

# Design, synthesis and *in vitro* evaluation of new thiosemicarbazone derivatives as potential anticancer agents

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**ABSTRACT:** Thiosemicarbazones play a pivotal role as potential therapeutic agents for the management of lung cancer. 4-(4-Cyanophenyl)-1-[(5-arylfuran-2-yl)methylene]thiosemicarbazides (**1-10**) were obtained *via* the reaction of 4-(4-cyanophenyl)thiosemicarbazide with 5-arylfurfurals. MTT assay was performed to assess their cytotoxic effects on A549 human lung adenocarcinoma and L929 mouse fibroblast cell lines. Compounds **1**, **5**, **6** and **7** were identified as the most effective anticancer agents on A549 cell line with IC<sub>50</sub> values of 12.75±0.35 µg/mL, 4.30±0.61 µg/mL, 5.50±2.12 µg/mL and 5.90±0.57 µg/mL, respectively compared to cisplatin (IC<sub>50</sub>= 12.00±0.71 µg/mL). The IC<sub>50</sub> values of these compounds for L929 cell line were higher than their IC<sub>50</sub> values for A549 cell line indicating that their anticancer effects were selective. The apoptotic effects of these compounds were also analyzed based on Annexin V-PI binding capacities in flow cytometry. According to flow cytometric analyses, the early apoptotic effects of compounds **1**, **5**, **6** and **7** on A549 cell line were determined as 4.7, 5.1, 7.3 and 5.1%, whereas their late apoptotic effects were determined as 3.7, 2.0, 4.9 and 3.3%, respectively. Compound **6** showed more apoptotic activity than compounds **1**, **5** and **7**. According to these findings, compounds **5** and **6** stand out as promising anticancer agents for further *in vitro* and *in vivo* studies.

**KEYWORDS:** Apoptosis; cytotoxicity; furan; lung cancer; thiosemicarbazone.

## 1. INTRODUCTION

Lung cancer remains the leading cause of cancer-related death across the globe with a 5-year survival rate of less than 15% [1,2]. As the most common lung cancer, non-small cell lung cancer (NSCLC) poses a major threat to public health. Despite substantial advances in diagnostic and therapeutic approaches, the overall survival for NSCLC patients still remains poor [3]. One main impediment for the treatment of NSCLC is that most patients are diagnosed at advanced or metastatic stages (stage III/IV) when the prognosis is poor and therapeutic options are limited [2-4].

Apoptosis is a tightly regulated cellular process that is essential for removal of damaged or unwanted cells and maintenance of cellular homeostasis [5,6]. In pathological conditions, particularly cancer, cells lose their ability to undergo apoptosis leading to uncontrolled proliferation [7]. Mounting evidence has shown that most of the anticancer agents trigger the induction of apoptosis and related cell death networks to eliminate tumor cells [7] and therefore the discovery of new apoptosis-inducing antitumor agents [8] is of great importance for the management of many types of cancer including lung cancer [6-10].

Thiosemicarbazones (TSCs) have attracted a great deal of attention for many years owing to their pharmacological utility as therapeutic agents and versatility as ligands allowing them to give rise to a great variety of coordination modes [11,12]. In particular, TSCs have been identified as promising antiproliferative agents against a variety of tumors through the inhibition of diverse molecular targets including ribonucleotide reductase (RNR) [11-20]. Among them, triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP), a potent RNR inhibitor, is an anticancer agent currently in clinical trials (Figure 1) [11-14].

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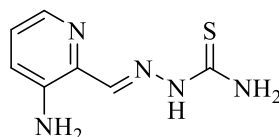
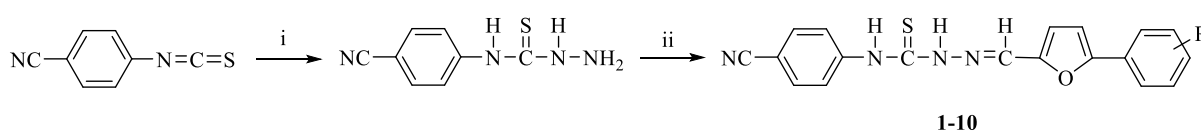


Figure 1. Triapine.

On the basis of the aforementioned findings and in the continuation of our ongoing research efforts to identify new thiosemicarbazone derivatives as anticancer agents [18-20], herein we reported the preparation of a new series of thiosemicarbazones and focused on their cytotoxic effects on A549 human lung adenocarcinoma and L929 mouse fibroblast cell lines. The most active compounds were also evaluated for their apoptotic effects on A549 cell line.

