Marker	% Gated
All	100.00
Apo	8.22
G0/G1	53.19
S	19.83
G2 + M	19.41

Marker	% Gated
All	100.00
Apo	66.84
G0/G1	24.00
S	8.14
G2 + M	1.64

Figure 2. Cell cycle specific blocking activity of compound **12** on SKBr-3 cells.

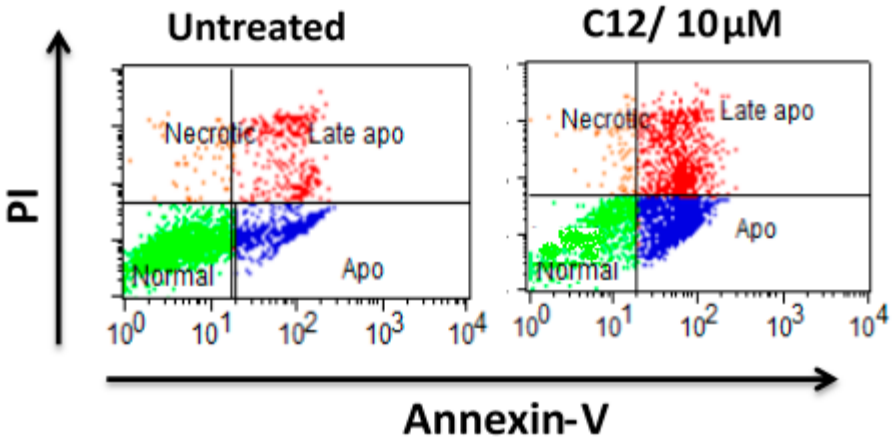


Figure 3. The apoptotic effect of compound **12** using Annexin-V staining of SKBr-3 cells.

The apoptotic population increased from 16.3% in the control group to 49.14% and late apoptosis population increased from 10% to 26% increased after 48 h exposure time using 10 µM of compound **12** for incubation. The cytotoxic effect of compound was not correlated to the increase of necrotic cell population.

The most active compound **12** presented apoptotic effects on overexpressed HER-2 oncogene and little sensitivity was measured on HER-2 negative MDA-MB-231 cells (Figures 4 and 5). Cell adhesion inhibition studies were also carried out using the HER-2 positive SKBr-3 and BT-474 cancer cell lines. Compound **12** at tested concentrations significantly inhibited cell adhesion of SKBr-3 and BT-474 ($p < 0.05$), (Figure 6). The results shown in Figure 7 demonstrate that compound **12** had a maximum effect on cell migration of SKBr-3 and BT-474 cancer cells. It significantly inhibited cell migration of SKBr-3 and BT-474 ($p < 0.05$). Percentages of viable/proliferative BT-474 cells treated with different concentration of compound **12** were determined (Figures 8 and 9). Cell proliferation inhibition was found to be significant at 10 µM concentration of compound **12**.

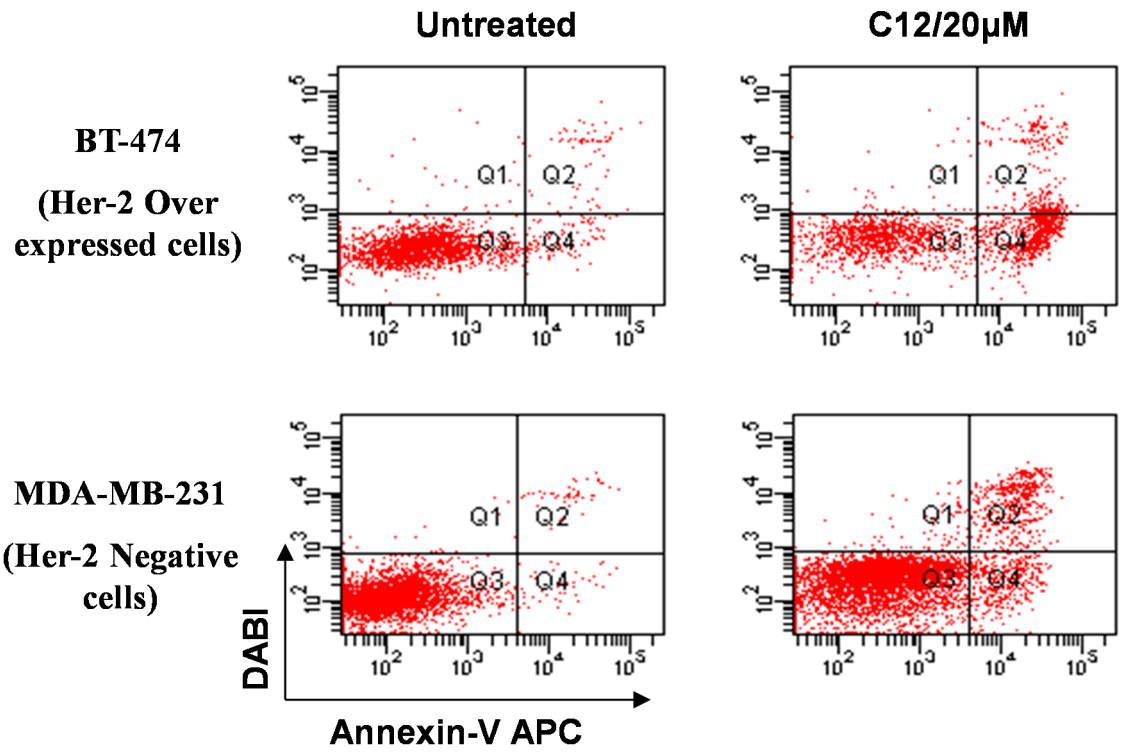


Figure 4. The apoptotic effect of compound **12** on HER-2 positive BT-474 and Her-2 negative MDA-MB-231 cells

