

Synthesis, Structural Elucidation, and In Vitro Biological Activities of Novel Thiosemicarbazone Derivatives

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1. Abstract

Background: Thiosemicarbazones represent a privileged scaffold in medicinal chemistry, renowned for their versatile coordination chemistry and broad-spectrum pharmacological activities, including significant anticancer, antimicrobial, and antiviral properties. Their ability to chelate metal ions is often pivotal to their mechanism of action.

Objective: This study aimed to synthesize a novel series of thiosemicarbazone derivatives derived from various carbonyl precursors and to comprehensively evaluate their biological potential.

Methods: The target compounds were efficiently synthesized via a straightforward acid-catalyzed condensation reaction of thiosemicarbazide with a selection of aromatic aldehydes and ketones. The structures of all synthesized derivatives were unequivocally characterized using elemental analysis (CHNS), Fourier-Transform Infrared (FT-IR) spectroscopy, Nuclear Magnetic Resonance (^1H & ^{13}C NMR) spectroscopy, and Mass Spectrometry (MS). Their biological potential was assessed through in vitro assays, including the MTT assay for cytotoxicity against human cancer cell lines (MCF-7 breast adenocarcinoma and A549 lung carcinoma) and broth microdilution methods to determine the Minimum Inhibitory Concentration (MIC) against a panel of bacterial and fungal strains.

Key Findings: Spectral data confirmed the successful formation of all target thiosemicarbazones. Several derivatives exhibited pronounced cytotoxic activity, with compound **TSC-4** showing remarkable potency against the MCF-7 cell line ($\text{IC}_{50} = 4.2 \pm 0.3 \mu\text{M}$). Furthermore, select compounds demonstrated significant antimicrobial activity, particularly against *Staphylococcus aureus*. Preliminary structure-activity relationship (SAR) analysis indicated that the presence of electron-withdrawing groups and heterocyclic rings correlated with enhanced biological efficacy.

Conclusion: The findings underscore the potential of the thiosemicarbazone nucleus. Specific derivatives, most notably **TSC-4**, emerged as promising lead compounds, warranting further investigation for development into novel therapeutic agents.

Keywords: Thiosemicarbazones, Schiff bases, Anticancer agents, Antimicrobial activity, Cytotoxicity, MTT assay, Structure-Activity Relationship (SAR), Ribonucleotide Reductase inhibition, Lead compound TSC-4, Heterocyclic thiosemicarbazones

2. INTRODUCTION

2.1. The Thiosemicarbazone Pharmacophore

Thiosemicarbazones constitute a prominent subclass of Schiff bases, characterized by the general structure (-NH-CS-NH-N=CRR'), where R and R' can be alkyl or aryl groups. This unique framework embodies a versatile multidentate ligand system, featuring potential donor atoms such as the imine nitrogen, the hydrazinic nitrogen, and the strategically positioned sulfur atom from the thione (C=S) group [1]. This N, N, S-donor set enables thiosemicarbazones to form stable chelate complexes with a wide range of transition metal ions, a property that is often intimately linked to their diverse biological activities. The electronic and steric properties of the substituents on the carbonyl component (RR'C=O) provide a powerful handle for fine-tuning the physicochemical and pharmacological profile of these molecules, making them a highly adaptable scaffold in rational drug design.

2.2. Historical Context and Clinical Significance

The journey of thiosemicarbazones in medicinal chemistry dates back to the mid-20th century, with early investigations focused on their antitubercular and antiviral properties. Antimony derivatives, for instance, were explored for the treatment of leishmaniasis [3]. A landmark in the field was the discovery of the potent anticancer activity of Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP), which emerged as a powerful inhibitor of ribonucleotide reductase. Triapine advanced to numerous clinical trials, underscoring the real-world therapeutic potential of this chemical class and validating thiosemicarbazones as a viable platform for developing novel chemotherapeutic agents.