## 2. RESULTS AND DISCUSSION

The synthesis of thiosemicarbazone derivatives (**1-10**) was carried out according to the steps depicted in Figure 2. In the initial step, 4-(4-cyanophenyl)thiosemicarbazide was synthesized *via* the reaction of 4-cyanophenyl isothiocyanate with hydrazine hydrate. The reaction of 4-(4-cyanophenyl)thiosemicarbazide with 5-arylfurfurals afforded 4-(4-cyanophenyl)-1-[(5-arylfuran-2-yl)methylene]thiosemicarbazides (**1-10**). The IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, and HRMS data were in agreement with the proposed structures of compounds **1-10**.



Compound	R
1	2-Cl
2	3-Cl
3	4-Cl
4	2-NO <sub>2</sub>
5	3-NO <sub>2</sub>
6	4-NO <sub>2</sub>
7	2,4-Cl <sub>2</sub>
8	3,4-Cl <sub>2</sub>
9	2,5-Cl <sub>2</sub>
10	2-NO <sub>2</sub> -4-Cl

Figure 2. The synthetic route for the preparation of the thiosemicarbazone derivatives (**1-10**). Reagents and conditions: (i) NH<sub>2</sub>NH<sub>2</sub>·H<sub>2</sub>O, ethanol, rt, 4h; (ii) ArCHO, ethanol, reflux, 8h.

MTT assay was performed to determine the antiproliferative effects of compounds **1-10** on A549 human lung adenocarcinoma cell line. Compounds **5**, **6** and **7** were more effective on A549 cell line than cisplatin, whereas compound **1** showed similar anticancer activity against A549 cell line compared to cisplatin (IC<sub>50</sub>= 12.00±0.71 µg/mL). IC<sub>50</sub> Values of compounds **1**, **5**, **6** and **7** for A549 cell line were found as 12.75±0.35 µg/mL, 4.30±0.61 µg/mL, 5.50±2.12 µg/mL and 5.90±0.57 µg/mL, respectively. This outcome pointed out the importance of the position of chloro and nitro substituents for anticancer activity against A549 cell line. In particular, 2-chloro, 3-nitro, 4-nitro and 2,4-dichloro substitutions significantly enhanced anticancer activity, whereas other substitutions significantly decreased anticancer activity.

In order to determine whether the compounds were toxic or nontoxic to healthy cells, the effects of compounds **1-10** on L929 mouse fibroblast cells were investigated using MTT test. The selectivity index (SI) values of compounds **1**, **5**, **6** and **7** were also determined to compare their selectivity (Table 1). The IC<sub>50</sub> values of these compounds for L929 cell line were higher than their IC<sub>50</sub> values for A549 cell line. This outcome indicated that their anticancer effects were selective. In particular, compounds **5** and **6** showed more selective anticancer activity than other compounds.

**Table 1.** IC<sub>50</sub> values of the compounds against A549 and L929 cells after 24 h.

Compound	IC <sub>50</sub> (µg/mL)		SI*
	A549 Cell line	L929 Cell line	
1	12.75±0.35	21.00±1.41	1.65
2	>500	39.00±3.61	ND
3	>500	26.00±1.73	ND
4	>500	20.33±1.53	ND
5	4.30±0.61	20.00±5.00	4.65
6	5.50±2.12	23.33±1.53	4.24
7	5.90±0.57	9.67±2.08	1.64
8	>500	14.00±3.46	ND
9	>500	6.90±0.36	ND
10	>500	9.00±1.00	ND
<b>Cisplatin</b>	12.00±0.71	ND	ND

ND: Not Determined.

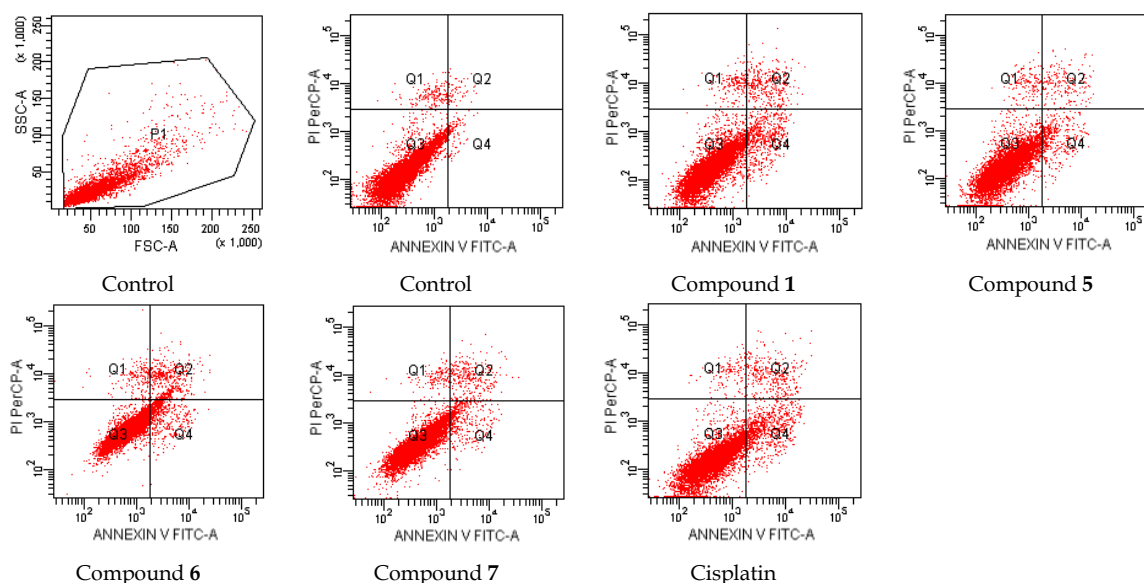
\*SI= IC<sub>50</sub> for L929 cell line / IC<sub>50</sub> for A549 cell line.

