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Synthesis and Cytotoxic Effects of Various Thiosemicarbazide Compounds on Primary and Metastatic Breast Cancer Cell Lines

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Abstract

BACKGROUND/AIMS: Thiosemicarbazides and their metal complexes are known for their antiviral, antibacterial, and antitumor properties. This research describes the synthesis of four novel thiosemicarbazide derivatives (5, 8, 13, and 14) and investigates their cytotoxic effects on primary (MCF-7) and metastatic (M4A4) breast cancer cells. The study explored the Wnt/β-catenin signaling pathway as well as key factors associated with proliferation, and stemness, that play a vital role in breast cancer development.

MATERIALS AND METHODS: Hydrazide derivatives containing imidazo[2,1-b]thiazole ring were synthesized through a conventional method. The MCF-7 and M4A4 cells were cultivated and exposed to different concentrations (200 μM, 400 μM, 800 μM) of the all compounds for 24 and 48 hours. Cytotoxicity was investigated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The distribution of β-catenin, GSK 3β, LGR5, Wnt 5a, Ki-67, Cxcl1, and CD44 were analyzed using indirect immunoperoxidase staining.

RESULTS: A 200 μM dose for all compounds for 24 hours was selected based on MTT assay for further analyses. Immunohistochemical analysis exhibited that compounds 5, 8, and 14 stimulated the Wnt/β-catenin pathway in both cell lines, with an elevation in the immunoreactivity of β-catenin, GSK 3β, and LGR5 also observed. Compound 13 reduced the proliferation of M4A4 cells, but an increase in stemness was also observed.

CONCLUSION: Various thiosemicarbazide compounds affected primary and metastatic breast cancer cell lines differently. Among these, compound 13 showed an ability to reduce the growth of metastatic breast cancer cells. However, additional research about the stemness properties of breast cancer should be evaluated in further investigations.

Keywords: Thiosemicarbazide, Wnt/β-catenin, stemness, breast cancer

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INTRODUCTION

Breast cancer is the most prevalent type of cancer in females.[1] Thiosemicarbazides and their metal complexes are known for their antiviral, antibacterial, and antitumor properties. Levamisole featuring an imidazo[2,1-b]thiazole structure was initially authorized as an anticancer agent by Başoğlu et al.[2,3] which led to a significant increase in global research on this chemical framework. This trend continued with thiosemicarbazide, which followed the development of Triapine, thioacetazone, and methisazone. The Wnt/β-catenin signaling pathway plays an important role in regulating key biological functions of cells such as proliferation and differentiation.[4-6] Dysregulation of Wnt signaling is a feature of oncogenic transformation. Knockdown of Wnt5a has been demonstrated to reduce cellular senescence by reducing p16INK4A levels, reducing β-gal-positive cells counts, and suppressing the senescence associated secretory phenotype (SASP), which encompasses key *SASP* genes such as IL6, IL16, Cxcl1, Cxcl5, Cxcl12.7,8,9 The objective of this study is to develop four novel thiosemicarbazide compounds (5, 8, 13, and 14) and evaluate their cytotoxic effects on MCF-7 and M4A4 cell lines. The study also explored the involvement of this signaling pathway and essential factors associated with senescence, proliferation, and stemness in breast cancer progression.

MATERIALS AND METHODS

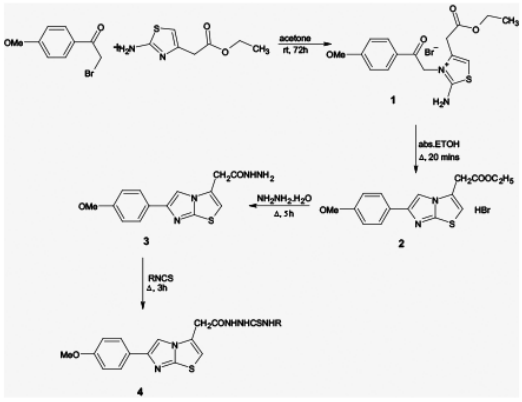
General synthesis pathway of 2-[(6-(4-methoxyphenyl)imidazo [2,1-b]thiazole-3-yl)acetyl]-N-alkyl/arylhydrazinecarbothioamides: the compounds were synthesized by heating 0.01 mol of each of compound 5, compound 8, compound 13, and compound 14 under reflux in 30 mL of ethanol on a water bath until a clear solution was obtained. Subsequently, 0.01 mol of alkyl isothiocyanate is added, and the mixture is heated for 3 hours. After the reaction is complete and the mixture has cooled to room temperature, the resulting precipitate is filtered and purified by washing with hot ethanol. The characterization of all synthesized compounds was performed using various spectroscopic techniques, including fourier transform infrared spectroscopy (FT-IR), 1H-NMR, and MS. In this study, only precisely characterized compounds were used for further analysis.