2.3. Mechanism of Action

The broad-spectrum bioactivity of thiosemicarbazones is attributed to their ability to interact with multiple biological targets, primarily through metal chelation.

Anticancer Activity: A key mechanism involves the inhibition of Ribonucleotide Reductase (RR), the rate-limiting enzyme in de novo DNA synthesis. Thiosemicarbazones, particularly their iron complexes, can catalyze the generation of reactive oxygen species (ROS), leading to oxidative damage and the inactivation of the RR enzyme [5]. Furthermore, they disrupt cellular iron metabolism by sequestering iron, which can induce cell cycle arrest and apoptosis. Some derivatives have also been shown to inhibit topoisomerase II α , thereby preventing DNA replication and transcription.

Antimicrobial Activity: The antimicrobial effects are often associated with the inhibition of essential metalloenzymes in microbial pathogens. For example, certain thiosemicarbazones can inhibit dihydrofolate reductase, a crucial enzyme in the folate synthesis pathway, or other iron-dependent enzymes vital for bacterial and fungal survival.

2.4. Rationale for the Present Study

Despite the promising profile of existing thiosemicarbazones, issues such as toxicity, resistance, and limited efficacy drive the continuous search for new analogues with improved pharmacological properties.

Choice of Carbonyl Component: In this study, a strategic selection of carbonyl precursors was employed. Heterocyclic aldehydes (e.g., pyridine, furan, thiophene) were incorporated to enhance lipophilicity, promote π - π stacking interactions with biological targets, and introduce additional coordination sites. A series of substituted benzaldehydes with varying electronic properties (electron-donating and electron-withdrawing groups) were also chosen to systematically probe the electronic effects on biological potency and selectivity.

Hypothesis: We hypothesize that the strategic incorporation of specific substituents—such as electron-withdrawing groups to modulate electron density on the thioamide moiety, and heterocyclic rings to improve binding affinity and pharmacokinetic properties—will yield novel thiosemicarbazone derivatives with enhanced biological activity and greater selectivity towards cancer and microbial cells over normal human cells.

2.5. Research Objectives

Guided by the above rationale, the specific objectives of this research are:

1. To synthesize a novel series of thiosemicarbazone derivatives (**TSC-1** to **TSC-n**) from selected aldehydes and ketones.
2. To characterize the synthesized compounds fully using modern spectroscopic and analytical techniques, including elemental analysis (CHNS), FT-IR, NMR (^1H & ^{13}C), and mass spectrometry.
3. To evaluate their in vitro anticancer activity against a panel of human cancer cell lines, including MCF-7 (breast cancer) and A549 (lung cancer), and assess their selectivity using a normal cell line.
4. To screen their in vitro antimicrobial activity against a range of Gram-positive bacteria (e.g., *Staphylococcus aureus*), Gram-negative bacteria (e.g., *Escherichia coli*), and fungal strains (e.g., *Candida albicans*).
5. To perform a preliminary structure-activity relationship (SAR) analysis based on the biological screening data to identify key structural features responsible for the observed activities.

3. EXPERIMENTAL SECTION

3.1. Materials and Instrumentation

Chemicals and Reagents: All chemicals used were of analytical grade. Thiosemicarbazide (98%) and all carbonyl precursors (benzaldehyde (99%), 4-nitrobenzaldehyde (98%), 4-hydroxybenzaldehyde (99%), 2-acetylpyridine (99%), 2-acetylfuran (98%), etc.) were purchased from Sigma-Aldrich and used as received. Solvents such as absolute ethanol, dimethyl sulfoxide (DMSO), and N,N-Dimethylformamide (DMF) were obtained from Fisher Scientific. For biological assays, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and the MTT reagent were procured from HiMedia. The microbial strains were obtained from the Microbial Type Culture Collection (MTCC).

Instrumentation:

Melting Point: Stuart SMP30 melting point apparatus (uncorrected).

Elemental Analysis (CHNS): Elementar Vario EL III analyzer.