After 24 h incubation, the apoptotic effects of compounds **1**, **5**, **6**, **7** and cisplatin on A549 cells were analyzed based on Annexin V-Propidium iodide (PI) binding capacities in flow cytometry as depicted in Figure 3. The early apoptotic effects of compounds **1**, **5**, **6**, **7** and cisplatin on A549 cell line (at IC<sub>50</sub> values) were determined as 4.7, 5.1, 7.3, 5.1 and 9.8% respectively, whilst their late apoptotic effects were determined as 3.7, 2.0, 4.9, 3.3 and 3.5%, respectively (Table 2 and Figure 3). According to these findings, compound **6** showed more apoptotic (early and late) activity than compounds **1**, **5** and **7**. The results indicated that *p*-nitro substituent significantly enhanced apoptotic activity against A549 cell line.

**Table 2.** Percents of typical quadrant analysis of Annexin V FITC/PI flow cytometry of A549 cells treated with compounds **1**, **5**, **6**, **7** and cisplatin.

Groups	Early apoptotic cells%	Late apoptotic cells%	Viability%	Necrosis%
Control	1.4	0.6	96.4	1.6
Cells treated with compound <b>1</b>	4.7	3.7	86.8	2.1
Cells treated with compound <b>5</b>	5.1	2.0	91.1	1.8
Cells treated with compound <b>6</b>	7.3	4.9	85.8	2.0
Cells treated with compound <b>7</b>	5.1	3.3	89.9	1.7
Cells treated with cisplatin	9.8	3.5	85.2	1.5

A549 cells were cultured for 24 hours in medium with compounds **1**, **5**, **6**, **7** and cisplatin at IC<sub>50</sub> values. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.



**Figure 3.** Flow cytometric analysis of A549 cells treated with IC<sub>50</sub> values of compounds **1**, **5**, **6**, **7** and cisplatin. At least 10,000 cells were analyzed per sample, and quadrant analysis was performed.

### 3. CONCLUSION

In the current work, we described the synthesis of new thiosemicarbazone derivatives, which were investigated for their cytotoxic and apoptotic effects on A549 cell line. Compounds **5** and **6** were identified as the most potent anticancer agents in this series against A549 cell line with  $IC_{50}$  values of  $4.3 \pm 0.61 \mu\text{g/mL}$  and  $5.5 \pm 2.12 \mu\text{g/mL}$ , respectively. Compound **6** also induced apoptosis significantly when compared with other compounds. Further studies are needed to enlighten the exact mechanism of action for the evaluation of compounds **5** and **6** as therapeutic agents for the treatment of NSCLC.

### 4. MATERIALS AND METHODS

#### 4.1. Chemistry

All reagents purchased from commercial suppliers were used without further purification. The MP90 digital melting point apparatus (Mettler Toledo, Ohio, USA) was used to determine the melting points (M.p.) of the compounds. IR spectra were recorded on an IRPrestige-21 Fourier Transform Infrared spectrophotometer (Shimadzu, Tokyo, Japan).  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded on a Varian Mercury-400 FT-NMR spectrometer (Agilent, Palo Alto, CA, USA). HRMS spectra were recorded on a Shimadzu LCMS-IT-TOF system (Shimadzu, Kyoto, Japan).

##### 4.1.1. General procedure for the synthesis of the compounds

##### 4-(4-Cyanophenyl)thiosemicarbazide [21]

A mixture of 4-cyanophenyl isothiocyanate (0.1 mol) and hydrazine hydrate (0.2 mol) in ethanol (30 mL) was stirred at room temperature for 4 h and then filtered. The residue was crystallized from ethanol.