Cytotoxicity assay: Primary (MCF-7, HTB-22, ATCC) and metastatic (M4A4, CRL- 2914, ATCC) breast cancer cell lines were cultured in medium [RPMI-1640 (F-1213, Biochrom), 10% fetal bovine serum (10270-106, Gibco), 1% pen-strep (PS-B, Capricorn Scientific) and 2 mm L-glutamine (K0283, Biochrom, Berlin, Germany)] in a humidified atmosphere at 37 ℃ and 5% CO_2. Cells (2x103 cells/mL) in 96 well plates were then treated with various concentrations (10 μM, 25μM, 50μM, 100μM, 200μM) of compounds 5, 8, 13 and 14 for 24 and 48 h. MTT (5 mg/mL, GC4568, Glentham Life Sciences) solution was added to each well, and incubated for 4 h and 50 μL of dimethyl sulfoxide (DMSO) was added and reaction was evaluated using microplate reader (ELX800UV, BioTek Instruments Inc) at a reference wavelength of 540 nm.

Experimental groups and immunocytochemistry assay: The cells were grouped into five separate experimental groups: control (cell culture medium only), compound 5, compound 8, compound 13, and compound 14. After a 30 minutes (min) fixation with 4% paraformaldehyde (1.04004.0800, Merck), they were permeabilized with 0.1% Triton-X-100 (A4975,0100, Applichem) on ice for 15 min; then 3% hydrogen peroxide (H_2O_2, 1.08597.2500, Merck) was added. They were incubated with blocking solution (TA-125-UB, ThermoFisher) for 1 h at RT before being exposed to primary antibodies against β-catenin (9562, Cell Signaling Technology), GSK 3β (27C10, Cell Signaling Technology), LGR5 (HPA012530, Sigma Aldrich), Wnt 5a (2392S, Cell Signaling Technology), Ki-67 (PRM325AA, Biocare Medical), Cxcl1 (MBS422132, MyBiosource) and CD44 (15675-I-AP, Proteintech) for 18 h at 4°C. After the washing step, secondary antibodies (biotinylated rabbit anti-mouse, and streptavidin-conjugated hydrogen peroxidase-TP-125-UB, ThermoFisher) were applied. Diaminobenzidine (DAB, 38611, ScyTek Laboratories) was used as chromogen; Mayer's hematoxylin (Bio-Optica, 05-06002/L Milano, Italy) was used for counterstaining. Immunoreactivities were semi-quantitatively graded using the H-score. The Kruskal-Wallis test was utilized to perform the analysis, and statistical significance was evaluated at a p-value less than 0.05.

RESULTS

Chemistry



Compounds	R
5	Phenyl
8	Phenethyl
13	4-Chlorophenyl
14	4-Flourophenyl

Schema 1. The general synthesis pathway of the thiosemicarbazides

Compound-5: White solid, yield: 70% (1.84 g), mp: 201-202 °C. Anal. Calcd. C□□H□□N□O□S□: C 57.65, H 4.38, N 16.01%; Found: C 57.16, H 4.27, N 15.60%. FT-IR (cm□¹): 3176 (N-H), 1687 (C=O), 1172 (C=S). ¹H-NMR (DMSO-d□, 500 MHz): δ 10.40 (s, NH-Ph), 10.23 (s, CS-NH), 7.48-7.15 (m, 5H, ar. CH), 3.78 (s, 3H, OCH□). ESI (+) MS m/z (%): 437.9 M+, 438.9 (M+H)+