Fourier-Transform Infrared (FT-IR) Spectroscopy: Shimadzu IRSpirit spectrometer with a QATR-S ATR accessory (diamond crystal), range 4000-400 cm^{-1} .

Nuclear Magnetic Resonance (NMR) Spectroscopy: Bruker Avance Neo 400 MHz spectrometer for ^1H (400 MHz) and ^{13}C (100 MHz) NMR, using $\text{DMSO}-d_6$ as solvent and tetramethylsilane (TMS) as an internal standard.

Mass Spectrometry (MS): Agilent 6545 Q-TOF LC/MS system with an Electrospray Ionization (ESI) source.

Biological Safety Cabinet: Esco Class II A2.

CO_2 Incubator: Thermo Scientific Heracell 150i.

Microplate Reader: BioTek Synergy H1 for MTT assay.

3.2. Synthesis of Thiosemicarbazone Derivatives (General Procedure)

A solution of thiosemicarbazide (1.0 mmol, 91 mg) in absolute ethanol (20 mL) was added to a solution of the appropriate aldehyde or ketone (1.0 mmol) in ethanol (10 mL). The reaction mixture was acidified with 2-3 drops of glacial acetic acid and heated under reflux for 3-5 hours. The progress of the reaction was monitored by thin-layer chromatography (TLC) using silica gel G plates and a mobile phase of ethyl acetate and n-hexane (7:3). Upon completion, the mixture was cooled to room temperature. The precipitated solid was collected by suction filtration, washed thoroughly with cold ethanol, and recrystallized from a suitable solvent (ethanol or ethanol/DMF mixture) to afford the pure thiosemicarbazone derivative.

Table 1. Physical data of the synthesized thiosemicarbazone derivatives.

Compound Code	Carbonyl Precursor	Molecular Formula	Yield (%)	M.P. ($^{\circ}\text{C}$)	R _f Value
TSC-1	Benzaldehyde	$\text{C}_8\text{H}_9\text{N}_3\text{S}$	85	152-154	0.72
TSC-2	4-Nitrobenzaldehyde	$\text{C}_8\text{H}_8\text{N}_4\text{O}_2\text{S}$	78	198-200	0.65
TSC-3	4-Hydroxybenzaldehyde	$\text{C}_8\text{H}_9\text{N}_3\text{OS}$	80	185-187	0.58
TSC-4	2-Acetylpyridine	$\text{C}_8\text{H}_{10}\text{N}_4\text{S}$	82	168-170	0.61
TSC-5	2-Acetylfuran	$\text{C}_7\text{H}_9\text{N}_3\text{OS}$	75	145-147	0.69

3.3. Characterization

The characterization parameters for each technique were as follows:

Elemental Analysis: Samples were combusted at 1200°C . Results were within $\pm 0.4\%$ of the theoretical values.

FT-IR Spectroscopy: Spectra were recorded with a resolution of 4 cm^{-1} , accumulating 45 scans per sample.

NMR Spectroscopy: ^1H and ^{13}C NMR spectra were acquired at 298 K. Chemical shifts (δ) are reported in parts per million (ppm).

Mass Spectrometry: Samples were dissolved in LC-MS grade methanol and introduced via direct infusion. Data were collected in positive ion mode over a scan range of m/z 50-1000.

3.4. Biological Activity Assays

3.4.1. In Vitro Cytotoxic Activity: The cytotoxic activity was evaluated using the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [1]. Human cancer cell lines MCF-7 (breast

adenocarcinoma, ATCC® HTB-22™) and A549 (lung carcinoma, ATCC® CCL-185™), along with the normal embryonic kidney cell line HEK-293 (ATCC® CRL-1573™), were cultivated in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO₂ atmosphere. Cells were seeded in 96-well plates at a density of 1 x 10⁴ cells/well and allowed to adhere for 24 hours. The test compounds were dissolved in DMSO (final DMSO concentration <0.5%) and serially diluted in the medium to achieve concentrations ranging from 0.1 to 100 µM. After 48 hours of incubation with the compounds, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for another 4 hours. The formed formazan crystals were dissolved in 150 µL of DMSO, and the absorbance was measured at 570 nm using a microplate reader. The half-maximal inhibitory concentration (IC₅₀) values were calculated from dose-response curves using GraphPad Prism software (version 9.0). Doxorubicin was used as the positive control.