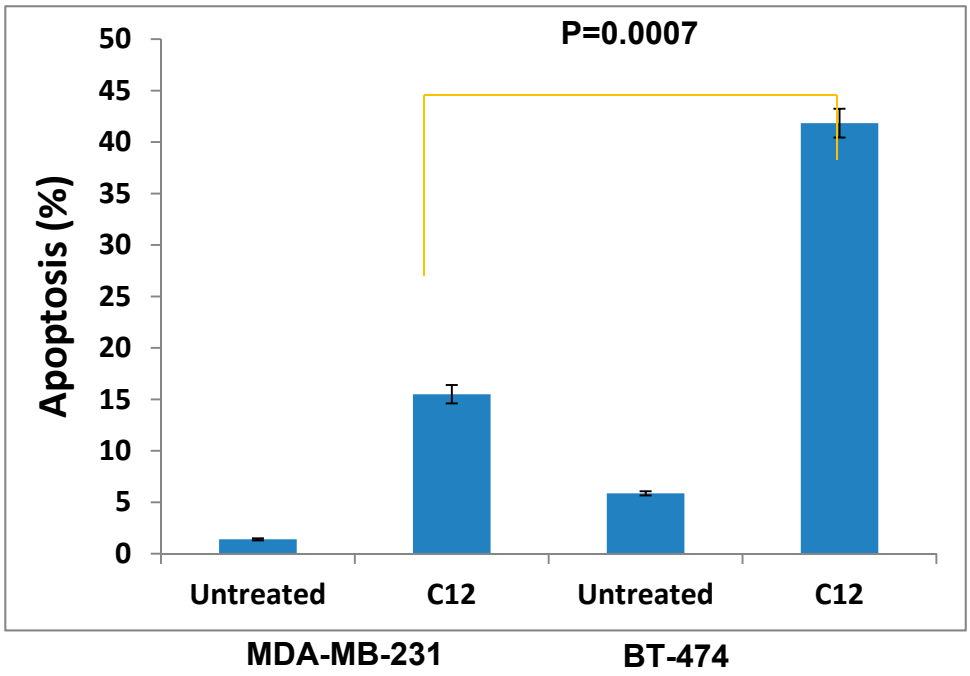


Figure 5. Histogram showing the % apoptosis of compound **12** on HER-2 negative MDA-MB-231 cells and HER-2 positive BT-474.

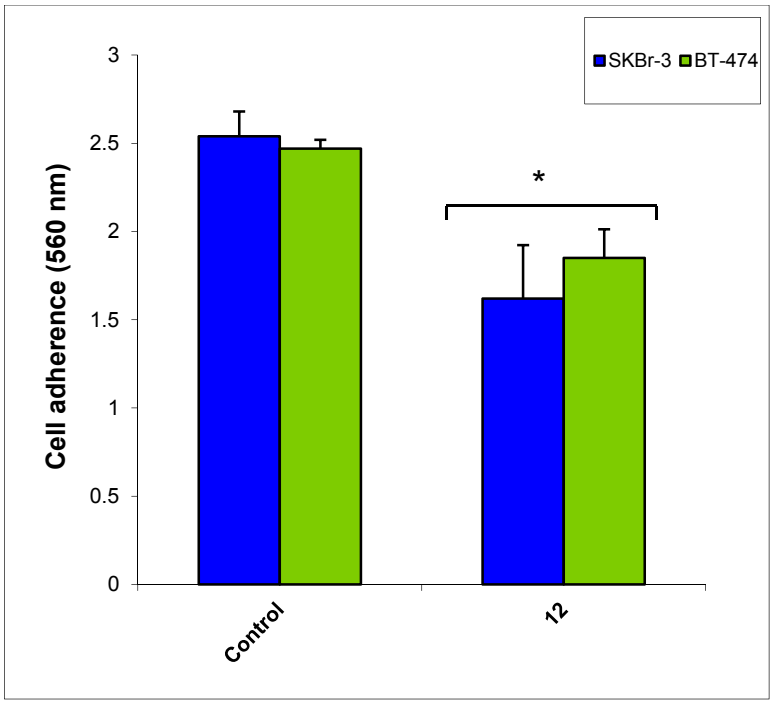


Figure 6. Effect of compound **12** on cell adherence of HER-2 positive cancer cell lines SKBr-3 and BT-474.