Compound-8: White solid, yield: 74% (2.06 g), mp: 200 °C. Anal. Calcd. C□□H□□N□O□S□: C 59.33, H 4.98, N 15.04%; Found: C 58.86, H 4.73, N 14.76%. FT-IR (cm□¹): 3159 (N-H), 1699 (C=O), 1172 (C=S). ¹H-NMR (DMSO-d□, 500 MHz): δ 10.18 (s, NH-Ph), 9.54 (s, CS-NH), 7.31-7.19 (m, 5H, ar. CH), 3.76 (s, 3H, OCH□).

Compound-13: White solid, yield: 79% (2.06 g), mp: 204 °C. Anal. Calcd. C□□H□□N□O□S□: C 53.44, H 3.84, N 14.84%; Found: C 52.81, H 3.71, N 14.53%. FT-IR (cm□¹): 3211, 3130 (N-H), 1682 (C=O), 1166 (C=S). ¹H-NMR (DMSO-d□, 500 MHz): δ 10.51 (s, NH-Ph), 10.40, 10.29 (s, CS-NH), 7.57-7.38 (m, 4H, ar. CH), 3.78 (s, 3H, OCH□).

Compound-14: White solid, yield: 93% (2.53 g), mp: 200 °C. Anal. Calcd. C□□H□□N□O□S□·C□H□OH: C 55.08, H 4.82, N 13.96%; Found: C 54.37, H 4.50, N 14.21%. FT-IR (cm□¹): 3122 (N-H), 1681 (C=O), 1167

(C=S). ^{1}H-NMR (DMSO-d□, 500 MHz): δ 10.39 (s, NH-Ph), 10.21 (s, CS-NH), 7.44-7.16 (m, 4H, ar. CH), 3.78 (s, 3H, OCH□).

Cell viability and cytotoxicity: MCF-7 exhibited adherence characteristics and an epithelial morphology, as depicted in Figure 1A. The metastatic breast cancer cell line M4A4 also displayed an epithelial morphology, with observations of a fusiform structure, highlighted in Figure 1B. After treated of cells with different concentrations of compound 5, 8, 13 and 14 for 24 (A, C) and 48 (B, D) hours, 200 µM dose and 24 h application were determined in both MCF-7(Figure 2 A, B) and M4A4 (Figure 2 C, D) cells.

Experimental groups and immunocytochemistry assay: β-catenin immunoreactivity was moderate in the control group, strongly positive in compound 5 and compound 14 applied groups, and it was weak in the compound 13 group in MCF -7 cell line (Figure 3A). Statistical analyses revealed that only the group treated with compound 13 exhibited a significant difference from the control group (p=0.029) (Figure 4A). In M4A4 cells, β-catenin immunoreactivity was moderate in control, groups treated with compound 13 and compound 14, while it was strongly positive and weak in groups treated with compound 8 and compound 5, respectively (Figure 3A). Immunoreactivity for GSK 3β was strongly positive in all groups where compounds were applied except the compound 13 group, which was moderate in both cell lines (Figure 3A), and it was statistically significant in MCF-7 cells for GSK 3β (p=0.015) when compared to the control group. Wnt5a and Cxcl1 immunoreactivities were weak or negative in all groups for MCF-7 and M4A4 cells (Figure 3B). Ki67 immunoreactivity was strongly positive in control and compound 14 applied groups, and it was moderate in compound 5 and 8 groups, while it was weak in compound 13 group for both type of cells (Figure 3C). Furthermore, in MCF-7 cells, the compound 13-applied group was statistically significantly different (p value is 0.008) compared to the control group. CD44 immunoreactivity was strongly positive in all groups where compounds were applied,

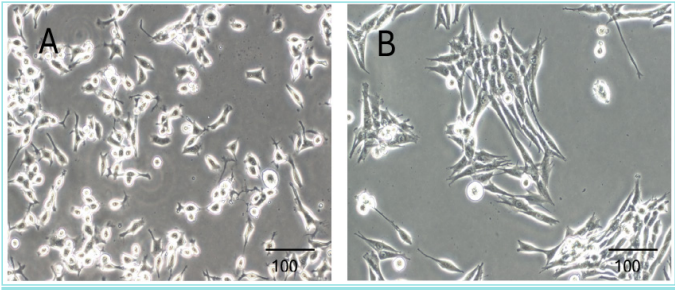


Figure 1. Cell culture photographs of MCF-7 (A) and M4A4 (B). Scale bars: 100µm.