##### 4-(4-Cyanophenyl)-1-[(5-arylfuran-2-yl)methylene]thiosemicarbazides (**1-10**)

A mixture of 4-(4-cyanophenyl)thiosemicarbazide (0.01 mol) and 5-arylfurfural (0.01 mol) was refluxed in ethanol for 8 h, filtered and crystallized from ethanol.

##### 4-(4-Cyanophenyl)-1-[(5-(2-chlorophenyl)furan-2-yl)methylene]thiosemicarbazide (**1**)

M.p. 192.8 °C. Yield 80%.

IR  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3257.77 (N-H stretching), 3138.18 (Aromatic C-H stretching), 2970.38 (Aliphatic C-H stretching), 2222.00 ( $\text{C}\equiv\text{N}$  stretching), 1602.85, 1579.70, 1537.27, 1519.91, 1492.90, 1467.83 (N-H bending,  $\text{C}=\text{N}$  and  $\text{C}=\text{C}$  stretching), 1411.89 (C-H bending), 1319.31, 1271.09, 1220.94, 1201.65, 1176.58, 1097.50, 1083.99, 1024.20 (C-N, C-O stretching and aromatic C-H in plane bending), 921.97, 910.40, 840.96, 800.96, 775.38, 756.10, 729.09 (Aromatic C-H out of plane bending).

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  (ppm): 7.30 (d,  $J = 3.60$  Hz, 1H, aromatic proton), 7.34 (d,  $J = 3.60$  Hz, 1H, aromatic proton), 7.37-7.41 (m, 1H, aromatic proton), 7.47-7.50 (m, 1H, aromatic proton), 7.58 (m, 1H, aromatic proton), 7.76 (d,  $J = 7.60$  Hz, 1H, aromatic proton), 7.85 (d,  $J = 8.80$  Hz, 2H, aromatic protons), 7.99 (d,  $J = 8.80$  Hz, 2H, aromatic protons), 8.17 (s, 1H,  $-\text{CH}=\text{N}$ ), 10.25 (s, 1H,  $\text{S}=\text{C}-\text{NH}$ ), 12.22 (s, 1H,  $\text{C}=\text{N}-\text{NH}$ ).

$^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO}-d_6$ )  $\delta$  (ppm): 102.44 (C), 106.72 (CH), 113.46 (CH), 115.64 (CH), 116.95 (C), 118.91 (C), 124.70 (2CH), 127.62 (CH,  $d, J = 6.5$  Hz), 128.32 (CH), 129.45 (CH,  $d, J = 20.5$  Hz), 130.83 (CH), 132.01 (2CH), 133.27 (CH,  $d, J = 40.4$  Hz), 143.36 and 144.60 (CH), 148.94 (C), 151.01 (C), 175.13 (C).

HRMS (ESI) ( $m/z$ ):  $[\text{M}+\text{H}]^+$  calcd. for  $\text{C}_{19}\text{H}_{13}\text{ClN}_4\text{OS}$ : 381.0571, found: 381.0582.

##### 4-(4-Cyanophenyl)-1-[(5-(3-chlorophenyl)furan-2-yl)methylene]thiosemicarbazide (**2**)

M.p. 176.6 °C. Yield 82%.

IR  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3346.50, 3278.99 (N-H stretching), 3126.61 (Aromatic C-H stretching), 2972.31 (Aliphatic C-H stretching), 2220.07 ( $\text{C}\equiv\text{N}$  stretching), 1635.64, 1606.70, 1581.63, 1541.12, 1527.62, 1508.33, 1456.26 (N-H bending,  $\text{C}=\text{N}$  and  $\text{C}=\text{C}$  stretching), 1417.68 (C-H bending), 1321.24, 1273.02, 1251.80, 1201.65, 1176.58, 1099.43, 1078.21, 1060.85, 1028.06 (C-N, C-O stretching and aromatic C-H in plane bending), 997.20, 981.77, 962.48, 933.55, 871.82, 831.32, 773.46, 756.10, 684.73, 671.23 (Aromatic C-H out of plane bending).

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  (ppm): 7.23 (d,  $J = 4.00$  Hz, 1H, aromatic proton), 7.29 (d,  $J = 3.60$  Hz, 1H, aromatic proton), 7.37-7.41 (m, 1H, aromatic proton), 7.46-7.51 (m, 1H, aromatic proton), 7.71-7.80 (m, 1H,

aromatic proton), 7.83 (d,  $J = 8.80$  Hz, 2H, aromatic protons), 7.90 (t,  $J = 3.60, 2.00, 1.60$  Hz, 1H, aromatic proton), 7.98 (d,  $J = 8.80$  Hz, 2H, aromatic protons), 8.12 (s, 1H,  $-CH=N$ ), 10.24 (s, 1H,  $S=C-NH$ ), 12.18 (s, 1H,  $C=N-NH$ ).