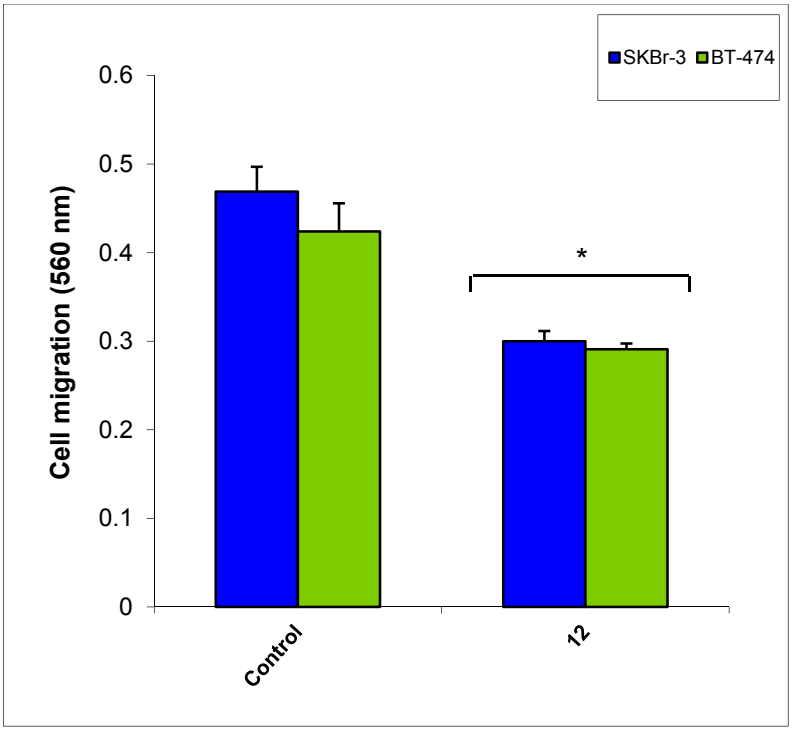


Figure 7. Effect of compound **12** on cell migration of HER-2 positive cancer cell lines SKBr-3 and BT-474.

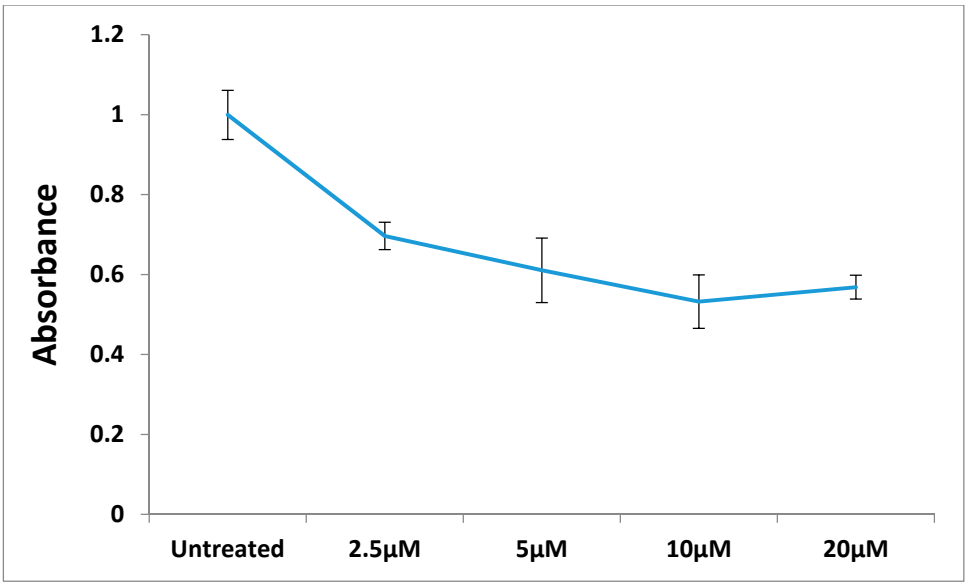


Figure 8. The absorbance of formazan dye produced by viable BT-474 cells treated with different concentrations of compound **12**.

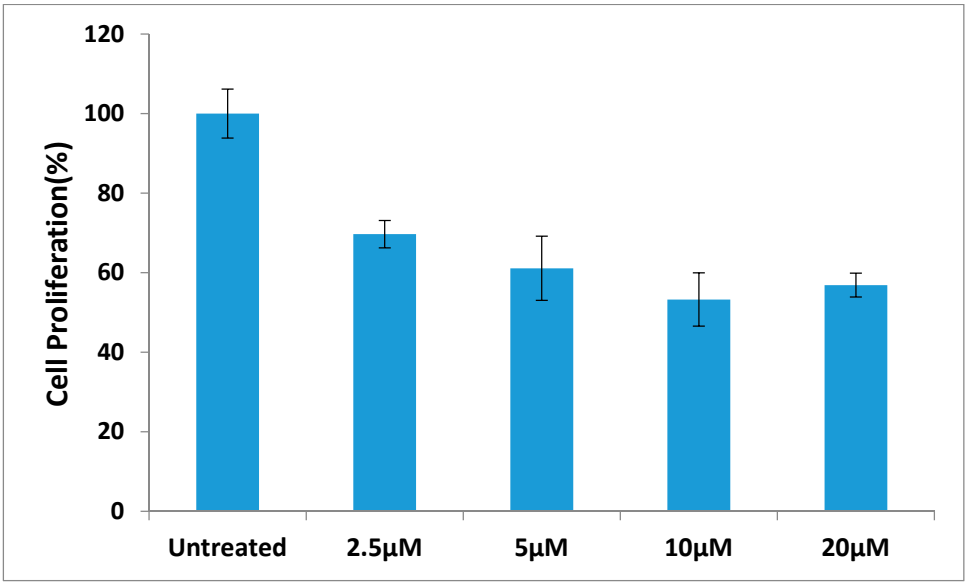


Figure 9. The percentage of viable/proliferative BT-474 cells treated with different concentrations of compound **12**.