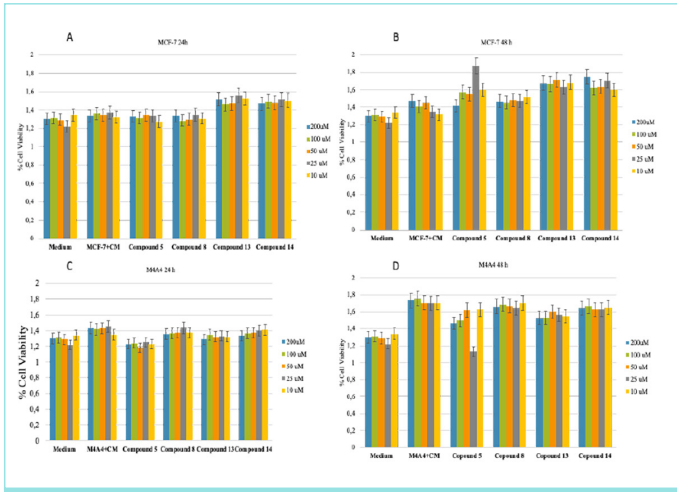


Figure 2. Cell viability assay of compound 5, compound 8, compound 13 and compound 14 for 24 (A,C) and 48 (B,D) h for MCF-7 and M4A4 cells. CM: Culture medium.

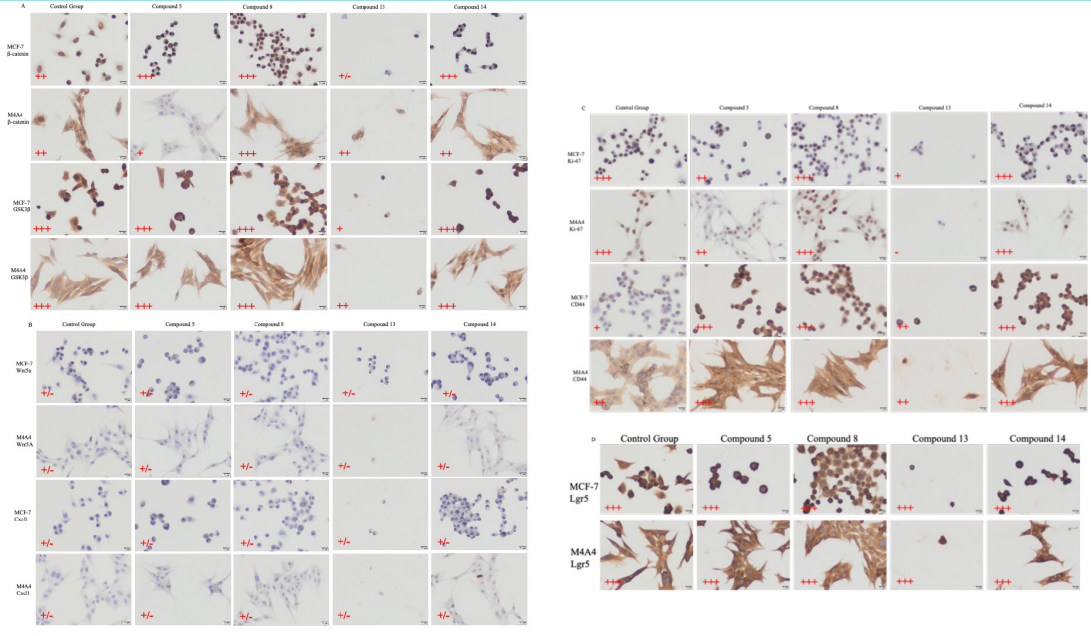


Figure 3. Distribution of β-catenin (A), GSK3β (A), Wnt5a (B), Cxcl1 (B), Ki-67 (C), CD44 (C) and LGR5 (D) after immunocytochemistry of control, compound 5, compound 8, compound 13 and compound 14. Scale bars: 20 µm.