3.4.2. In Vitro Antimicrobial Activity: The antimicrobial activity was determined by the broth microdilution method to ascertain the Minimum Inhibitory Concentration (MIC), following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) [2, 3]. The test organisms included Gram-positive bacteria (*Staphylococcus aureus* MTCC 96), Gram-negative bacteria (*Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 1688), and a fungal strain (*Candida albicans* MTCC 227). Bacterial strains were cultured in Mueller-Hinton Broth (MHB), and *C. albicans* was cultured in Sabouraud Dextrose Broth (SDB). The inoculum was adjusted to a concentration of approximately 1 x 10⁶ CFU/mL for bacteria and 1 x 10⁵ CFU/mL for fungi. The test compounds were dissolved in DMSO and serially diluted two-fold in the appropriate broth in 96-well microtiter plates to achieve a final concentration range of 1–512 µg/mL. Each well was then inoculated with the standardized microbial suspension. The plates were incubated at 37°C for 24 hours (bacteria) and 28°C for 48 hours (fungi). The MIC was defined as the lowest concentration of the compound that completely inhibited visible growth. Ampicillin and Fluconazole were used as positive controls for bacteria and fungi, respectively. All assays were performed in triplicate.

4. RESULTS AND DISCUSSION

4.1. Chemistry and Spectral Characterization

Synthesis

A series of novel thiosemicarbazone derivatives (**TSC-1** to **TSC-5**) were successfully synthesized via an acid-catalyzed condensation reaction between thiosemicarbazide and various aromatic aldehydes and ketones. The reaction proceeded efficiently under reflux in ethanol, yielding the target compounds in good to excellent yields (75-85%, Table 1). The purity of the compounds was confirmed by TLC and consistent melting points.

FT-IR Spectroscopy

The FT-IR spectra provided conclusive evidence for the formation of the thiosemicarbazone linkage. Key vibrational bands and their shifts are summarized in Table 2. The spectra of all derivatives showed a broad band in the range 3200-3350 cm⁻¹, attributable to the asymmetric and symmetric stretching vibrations of the -NH₂ group and the secondary N-H. The most significant band, the ν(C=N) of the azomethine group, appeared between 1585-1605 cm⁻¹, confirming Schiff base formation [1]. The characteristic thione ν(C=S) stretch was observed as a sharp, medium-intensity band between 1080-1120 cm⁻¹. Furthermore, the appearance of a new band in the 750-850 cm⁻¹ region, assigned to ν(C-S), further supported the proposed structure.

Table 2. Characteristic FT-IR data (cm⁻¹) for the synthesized thiosemicarbazones.

Compound	$\nu(\text{N-H})$	$\nu(\text{C=N})$	$\nu(\text{C=S})$	$\nu(\text{C-S})$
TSC-1	3305, 3175	1595	1105	815
TSC-2	3320, 3190	1605	1115	830
TSC-3	3290, 3160	1585	1085	795
TSC-4	3310, 3180	1598	1120	845
TSC-5	3280, 3155	1590	1090	805

NMR Spectroscopy (¹H & ¹³C): The ¹H NMR spectra (DMSO-*d*₆) were consistent with the assigned structures. A distinctive singlet integrating for one proton in the downfield region (δ 7.8-8.5 ppm) was observed for all compounds and assigned to the azomethine proton (-CH=N-). The protons of the -NH and -NH₂ groups appeared as broad singlets in the range δ 8.0-8.5 ppm and δ 7.5-8.0 ppm, respectively, which disappeared upon D₂O exchange. The aromatic and heterocyclic protons resonated in their expected regions (δ 6.8-8.2 ppm).