3. Experimental Section

3.1. General Information

All the solvents were obtained from Merck (Kenilworth, NJ, USA). The homogeneity of the compounds was checked by TLC performed on Silica gel G coated plates (Merck). An iodine chamber was used for visualization of TLC spots. The FT-IR spectra were recorded in KBr pellets on a Spectrum BX FT-IR spectrophotometer (Perkin Elmer, Hopkinton, MA, USA). The elemental analysis for C, H, N and S were within the limit of ±0.4% and ±0.3% of the theoretical values respectively. Melting points were determined on a Gallenkamp melting point apparatus, and the thermometer was uncorrected. NMR

Spectra were scanned in DMSO-d_6 on a Bruker NMR spectrophotometer operating at 500 MHz for ^{1}H and 125.76 MHz for ^{13}C. Chemical shifts δ are expressed in parts per million (ppm) relative to TMS as an internal standard and D_2O was added to confirm the exchangeable protons. Coupling constants (*J*) are in hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), m (multiplet). Mass spectra were measured on triple quadruple mass spectroscopy (Waters, Corp. Milford, MA, USA).

3.1.1. Representative Procedure for Synthesis of **1**–**18**

To a solution of thiosemicarbazide (0.0119 mol) in ethanol (11 mL), water (22 mL) was added. To this solution, 3-methoxybenzaldehyde/3-hydroxybenzaldehyde (0.0125 mol) and acetic acid (0.55 mL) were added. The mixture was stirred under reflux for 1 h and cooled to ambient temperature. After, the precipitate was collected with filter under vacuum and washed with water.

(2Z)-2-(3-Methoxybenzylidene)-N-phenylhydrazinecarbothioamide (**1**): Yield: 70%; 161–162; m.p.: 3143 °C; IR (KBr): (NH str.), 1578 (C=N str.), 1207 (NCSN str.), 1155 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (3H, s, -OCH_3), 6.9–7.5 (9H, m, Ar-H), 8.1 (1H, s, N=CH), 10.1 (1H, s, NH, D_2O exchg.), 11.8 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.78 (OCH_3), 112.36, 116.56, 121.20, 125.89, 126.59, 128.55, 130.20, 135.88, 139.58, 143.28, 160.05 (N=C), 176.58 (C=S); MS: *m*/*z* = 284.24 $[M-1]^+$; Analysis: for $C_{15}H_{15}N_3OS$, calcd. C 63.13, H 5.30, N 14.73, S 11.24%; found C 63.25, H 5.20, N 14.53, S 11.21%.

(2Z)-N-(4-Chlorophenyl)-2-(3-methoxybenzylidene) hydrazinecarbothioamide (**2**): Yield: 75%; m.p.: 197–198 °C; IR (KBr): 3331 (NH str.), 1504 (C=N str.), 1273 (NCSN str.), 1155 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (3H, s, -OCH_3), 7.0–7.6 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 10.1 (1H, s, NH, D_2O exchg.), 11.9 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.78, 112.52, 116.56, 121.20, 128.16, 128.43, 129.88, 130.21, 131.20, 135.78, 138.56, 143.61, 160.04, 176.55. MS: *m*/*z* = 318.91 $[M-1]^+$; Analysis: for $C_{15}H_{14}ClN_3OS$, calcd. C 56.33, H 4.41, N 13.14, S 10.03%; found C 56.12, H 4.40, N 13.12, S 10.00%.

(2Z)-2-(3-Methoxybenzylidene)-N-(prop-2-en-1-yl)hydrazinecarbothioamide (**3**): Yield: 72%; m.p.: 115–116 °C; IR (KBr): 3357 (NH str.), 1527 (C=N str.), 1286 (NCSN str.), 1156 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (3H, s, -OCH_3), 4.2 (2H, d, -CH_2), 5.1 (2H, m, CH_2), 5.9 (1H, m, CH), 6.9–7.4 (4H, m, Ar-H), 8.0 (1H, s, N=CH), 8.7 (1H, s, NH, D_2O exchg.), 11.5 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.74, 112.46, 115.94, 116.05, 116.05, 120.67, 130.19, 135.58, 136.06, 142.44, 160.2, 177.74; MS: *m*/*z* = 251.00 $[M-2]^+$; Analysis: for $C_{12}H_{15}N_3OS$, calcd. C 57.81, H 6.06, N 6.85, S 12.86%; found C 57.60, H 6.04, N 6.80, S 12.83%.

(*2Z)-N-Benzyl-2-(3-methoxybenzylidene)hydrazinecarbothioamide* (**4**): Yield: 68%; m.p.: 127–129 °C; IR (KBr): 3148 (NH str.), 1545 (C=N str.), 1254 (NCSN str.), 1041 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.79 (3H, s, -OCH_3), 4.8 (2H, s, -CH_2), 6.9–7.4 (9H, m, Ar-H), 8.0 (1H, s, N=CH), 9.1 (1H, s, NH, D_2O exchg.), 11.6 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 47.05, 55.74, 112.55, 116.04, 120.04, 120.68, 127.17, 127.63, 128.63, 130.21, 136.04, 139.91, 142.62, 160.02, 178.14; MS: *m*/*z* = 300.09

$[M + 1]^+$; Analysis: for $C_{16}H_{17}N_3OS$, calcd. C 62.92, H 7.59, N 3.76, S 10.50%; found C 62.87, H 7.57, N 3.75, S 10.53%.