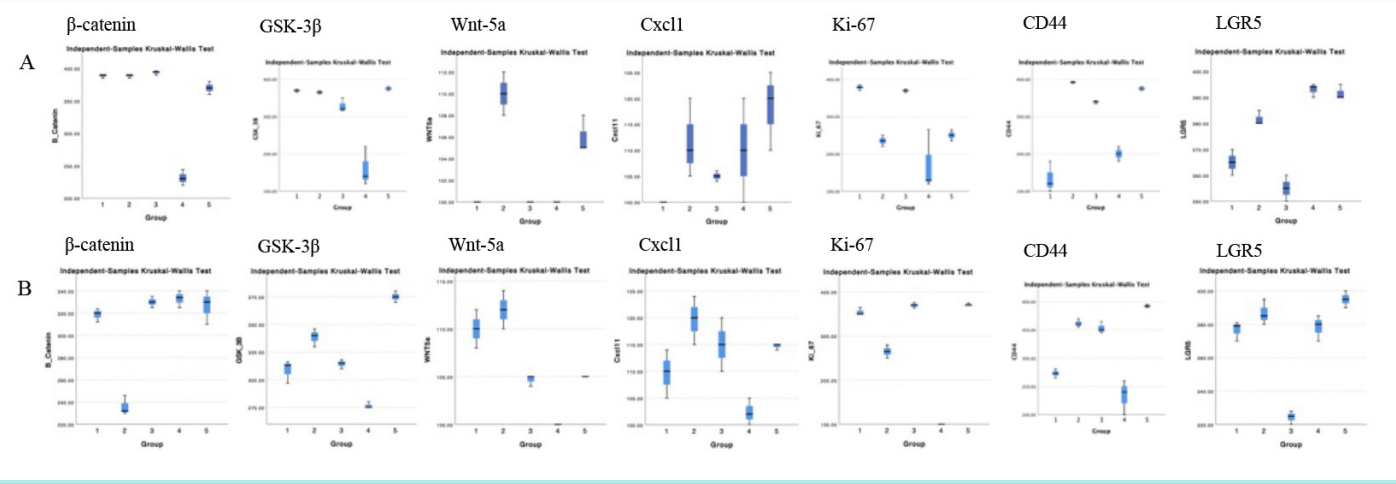


Figure 4. Statistical analyses of β-catenin, GSK-3β, Wnt-5a, Cxcl1, Ki-67, CD44 and LGR5 in MCF-7 (A) and M4A4 (B) cells in control (1), compound 5 (2), compound 8 (3), compound 13 (4) and compound 14 (5) groups.

except in the control group, and was weak or moderate in MCF-7 and M4A4 cells, respectively (Figure 3C). Finally, immunoreactivity for LGR-5 was strongly positive in all groups where compounds were applied except the compound 13 group, which was moderate in both cell lines (Figure 3D), and it was statistically significant in MCF-7 cells for LGR-5 ($p=0.027$) when compared to the control group. All statistical analyses were shown in Figure 4.

DISCUSSION

After synthesizing the hydrazide compound containing the 4-methoxy-substituted imidazo[2,1-b]thiazole core structure, we prepared thiosemicarbazide derivatives with yields ranging from 70% to 90% by reacting the hydrazide with four different aryl isothiocyanates using our previously reported method from our earlier study. The synthesized compounds were characterized by various spectroscopic techniques.[3] The most significant evidence confirming the formation of the desired compounds was observed in the FT-IR spectra, where the characteristic double band associated with the NH^2 group disappeared, and a new band in the range of 1200-1050 cm^{-1}, corresponding to the C = S stretching vibration, appeared. In the ^{1}H-NMR spectra, the absence of signals attributed to NH^2 protons further supported the conversion of NH^2 to N = C. Additionally, an increase in integral values from 7 to 8 ppm, corresponding to aromatic protons, was observed, with distinct peaks assigned to the hydrogens of phenyl, phenethyl, 4-chlorophenyl, and 4-fluorophenyl groups. Mass spectroscopy of the phenyl derivative selected as the prototype was conducted, and the M+ peak of the compound with a molecular weight of 437 was observed as the base peak with an abundance of 100%. The spectra of the synthesized compounds are provided in the supplementary material file.