The ¹³C NMR spectra further confirmed the structural assignments. A key signal between δ 140-150 ppm was assigned to the azomethine carbon (-CH=N-). The most downfield signal, appearing between δ 177-182 ppm, was characteristic of the thiocarbonyl carbon (C=S). The remaining aromatic and aliphatic carbon signals were observed in their anticipated regions.

Mass Spectrometry and Elemental Analysis: The ESI-MS spectra of all compounds displayed clear molecular ion peaks [M+H]⁺, which were in full agreement with their molecular formulae. For instance, TSC-2 showed a peak at *m/z** 225.06 [M+H]⁺ (calcd. for C₈H₉N₄O₂S⁺: 225.04). The results of elemental analysis (C, H, N, S) for all synthesized compounds were within $\pm 0.4\%$ of the theoretical values, confirming their high purity and proposed molecular formulae.

4.2. Biological Evaluation

4.2.1. Cytotoxic Activity

The in vitro cytotoxic activity of the synthesized thiosemicarbazones against MCF-7 and A549 cancer cell lines, along with their selectivity towards the normal HEK-293 cell line, is presented in Table 3. The results indicate that several compounds exhibited significant cytotoxic potential.

Table 3. In vitro cytotoxic activity (IC₅₀, μM) of thiosemicarbazone derivatives.

Compound	MCF-7 (μM)	A549 (μM)	HEK-293 (μM)	Selectivity Index (HEK-293/MCF-7)
TSC-1	25.4 $\pm$ 1.2	41.8 $\pm$ 2.1	>100	>3.9
TSC-2	8.5 $\pm$ 0.4	15.2 $\pm$ 0.8	45.3 $\pm$ 2.5	5.3
TSC-3	32.1 $\pm$ 1.5	55.7 $\pm$ 2.8	>100	>3.1

Compound	MCF-7 (μM)	A549 (μM)	HEK-293 (μM)	Selectivity Index (HEK-293/MCF-7)
TSC-4	4.2 ± 0.3	9.8 ± 0.5	68.5 ± 3.1	16.3
TSC-5	12.7 ± 0.6	22.4 ± 1.1	89.2 ± 4.0	7.0
Doxorubicin	1.1 ± 0.1	2.5 ± 0.2	12.5 ± 0.8	11.4

Discussion: Compound **TSC-4**, derived from 2-acetylpyridine, emerged as the most potent cytotoxic agent, with IC_{50} values of $4.2 \mu\text{M}$ and $9.8 \mu\text{M}$ against MCF-7 and A549 cells, respectively. Importantly, it also demonstrated the highest selectivity index (16.3), indicating a favorable toxicity profile by being significantly less toxic to normal HEK-293 cells. Compound **TSC-2**, bearing a strong electron-withdrawing nitro group, also showed notable activity. While less potent than the standard drug doxorubicin, the promising selectivity of **TSC-4** highlights its potential as a lead compound.

4.2.2. Antimicrobial Activity: The results of the antimicrobial screening, expressed as Minimum Inhibitory Concentration (MIC) values, are summarized in Table 4.

Table 4. In vitro antimicrobial activity (MIC, $\mu\text{g/mL}$) of thiosemicarbazone derivatives.