$^{13}C$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$  (ppm): 102.45 (C), 106.72 (CH), 109.92 (CH), 116.66 (CH, d,  $J = 58.4$  Hz), 118.93 (C), 122.56 (C), 123.57 (CH), 124.78 (2CH), 127.98 (CH), 131.11 (CH, d,  $J = 46.1$  Hz), 132.30 (2CH), 132.69 (CH, d,  $J = 32.7$  Hz), 133.69 (CH, d,  $J = 39.7$  Hz), 143.41 and 144.61 (CH), 149.27 (C), 153.37 (C), 175.10 (C).

HRMS (ESI) (m/z):  $[M+H]^+$  calcd. for  $C_{19}H_{13}ClN_4OS$ : 381.0571, found: 381.0564.

#### 4-(4-Cyanophenyl)-1-[(5-(4-chlorophenyl)furan-2-yl)methylene]thiosemicarbazide (3)

M.p. 195.1 °C. Yield 85%.

IR  $\nu_{max}$  (cm $^{-1}$ ): 3363.86, 3288.63 (N-H stretching), 3070.68 (Aromatic C-H stretching), 2970.38 (Aliphatic C-H stretching), 2218.14 (C $\equiv$ N stretching), 1600.92, 1585.49, 1521.84, 1506.41, 1475.54 (N-H bending, C=N and C=C stretching), 1444.68, 1413.82, 1398.39 (C-H bending), 1321.24, 1253.73, 1209.37, 1174.65, 1093.64, 1024.20 (C-N, C-O stretching and aromatic C-H in plane bending), 979.84, 921.97, 827.46, 802.39, 783.10, 731.02, 680.87 (Aromatic C-H out of plane bending).

$^1H$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  (ppm): 7.15-7.24 (m, 3H, aromatic protons), 7.50-7.56 (m, 2H, aromatic protons), 7.72-7.89 (m, 3H, aromatic protons), 7.99 (d,  $J = 8.40$  Hz, 2H, aromatic protons), 8.13 (s, 1H,  $-CH=N$ ), 10.22 (s, 1H,  $S=C-NH$ ), 12.17 (s, 1H,  $C=N-NH$ ).

$^{13}C$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$  (ppm): 102.37 (C), 106.62 (CH), 109.17 (CH), 116.65 (CH, d,  $J = 46.8$  Hz), 118.86 (C), 119.29 (C), 124.67 (2CH), 125.64 (CH, d,  $J = 6.4$  Hz), 128.22 (CH, d,  $J = 9.6$  Hz), 128.94 (CH, d,  $J = 4.5$  Hz), 132.23 (2CH), 132.62 (CH, d,  $J = 10.3$  Hz), 133.25 (CH, d,  $J = 34.6$  Hz), 143.34 and 144.54 (CH), 148.47 (C, d,  $J = 11.5$  Hz), 153.63 (C, d,  $J = 32.7$  Hz), 174.97 (C).

HRMS (ESI) (m/z):  $[M+H]^+$  calcd. for  $C_{19}H_{13}ClN_4OS$ : 381.0571, found: 381.0590.

#### 4-(4-Cyanophenyl)-1-[(5-(2-nitrophenyl)furan-2-yl)methylene]thiosemicarbazide (4)

M.p. 201.1 °C. Yield 86%.

IR  $\nu_{max}$  (cm $^{-1}$ ): 3282.84 (N-H stretching), 3118.90, 3066.82 (Aromatic C-H stretching), 2970.38 (Aliphatic C-H stretching), 2220.07 (C $\equiv$ N stretching), 1604.77, 1516.05 (N-H bending and C=N stretching), 1438.90, 1411.89 (C-H bending), 1344.38, 1325.10, 1269.16, 1255.66, 1207.44, 1176.58, 1026.13 (C-N, C-O stretching and aromatic C-H in plane bending), 981.77, 925.83, 835.18, 781.17, 744.52, 702.09 (Aromatic C-H out of plane bending).

$^1H$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  (ppm): 7.14 (d,  $J = 3.60$  Hz, 1H, aromatic proton), 7.27 (d,  $J = 3.20$  Hz, 1H, aromatic proton), 7.60-7.65 (m, 1H, aromatic proton), 7.73-7.80 (m, 2H, aromatic protons), 7.86 (d,  $J = 8.40$  Hz, 2H, aromatic protons), 7.92-7.98 (m, 1H, aromatic proton), 8.05 (d,  $J = 8.80$  Hz, 2H, aromatic protons), 8.08 (s, 1H,  $-CH=N$ ), 10.09 (s, 1H,  $S=C-NH$ ), 12.29 (s, 1H,  $C=N-NH$ ).