(2Z)-N-Ethyl-2-(3-methoxybenzylidene)hydrazinecarbothioamide (**5**): Yield: 72%; m.p.: 122–123 °C; IR (KBr): 3351 (NH str.), 1544 (C=N str.), 1272 (NCSN str.), 1153 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 1.15 (3H, t, -CH_3), 3.6 (2H, q, CH_2), 3.8 (3H, s, -OCH_3), 6.9–7.3 (4H, m, Ar-H), 8.0 (1H, s, N=CH), 8.5 (1H, s, NH, D_2O exchg.), 11.4 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 15.07, 55.72, 112.35, 116.05, 120.62, 130.21, 136.07, 142.22, 160.01, 177.16; MS: *m*/*z* = 237.07 $[M]^+$; Analysis: for $C_{11}H_{15}N_3OS$, calcd. C 55.67, H 6.37, N 17.71, S 13.51%; found C 55.87, H 6.35, N 17.73, S 13.48%.

(2Z)-2-(3-Methoxybenzylidene)-N-(3-methoxyphenyl)hydrazinecarbothioamide (**6**): Yield: 65%; m.p.: 148–150 °C; IR (KBr): 3308 (NH str.), 1599 (C=N str.), 1286 (NCSN str.), 1156 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (6H, s, -OCH_3), 6.9–7.5 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 10.0 (1H, s, NH, D_2O exchg.), 11.8 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.77, 104.15, 107.40, 110.66, 111.98, 112.44, 115.42, 116.55, 118.48, 121.17, 129.88, 130.69, 134.06, 135.83, 140.66, 141.79, 143.35, 148.55, 159.49, 160.11, 165.69, 176.31. MS: *m*/*z* = 315.44 $[M]^+$; Analysis: for $C_{16}H_{17}N_3O_2S$, calcd. C 60.93, H 5.43, N 3.32, S 10.17%; found C 60.70, H 5.40, N 3.30, S 10.15%.

(2Z)-2-(3-Methoxybenzylidene)-N-(4-methoxyphenyl)hydrazinecarbothioamide (**7**): Yield: 70%; m.p.: 156–157 °C; IR (KBr): 3304 (NH str.), 1542 (C=N str.), 1236 (NCSN str.), 1152 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (6H, s, -OCH_3), 6.9–7.5 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 10.0 (1H, s, NH, D_2O exchg.), 11.7 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.69, 55.71, 55.78, 112.27, 113.76, 114.11, 116.50, 121.17, 126.58, 128.27, 130.18, 132.48, 132.71, 135.96, 143.03, 156.99, 157.51, 160.06, 176.98, 180.69; MS: *m*/*z* = 315.00 $[M]^+$; Analysis: for $C_{16}H_{17}N_3O_2S$, calcd. C 60.93, H 5.43, N 13.32, S 10.17%; found C 60.71, H 5.42, N 13.34, S 10.15%.

(2Z)-N-(4-Ethoxyphenyl)-2-(3-methoxybenzylidene)hydrazinecarbothioamide (**8**): Yield: 68%; m.p.: 160–161 °C; IR (KBr): 3155 (NH str.), 1540 (C=N str.), 1274 (NCSN str.), 1115 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 1.3 (3H, t, -CH_3), 3.8 (3H, s, -OCH_3), 4.0 (2H, q, -CH_2), 6.9–7.5 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 10.0 (1H, s, NH, D_2O exchg.), 11.7 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 15.16, 55.78, 63.63, 112.23, 114.23, 114.59, 116.51, 121.17, 128.24, 130.17, 132.35, 135.96, 142.99, 156.77, 160.05, 176.93; MS: *m*/*z* = 329.23 $[M]^+$; Analysis: for $C_{17}H_{19}N_3O_2S$, calcd. C 61.98, H 5.81, N 12.76, S 9.73%; found C 61.75, H 5.84, N 12.74, S 9.75%.

(2Z)-N-(3-Chlorophenyl)-2-(3-methoxybenzylidene)hydrazinecarbothioamide (**9**): Yield: 70%; m.p.: 138–140 °C; IR (KBr): 3421 (NH str.), 1545 (C=N str.), 1269 (NCSN str.), 1152 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (3H, s, -OCH_3), 7.0–7.7 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 10.1 (1H, s, NH, D_2O exchg.), 11.9 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.78, 112.63, 116.57, 121.20, 124.88, 125.52, 125.84, 130.04, 130.21, 132.65, 135.74, 141.06, 143.80, 160.05, 176.40; MS: *m*/*z* = 319.00 $[M]^+$; Analysis: for $C_{15}H_{14}ClN_3OS$, calcd. C 56.33, H 4.41, N 13.14, S 10.03%; found C 56.50, H 4.40, N 13.15, S 10.00%.

(2Z)-2-(3-Hydroxybenzylidene)-N-phenylhydrazinecarbothioamide (**10**): Yield: 78%; m.p.: 212–213 °C; IR (KBr): 3311 (NH str.), 1542 (C=N str.), 1275 (NCSN str.), 1163 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 6.8–7.6 (9H, m, Ar-H), 8.1 (1H, s, N=CH), 9.5 (1H, s, -OH, D_2O exchg.), 10.1 (1H, s, NH, D_2O exchg.), 11.7 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 114.31, 117.78, 119.39, 125.74, 126.19, 128.31, 128.53, 128.75, 130.13, 135.76, 139.56, 143.69, 158.08, 176.43; MS: *m*/*z* = 265.30 $[M - 6]^+$; Analysis: for $C_{14}H_{13}N_3OS$, calcd. C 61.97, H 4.83, N 15.49, S 11.82%; found C 61.97, H 4.83, N 15.49, S 11.82%.