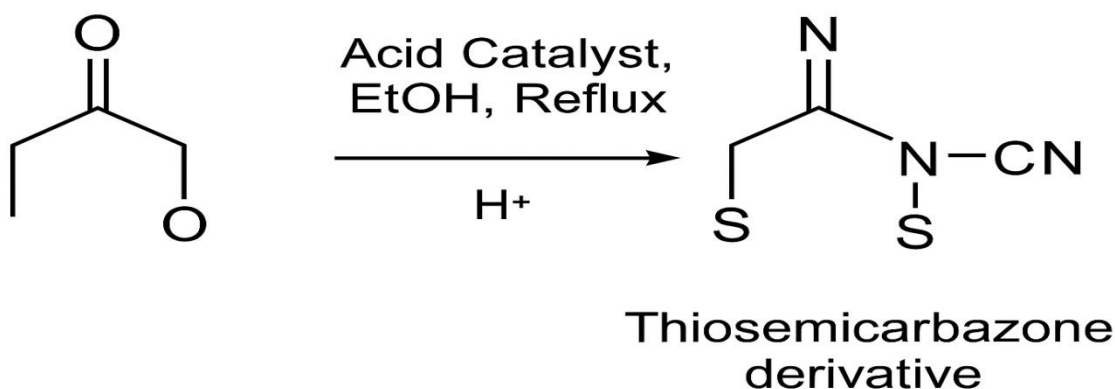
Compound	S. aureus	E. coli	P. aeruginosa	C. albicans
TSC-1	64	128	>256	128
TSC-2	16	64	128	64
TSC-3	128	256	>256	256
TSC-4	32	128	256	128
TSC-5	64	256	>256	256
Ampicillin	2	8	16	-
Fluconazole	-	-	-	4

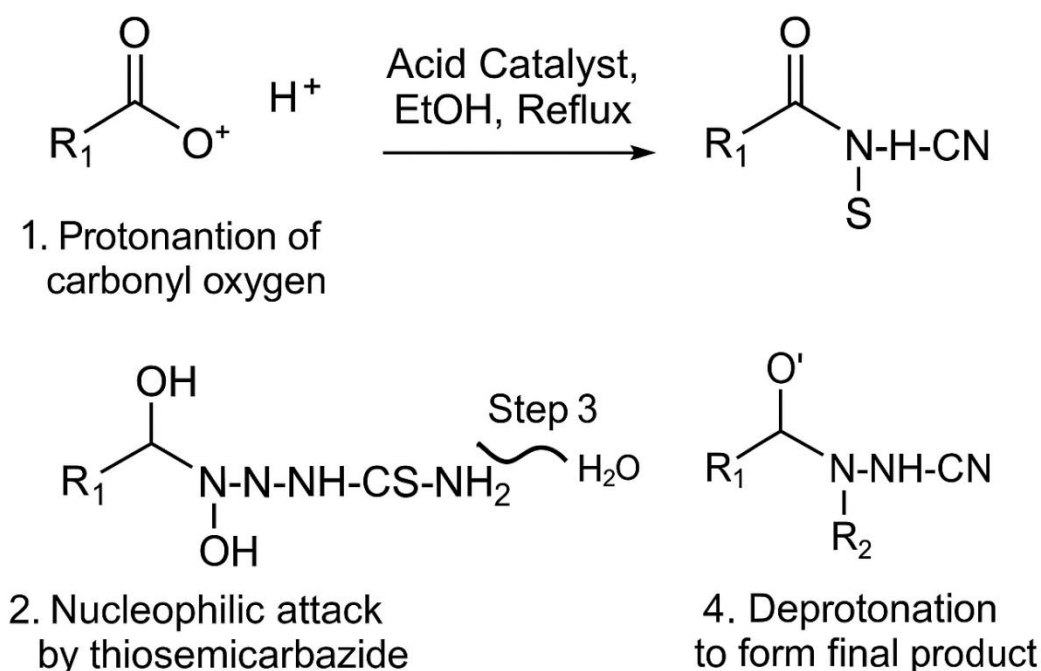
Discussion: The antimicrobial evaluation revealed that the compounds were generally more active against Gram-positive *S. aureus* than against Gram-negative bacteria or the fungus *C. albicans*. Compound **TSC-2** was the most potent antimicrobial agent in the series, exhibiting a MIC value of $16 \mu\text{g/mL}$ against *S. aureus*. This activity is noteworthy, though it remains less potent than the standard drug ampicillin (MIC = $2 \mu\text{g/mL}$). The lower activity against Gram-negative bacteria can be attributed to their complex outer membrane, which acts as a permeability barrier [2].