$^{13}C$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$  (ppm): 102.40 (C), 106.65 (CH), 112.18 (CH), 115.39 (CH), 116.91 (C), 118.82 (C), 121.69 (CH), 123.80 (2CH, d,  $J = 10.3$  Hz), 128.67 (CH), 129.69 (CH), 132.39 (2CH, d,  $J = 11.6$  Hz), 133.43 (CH), 142.99 and 144.56 (CH), 146.89 (CH), 149.49 (C), 150.14 (C), 174.78 (C).

HRMS (ESI) (m/z):  $[M+H]^+$  calcd. for  $C_{19}H_{13}N_5O_3S$ : 392.0812, found: 392.0791.

#### 4-(4-Cyanophenyl)-1-[(5-(3-nitrophenyl)furan-2-yl)methylene]thiosemicarbazide (5)

M.p. 211.3 °C. Yield 88%.

IR  $\nu_{max}$  (cm $^{-1}$ ): 3321.42, 3261.63 (N-H stretching), 3163.26, 3068.75 (Aromatic C-H stretching), 2953.02 (Aliphatic C-H stretching), 2223.92 (C $\equiv$ N stretching), 1589.34, 1517.98, 1494.83 (N-H bending, C=N and C=C stretching), 1440.83 (C-H bending), 1346.31, 1323.17, 1265.30, 1253.73, 1190.08, 1174.65, 1026.13 (C-N, C-O stretching and aromatic C-H in plane bending), 979.84, 947.05, 923.90, 896.90, 862.18, 837.11, 798.53, 781.17, 738.74, 731.02, 680.87 (Aromatic C-H out of plane bending).

$^1H$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  (ppm): 7.28 (d,  $J = 3.60$  Hz, 1H, aromatic proton), 7.45 (d,  $J = 3.60$  Hz, 1H, aromatic proton), 7.72-7.76 (m, 2H, aromatic protons), 7.83 (d,  $J = 8.40$  Hz, 2H, aromatic protons), 7.99 (d,  $J = 8.40$  Hz, 2H, aromatic protons), 8.14 (s, 1H,  $-CH=N$ ), 8.24-8.27 (m, 1H, aromatic proton), 8.55-8.56 (m, 1H, aromatic proton), 10.25 (s, 1H,  $S=C-NH$ ), 12.20 (s, 1H,  $C=N-NH$ ).

$^{13}C$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$  (ppm): 102.42 (C), 106.69 (CH), 110.85 (CH), 116.06 (CH), 116.92 (C), 118.19 and 118.88 (CH), 122.48 (CH), 124.55 (2CH), 129.95 (CH), 130.61 and 130.85 (CH), 132.29 (2CH), 132.90 (CH), 143.35 (CH), 148.44 (C), 149.81 (C), 152.50 (C), 175.11 (C).

HRMS (ESI) (m/z):  $[M+H]^+$  calcd. for  $C_{19}H_{13}N_5O_3S$ : 392.0812, found: 392.0807.

4-(4-Cyanophenyl)-1-[(5-(4-nitrophenyl)furan-2-yl)methylene]thiosemicarbazide (6)

M.p. 217.4 °C. Yield 92%.

IR  $\nu_{\max}$  (cm<sup>-1</sup>): 3313.71 (N-H stretching), 3122.75 (Aromatic C-H stretching), 2976.16 (Aliphatic C-H stretching), 2223.92 (C≡N stretching), 1597.06, 1548.84, 1508.33, 1492.90 (N-H bending, NO<sub>2</sub>, C=N and C=C stretching), 1446.61, 1411.89 (C-H bending), 1323.17, 1298.09, 1274.95, 1215.15, 1174.65, 1107.14, 1020.34 (C-N, C-O stretching and aromatic C-H in plane bending), 925.83, 848.68, 833.25, 790.81, 777.31, 752.24, 738.74, 692.44 (Aromatic C-H out of plane bending).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 7.32 (d, *J* = 3.60 Hz, 1H, aromatic proton), 7.49 (d, *J* = 3.60 Hz, 1H, aromatic proton), 7.74-7.79 (m, 1H, aromatic proton), 7.86 (d, *J* = 8.40 Hz, 2H, aromatic protons), 8.01 (d, *J* = 8.80 Hz, 2H, aromatic protons), 8.05-8.09 (m, 1H, aromatic proton), 8.16 (s, 1H, -CH=N), 8.29 (d, *J* = 9.20 Hz, 2H, aromatic protons), 10.26 (s, 1H, S=C-NH), 12.27 (s, 1H, C=N-NH).

<sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 102.39 (C), 106.75 (CH), 112.55 (CH), 116.17 (CH), 116.90 (C), 118.86 (CH), 124.34 (CH), 124.62 (2CH, *d*, *J* = 7.7 Hz), 132.28 (2CH), 132.69 (CH), 133.40 (C), 135.02 (CH), 143.27 (CH), 146.24 (CH), 150.67 (C), 152.58 (C), 175.12 (C).

HRMS (ESI) (*m/z*): [M+H]<sup>+</sup> calcd. for C<sub>19</sub>H<sub>13</sub>N<sub>5</sub>O<sub>3</sub>S: 392.0812, found: 392.0815.

4-(4-Cyanophenyl)-1-[(5-(2,4-dichlorophenyl)furan-2-yl)methylene]thiosemicarbazide (7)

M.p. 197.9 °C. Yield 84%.

IR  $\nu_{\max}$  (cm<sup>-1</sup>): 3332.99, 3292.49 (N-H stretching), 3066.82 (Aromatic C-H stretching), 2214.28 (C≡N stretching), 1606.70, 1587.42, 1539.20, 1477.47, 1462.04 (N-H bending, C=N and C=C stretching), 1384.89 (C-H bending), 1323.17, 1290.38, 1265.30, 1176.58, 1099.43, 1031.92 (C-N, C-O stretching and aromatic C-H in plane bending), 987.55, 921.97, 869.90, 833.25, 813.96, 786.96, 686.66 (Aromatic C-H out of plane bending).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 7.28 (d, *J* = 3.60 Hz, 1H, aromatic proton), 7.34 (d, *J* = 3.60 Hz, 1H, aromatic proton), 7.52-7.54 (m, 1H, aromatic proton), 7.70-7.73 (m, 1H, aromatic proton), 7.82 (d, *J* = 9.20 Hz, 2H, aromatic protons), 7.97-8.01 (m, 3H, aromatic protons), 8.14 (s, 1H, -CH=N), 10.19 (s, 1H, S=C-NH), 12.17 (s, 1H, C=N-NH).

<sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 102.41 (C), 106.68 (CH), 113.76 (CH), 115.45 (CH), 116.89 (C), 118.78 (C), 124.51 (2CH), 126.54 (C), 127.79 (CH), 129.30 (CH), 130.09 (CH, *d*, *J* = 10.3 Hz), 132.22 (2CH), 132.09 (CH, *d*, *J* = 52.6 Hz), 143.25 and 144.57 (CH), 149.17 (C), 149.98 (C), 175.14 (C).

HRMS (ESI) (*m/z*): [M+H]<sup>+</sup> calcd. for C<sub>19</sub>H<sub>12</sub>Cl<sub>2</sub>N<sub>4</sub>OS: 415.0182, found: 415.0168.

4-(4-Cyanophenyl)-1-[(5-(3,4-dichlorophenyl)furan-2-yl)methylene]thiosemicarbazide (8)

M.p. 202 °C. Yield 82%.

IR  $\nu_{\max}$  (cm<sup>-1</sup>): 3495.01 (N-H stretching), 3147.83, 3010.88 (Aromatic C-H stretching), 2983.88 (Aliphatic C-H stretching), 2227.78 (C≡N stretching), 1625.99, 1602.85, 1585.49, 1546.91, 1519.91, 1456.26 (N-H bending, C=N and C=C stretching), 1411.89 (C-H bending), 1330.88, 1273.02, 1219.01, 1176.58, 1138.00, 1082.07, 1029.99 (C-N, C-O stretching and aromatic C-H in plane bending), 981.77, 935.48, 923.90, 867.97, 837.11, 815.89, 792.74, 771.53, 734.88, 696.30 (Aromatic C-H out of plane bending).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 7.24 (d, *J* = 4.00 Hz, 1H, aromatic proton), 7.32 (d, *J* = 4.00 Hz, 1H, aromatic proton), 7.69-7.85 (m, 4H, aromatic protons), 8.00-8.08 (m, 3H, aromatic protons), 8.14 (s, 1H, -CH=N), 10.23 (s, 1H, S=C-NH), 12.19 (s, 1H, C=N-NH).

<sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  (ppm): 102.44 (C), 106.71 (CH), 110.37 (CH), 116.24 (CH), 116.93 (C), 118.86 (C), 123.94 (CH), 124.63 (2CH), 125.50 (CH), 131.17 (CH), 129.83 (C), 132.24 (2CH), 132.90 (CH), 143.33 (CH), 149.51 (C), 152.41 (C), 175.08 (C).