(2Z)-N-(4-Chlorophenyl)-2-(3-hydroxybenzylidene)hydrazinecarbothioamide (**11**): Yield: 65%; m.p.: 222–224 °C; IR (KBr): 3277 (NH str.), 1591 (C=N str.), 1276 (NCSN str.), 1196 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 6.8–7.6 (9H, m, Ar-H), 8.0 (1H, s, N=CH), 9.5 (1H, s, -OH, D_2O exchg.), 10.1 (1H, s, NH, D_2O exchg.), 11.8 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 114.35, 117.81, 119.41, 127.86, 128.39, 129.70, 130.11, 135.66, 138.57, 144.00, 158.07, 176.41; MS: *m*/*z* = 304.13 $[M - 1]^+$; Analysis: for $C_{14}H_{12}ClN_3OS$, calcd. C 54.99, H 3.96, N 13.74, S 10.49%; found C 54.78, H 3.95, N 13.73, S 10.47%.

(2Z)-2-(3-Hydroxybenzylidene)-N-(3-methoxyphenyl)hydrazinecarbothioamide (**12**): Yield: 70%; m.p.: 185–186 °C; IR (KBr): 3312 (NH str.), 1546 (C=N str.), 1277 (NCSN str.), 1155 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (6H, s, 2×-OCH_3), 6.5–7.5 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 9.6 (1H, s, -OH, D_2O exchg.), 10.0 (1H, s, NH, D_2O exchg.), 11.8 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.62, 111.16, 11.58, 114.29, 115.11, 117.81, 118.08, 119.41, 121.59, 129.22, 130.14, 130.77, 135.71, 138.13, 140.65, 143.76, 158.08, 159.48, 176.14; MS: *m*/*z* = 302.30 $[M + 1]^+$; Analysis: for $C_{15}H_{15}N_3O_2S$, calcd. C 59.78, H 5.02, N 13.94, S 10.64%; found C 59.57, H 5.00, N 13.96, S 10.62%.

(2Z)-2-(3-Hydroxybenzylidene)-N-(prop-2-en-1-yl)hydrazinecarbothioamide (**13**): Yield: 60%; m.p.: 94–97 °C; IR (KBr): 3357 (NH str.), 1585 (C=N str.), 1222 (NCSN str.), 1144 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 4.2 (2H, d, -CH_2), 5.1 (2H, m, =CH_2), 5.9 (1H, m, =CH), 6.9–7.2 (4H, m, Ar-H), 7.9 (1H, s, N=CH), 8.6 (1H, s, -OH, D_2O exchg.), 9.6 (1H, s, NH, D_2O exchg.), 11.4 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 46.19, 113.80, 116.03, 117.60, 119.14, 130.17, 135.42, 135.85, 142.98, 157.97, 177.59; MS: *m*/*z* = 235.12 $[M]^+$; Analysis: for $C_{11}H_{13}N_3OS$, calcd. C 56.15, H 5.57, N 17.86, S 13.63%; found C 56.26, H 5.55, N 17.87, S 13.60%.

(2Z)-N-Benzyl-2-(3-hydroxybenzylidene)hydrazinecarbothioamide (**14**): Yield: 65%; m.p.: 130–132 °C; IR (KBr): 3360 (NH str.), 1551 (C=N str.), 1301 (NCSN str.), 1147 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 4.8 (2H, s, -CH_2), 6.8–7.3 (9H, m, Ar-H), 8.0 (1H, s, N=CH), 9.0 (1H, s, -OH, D_2O exchg.), 9.6 (1H, s, NH, D_2O exchg.), 11.5 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 18.99, 47.05, 56.55, 114.00, 117.58, 119.10, 127.20, 127.67, 128.64, 130.13, 135.90, 139.90, 143.09, 158.03, 177.99; MS: *m*/*z* = 285 $[M]^+$; Analysis: for $C_{15}H_{15}N_3OS$, calcd. C 63.13, H 5.30, N 4.73, S 11.24%; found C 63.36, H 5.32, N 4.75, S 11.22%.

(2Z)-2-(3-Hydroxybenzylidene)-N-(4-methoxyphenyl)hydrazinecarbothioamide (**15**): Yield: 72%; m.p.: 182–183 °C; IR (KBr): 3169 (NH str.), 1581 (C=N str.), 1283 (NCSN str.), 1022 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.7 (6H, s, 2× -OCH_3), 6.8–7.4 (8H, m, Ar-H), 8.0 (1H, s, N=CH), 9.5 (1H, s, -OH,

D_2O exchg.), 9.9 (1H, s, NH, D_2O exchg.), 11.7 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 55.69, 113.72, 114.11, 114.28, 114.66, 117.66, 118.86, 119.31, 126.58, 127.96, 130.09, 132.09, 132.45, 132.70, 135.84, 143.39, 156.99, 157.39, 158.06, 176.79, 180.67; MS: *m*/*z* = 302.49 $[M + 1]^+$; Analysis: for $C_{15}H_{15}N_3O_2S$, calcd. C 59.78, H 5.02, N 13.94, S 10.64%; found C 59.55, H 5.00, N 13.92, S 10.66%.