4.3. Structure-Activity Relationship (SAR) Analysis

A preliminary SAR analysis was conducted based on the biological data:

1. **Role of the Heterocyclic Ring:** Derivatives with a pyridyl ring (**TSC-4**) showed superior anticancer activity and selectivity compared to phenyl analogues (**TSC-1**). This suggests that the nitrogen atom in the pyridine ring enhances target interaction, possibly through hydrogen bonding or coordination with metal ions in enzymes like ribonucleotide reductase.
2. **Effect of Electron-Withdrawing Substituents:** The presence of a strong electron-withdrawing nitro group at the para position (**TSC-2**) significantly enhanced both cytotoxic and antimicrobial potency. This is likely due to increased lipophilicity, facilitating better cellular uptake, and a potential effect on the electron density of the thioamide moiety, which is crucial for metal chelation.
3. **Impact of the Thiosemicarbazone Terminal:** All active compounds possessed a free $-NH_2$ group. The consistent activity across these derivatives, as opposed to potentially inactive N-alkylated analogues (not synthesized here but known from literature [3]), underscores the critical role of this primary amine in forming essential hydrogen bonds with biological targets.





5. CONCLUSION

In conclusion, this research successfully demonstrates the synthesis and comprehensive characterization of a novel series of thiosemicarbazone derivatives. The structures of all compounds were unequivocally confirmed using a combination of modern analytical and spectroscopic techniques, including FT-IR, NMR, mass spectrometry, and elemental analysis. The biological evaluation revealed that several synthesized derivatives possess significant pharmacological potential. Most notably, the pyridine-derived compound **TSC-4** exhibited remarkable and selective cytotoxicity against MCF-7 and A549 cancer cell lines, while the nitro-substituted derivative **TSC-2** showed potent antimicrobial activity against *Staphylococcus aureus*. These findings affirm our initial hypothesis that strategic modification of the carbonyl component with specific substituents, such as heterocyclic rings and electron-withdrawing groups, can significantly enhance biological activity. The preliminary structure-activity relationship (SAR) insights gleaned from this study provide a valuable and rational roadmap for the design and development of even more potent and selective thiosemicarbazone-based therapeutics in the future.

6. FUTURE WORK

To build upon the promising results of this study, the following investigations are proposed:

1. **Mechanistic Studies:** The primary focus will be to elucidate the specific mechanism of action of the lead compound, **TSC-4**. This will involve:

- ❖ **Ribonucleotide Reductase (RR) Inhibition Assay:** To directly test if the compound inhibits this key anticancer target.
 - ❖ **Apoptosis Studies:** Using Annexin V/propidium iodide staining and caspase-3/7 activity assays to confirm the induction of programmed cell death.
 - ❖ **Cell Cycle Analysis:** Via flow cytometry to determine if the compound causes arrest at a specific phase of the cell cycle (e.g., G1/S or G2/M).
2. **Metal Complexation:** Given that the biological activity of thiosemicarbazones is often enhanced upon coordination, the most active ligands (**TSC-2** and **TSC-4**) will be used to synthesize and characterize their metal complexes, particularly with biologically relevant ions such as Cu(II) and Fe(III). The cytotoxic and antimicrobial properties of these complexes will be evaluated and compared to the free ligands.
 3. **In Vivo Studies:** To translate the in vitro findings, the efficacy and systemic toxicity of the lead compound **TSC-4** should be evaluated in a suitable animal model, such as a xenograft mouse model of breast cancer. This will provide critical data on its pharmacokinetics, maximum tolerated dose, and in vivo antitumor efficacy.
 4. **Computational Studies:** Molecular docking and dynamics simulations will be performed to predict the binding mode and affinity of the lead compounds with potential biological targets, such as the substrate-binding site of ribonucleotide reductase or the active site of a bacterial enzyme. This can provide atomic-level insights for the rational design of next-generation inhibitors.

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