Our study investigated the effects of thiosemicarbazide compounds 5, 8, 13, and 14 on MCF-7 and M4A4 breast cancer cell lines. Notably, applying compounds 5, 8, and 14 resulted in an accumulation of β-catenin and GSK3β in the cytoplasm and nucleus. This observation supports the activation of the canonical Wnt/β-catenin pathway, which may drive cell proliferation and differentiation. However, in certain conditions, thiosemicarbazides might stimulate proliferation if they induce stress responses or activate pathways that promote cell survival or regeneration. In our study, if compounds like thiosemicarbazide derivatives (such as 5, 8, 13, and 14) are triggering increased proliferation in breast cancer cell lines (as indicated by Ki-67 levels), it may suggest that these compounds are activating signaling pathways that support cell growth, potentially through the Wnt/β-catenin pathway or other cell survival mechanisms. However, it is noteworthy that the application of compound 13 resulted in reduced immunoreactivity for investigated proteins, indicating a different mode of action or a potential inhibitory effect on this pathway. The proliferation marker Ki-67 showed increased immunoreactivity with compounds 8 and 14 in both MCF-7 and M4A4 cell lines, suggesting enhanced cell proliferation in response to these compounds. In contrast, compound 5, decreased Ki-67 levels compared to the control group. In addition, compound 13 reduced the number of cells. This reduction in Ki-67 supports the notion that these compounds may inhibit cell proliferation, with compound 13 showing particular effectiveness. Interestingly, all compounds appeared to enhance stemness properties in both MCF-7 and M4A4 cells. This weak intensity of Wnt5a and Cxcl1 may indicate a limited role for these factors in the context of the treatments applied. Furthermore, LGR5, a downstream target of the canonical Wnt pathway, showed decreased expression associated with increased senescence. However, our results suggest that none of the tested compounds significantly impacted senescence in MCF-7 and M4A4 cells, indicating that their effects may primarily be on proliferation and stemness rather than on senescent cell populations.

Study Limitations

An *in vitro* cell culture model was used in this study, but this does not capture the complexity of tumor microenvironments, including immune responses, stromal interactions, or pharmacokinetics. The effects of compounds may differ in a more complex in vivo setting. Future studies could focus on evaluating the efficacy of these thiosemicarbazide compounds in 3D spheroid models to better mimic the tumor microenvironment.

CONCLUSION

Overall, our findings highlight the diverse impacts of thiosemicarbazide compounds on both primary and metastatic breast cancer cell lines. In particular, compound 13 shows promise for reducing the proliferation

of metastatic breast cancer cells, although further investigation is warranted to evaluate its impact on stemness properties. The balance between proliferation and stemness should be controlled. Understanding the balance between proliferation, differentiation, and stemness in response to these compounds could inform future treatment strategies for breast cancer.

MAIN POINTS

- Synthesized thiosemicarbazide derivatives affected breast cancer cell lines.
- The cytotoxic effects of derivatives differed, and compounds 8 and 14 activated the canonical Wnt/β-catenin pathway, promoting cell proliferation, while compound 13 inhibited proliferation.
- All compounds were found to enhance stemness properties in both MCF-7 and M4A4 cells.

ETHICS

Ethics Committee Approval: Not available.

Informed Consent: Not available.

Footnotes

Authorship Contributions

Concept: H.S.V., E.B., H.K.E., F.B., N.U.G., Design: H.S.V, E.B., H.K.E., F.B., Data Collection and/or Processing: H.S.V., E.B., H.K.E., F.B., N.U.G., Analysis and/or Interpretation: H.S.V., E.B., H.K.E., F.B., N.U.G., Literature Search: H.S.V., E.B., H.K.E., F.B., N.U.G., Writing: H.S.V., E.B., H.K.E., F.B., N.U.G.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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