(2Z)-N-(3-Methoxyphenyl)-2-(3-hydroxybenzylidene)hydrazinecarbothioamide (**16**): Yield: 70%; m.p.: 200–202 °C; IR (KBr): 3138 (NH str.), 1510 (C=N str.), 1280 (NCSN str.), 1039 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 3.8 (3H, s, -OCH_3), 4.0 (2H, q, -CH_2), 6.9–7.5 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 9.5 (1H, s, -OH, D_2O exchg.), 10.0 (1H, s, NH, D_2O exchg.), 11.7 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 15.16, 55.78, 112.23, 114.23, 114.59, 116.51, 121.17, 128.24, 130.17, 132.35, 135.96, 142.99, 156.77, 160.05, 176.93; MS: *m*/*z* = 315.10 $[M]^+$; Analysis: for $C_{16}H_{17}N_3O_2S$, calcd. C 60.93, H 5.43, N 13.32, S 10.17%; found C 60.70, H 5.41, N 13.30, S 10.14%.

(2Z)-N-(3-Chlorophenyl)-2-(3-hydroxybenzylidene)hydrazinecarbothioamide (**17**): Yield: 75%; m.p.: 198–200 °C; IR (KBr): 3281 (NH str.), 1589 (C=N str.), 1276 (NCSN str.), 1172 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 6.8–7.7 (8H, m, Ar-H), 8.1 (1H, s, N=CH), 9.5 (1H, s, -OH, D_2O exchg.), 10.1 (1H, s, NH, D_2O exchg.), 11.9 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 114.38, 117.88, 119.46, 124.56, 125.37, 125.54, 130.02, 130.12, 132.60, 135.61, 141.06, 144.18, 158.07, 176.24; MS: *m*/*z* = 304.58 $[M - 1]^+$; Analysis: for $C_{14}H_{12}ClN_3OS$, calcd. C 54.99, H 3.96, N 13.74, S 10.49%; found C 54.76, H 3.94, N 13.76, S 10.46%.

(2Z)-N-Ethyl-2-(3-hydroxybenzylidene)hydrazinecarbothioamide (**18**): Yield: 60%; m.p.: 152–154 °C; IR (KBr): 3358 (NH str.), 1557 (C=N str.), 1239 (NCSN str.), 1168 (C=S str.); ^{1}H-NMR (DMSO-d_6); δ = 1.15 (3H, t, CH_3), 3.6 (2H, q, CH_2), 6.8–7.2 (4H, m, Ar-H), 7.4 (1H, s, N=CH), 8.4 (1H, s, NH, D_2O exchg.), 9.5 (1H, s, -OH, D_2O exchg.), 11.3 (1H, s, NHCS, D_2O exchg.); ^{13}C-NMR (DMSO-d_6): δ = 15.07, 113.96, 117.46, 118.95, 130.09, 135.97, 142.60, 158.04, 177.10; MS: *m*/*z* = 223.18 $[M]^+$; Analysis: for $C_{10}H_{13}N_3OS$, calcd. C 53.79, H 5.87, N 8.82, S 14.36%; found C 53.58, H 5.85, N 8.84, S 14.34%.

3.2. Cell Lines

SKBr-3, BT-474 and MDA-MB-231 breast cancer cell lines were purchased from the American Type Culture Collection (0801 University Boulevard, Manassas, VA, USA). SKBR-3 cells were cultured in McCoy's 5A (GIBCO, 8717, Grovement Cir, Gaitherberg, MD, USA), and BT-474, MDA-MB-231 cells were cultured in DMEM (Sigma, 82024 Taufkirchen, Germany). The media supplemented with 10% FBS (Cambrex Bio Science, Baltimore, MD, USA), 100 IU/mL penicillin and 100 mg/mL streptomycin. Cell viability was assessed by trypan blue exclusion analysis. Cell numbers were determined by using a hemacytometer.

3.2.1. WST-1 Cell Proliferation Assay

Cells were seeded into 96-well plates at 0.4×10^4/well and incubated overnight. The medium was replaced with fresh one containing the desired concentrations of the compounds. After 48 h, 10 μL of

the WST-1 reagent was added to each well and the plates were re-incubated for 4 h at 37 °C. The amount of formazan was quantified using an ELISA reader at 450 nm.

3.2.2. Measurement of IC_{50}

Cells were seeded into 96-well plates at 0.4×10^4/well and incubated overnight. The medium was replaced with fresh one containing the desired concentrations of the compounds. After 48 h, 10 μL of the WST-1 reagent was added to each well and the plates were re-incubated for 4 h at 37 °C. The amount of formazan was quantified using an ELISA reader at 450 nm. For the compounds and the reference chemotherapeutic agent 5-FU, cells were cultured one day before treatment. Fresh media with fixed dose of 20 μM were replaced. IC_{50} was mathematically calculated as IC_{50} = fixed dose (20) × 50/(formazan quantity of treated cells/formazan quantity of untreated cells) × 100.

3.2.3. Flow Cytometric Analysis of Cellular DNA Content

2×10^6 cells were fixed in 1 mL ethanol (70%) for 60 min at room temperature. Harvested cells were re-suspended in 1 mL Na citrate (50 mM) containing 250 μg RNase A and incubated at 50 °C for 60 min. Next, cells were re-suspended in the same buffer containing 4 μg propidium iodine (PI) and incubated for 30 min before being analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). The percentage of cells in various cell cycle phases was determined by using Cell Quest Pro software (Becton Dickinson).

3.2.4. Measurement of Annexin-V Binding by Flow Cytometry

It has been shown that loss of phospholipid asymmetry of the plasma membrane is an early event of apoptosis. The annexin-V binds to negatively charged phospholipids, like phosphatidylserine. During apoptosis, the cells react to annexin-V once chromatin condenses but before the plasma membrane loses its ability to exclude propidium iodide (PI). Hence, by staining cells with a combination of fluorescein isothiocyanate (FITC) annexin-V and PI, it is possible to detect non-apoptotic live cells, early apoptotic cells and late apoptotic or necrotic cells. Annexin-V staining was performed by using Vybrant Apoptosis Assay Kit # 2 (Molecular Probe, Eugene, Oregon, 97402-0469) following the manufacturer's recommendations. Annexin-V stained cells were analyzed by flow cytometry, measuring the fluorescence emission at 530 and less than 575 nm.

3.2.5. Cancer Cell Migration Assay

Cell migration assay was performed according to the standard protocol. Three concentration 25 μM of compound was taken for testing. The lower well of the migration plate was supplemented with 500 μL of DMEM containing 10% fetal bovine serum with or without (vehicle control ethanol only) test compound. To the inside of each insert 100 μL of $0.5–1.0 \times 10^6$ cells/mL of SKBr-3/BT-474 cell suspension was added separately. The plates were incubated for 8 h at 37 °C in a humidified CO_2 incubator. After incubation, the media from inside of the inserts was carefully aspirated and the non-migratory cells were removed using cotton-tipped swabs. The inserts were transferred to a clean well containing 400 μL of cell stain solution and incubated for 10 min at room temperature. The stained

inserts were gently washed several times and then transferred again to an empty well. Finally, 200 μL of extraction solution per well was added and incubated for 10 min on an orbital shaker. From each sample, 100 μL was taken in a 96-well microtiter plate and the absorbance at 560 nm was read in a plate reader.

3.2.6. Cancer Cell Adhesion Assay

Under sterile conditions, the adhesion plate was allowed to warm up at room temperature for 10 min. 150 μL of 0.1–1.0 × 10^6 cells/mL of SKBr-3/BT-474 cell suspension in serum free media with vehicle control ethanol only or test compound (25 μM) was added to the inside of each well. The plates were incubated for 30–90 min in a CO_2 incubator. The wells were washed three times with PBS and the adhered cells were stained with 200 μL of cell stain solution for 10 min at room temperature. The excess stain was removed by washing 4–5 times with distilled water. After air drying the wells, 200 μL of extraction solution per well was added and then incubated for 10 min on an orbital shaker. The 150 μL from each extracted sample was transferred to a 96-well microtiter plate and the absorbance at 560 nm was read in a plate reader. Absorbance of dye in the control (vehicle-treated) cells was regarded as 100% adherence and the percentage adherence of treated cells was calculated in comparison with that of the control cells. Cell Migration and Cell Adhesion Assay kits were obtained from Cell Biolabs, Inc. (San Diego, CA, USA).

3.2.7. The Effect of Different Concentration of Compound **12** on BT-474 Cells Proliferation

The cytotoxic effects of the compound **12** on BT-474 cell line was assayed by the MTT assay. The cells were seeded at a density of 5 × 10^4 cells/well. The compound was serially diluted to final concentration of 20 μM, 10 μM, 5 μM and 2.5 μM. 200 μL liquid of each concentration was applied to the wells of a 96-well plate containing confluent cell monolayers (six wells per concentration). The dilution medium without the sample served as a control. After 48 h of incubation, MTT solution (5 mg/mL) was then added to each well, and the formazan precipitate was dissolved in 200 μL dimethyl sulfoxide after 4 h incubation. The content of the wells was homogenized on a microplate shaker for 5 min. The optical densities (OD) were measured on a microplate ELISA reader at 492 nm. All tests and analyses were run in triplicate and mean values were recorded. The cell survival curves were calculated after comparing with the control. The percentage viability was calculated as follows:

$$\%\text{viability} = \text{mean absorbance of treated wells} \times 100 \text{ mean absorbance of untreated wells}$$

4. Conclusions

In conclusion, we focused on the design and synthesis of 2-cyclohexyl-*N*-[(*Z*)-(3-methoxyphenyl/3-hydroxyphenyl)methylidene]hydrazinecarbothioamides **1**–**18**, which were fully characterized by spectral analysis. The synthesized compounds were screened *in vitro* against HER-2 overexpressed breast cancer cell lines SKBr-3. Compound **12** presented the most significant activity against HER-2 over expressed breast cancer cell lines SKBr-3 and BT-474. Compound **12** significantly inhibited the cell migration and cell adhesion of breast cancer cell lines. Compound **12** was found to most active compound of this series and represents a good lead for development of drugs, targeting HER-2 over-expressed breast cancer cell lines.

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Author Contributions

Design and synthesis of the compounds was performed by MA Bhat; A Al-Dhfyan performed the anti-proliferative activity, AM Naglah contributed in the spectral analysis of the compounds, AA Khan performed the cell adhesion and cell migration assays, MA Al-Omar helped in the preparation of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds (**1**–**18**) are available from the authors. The compounds are solid, crystalline in nature with ≥ 99% purity.