HRMS (ESI) (*m/z*): [M+H]<sup>+</sup> calcd. for C<sub>19</sub>H<sub>12</sub>Cl<sub>2</sub>N<sub>4</sub>OS: 415.0182, found: 415.0185.

4-(4-Cyanophenyl)-1-[(5-(2,5-dichlorophenyl)furan-2-yl)methylene]thiosemicarbazide (9)

M.p. 201.8 °C. Yield 81%.

IR  $\nu_{\max}$  (cm<sup>-1</sup>): 3290.56 (N-H stretching), 3140.11, 3010.88 (Aromatic C-H stretching), 2968.45 (Aliphatic C-H stretching), 2227.78 (C≡N stretching), 1606.70, 1589.34, 1521.84, 1463.97 (N-H bending, C=N and C=C stretching), 1442.75, 1413.82, 1379.10 (C-H bending), 1323.17, 1269.16, 1201.65, 1176.58, 1134.14, 1097.50, 1024.20 (C-N, C-O stretching and aromatic C-H in plane bending), 987.55, 937.40, 914.26, 883.40, 835.18, 804.32, 783.10, 707.88, 675.09 (Aromatic C-H out of plane bending).

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  (ppm): 7.28 (d,  $J = 4.00$  Hz, 1H, aromatic proton), 7.38-7.42 (m, 1H, aromatic proton), 7.58 (d,  $J = 8.80$  Hz, 1H, aromatic proton), 7.71-7.75 (m, 1H, aromatic proton), 7.81 (d,  $J = 8.80$  Hz, 2H, aromatic protons), 7.95 (d,  $J = 8.80$  Hz, 2H, aromatic protons), 8.00-8.04 (m, 1H, aromatic proton), 8.12 (s, 1H,  $-\text{CH}=\text{N}$ ), 10.27 (s, 1H,  $\text{S}=\text{C}-\text{NH}$ ), 12.22 (s, 1H,  $\text{C}=\text{N}-\text{NH}$ ).

$^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$  (ppm): 102.38 (C), 106.68 (CH), 114.39 (CH), 115.45 (CH), 116.89 (C), 118.84 (C), 124.57 (2CH), 127.24 (CH), 127.77 (CH), 129.00 (CH), 132.30 (2CH, d,  $J = 7.7$  Hz), 132.60 (CH, d,  $J = 22.5$  Hz), 133.43 (C), 143.30 (CH), 149.47 (C, d,  $J = 3.8$  Hz), 155.69 (C), 175.15 (C).

HRMS (ESI) (m/z):  $[\text{M}+\text{H}]^+$  calcd. for  $\text{C}_{19}\text{H}_{12}\text{Cl}_2\text{N}_4\text{OS}$ : 415.0182, found: 415.0177.

4-(4-Cyanophenyl)-1-[(5-(4-chloro-2-nitrophenyl)furan-2-yl)methylene]thiosemicarbazide (**10**)

M.p. 196.8 °C. Yield 85%.

IR  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3298.28 (N-H stretching), 3124.68 (Aromatic C-H stretching), 2972.31 (Aliphatic C-H stretching), 2222.00 ( $\text{C}\equiv\text{N}$  stretching), 1627.92, 1606.70, 1591.27, 1552.70, 1519.91, 1460.11 (N-H bending,  $\text{NO}_2$ ,  $\text{C}=\text{N}$  and  $\text{C}=\text{C}$  stretching), 1442.75, 1402.25, 1375.25 (C-H bending), 1346.31, 1325.10, 1263.37, 1201.65, 1192.01, 1174.65, 1114.86, 1083.99, 1016.49 (C-N, C-O stretching and aromatic C-H in plane bending), 983.70, 925.83, 914.26, 879.54, 829.39, 783.10, 758.02, 696.30 (Aromatic C-H out of plane bending).

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  (ppm): 7.13-7.22 (m, 1H, aromatic proton), 7.27 (d,  $J = 3.20$  Hz, 1H, aromatic proton), 7.60-7.65 (m, 1H, aromatic proton), 7.72-7.76 (m, 1H, aromatic proton), 7.83-7.88 (m, 1H, aromatic proton), 7.95-8.08 (m, 3H, aromatic protons), 8.16-8.20 (m, 2H,  $-\text{CH}=\text{N}$  and aromatic proton), 10.08 (s, 1H,  $\text{S}=\text{C}-\text{NH}$ ), 12.30 (s, 1H,  $\text{C}=\text{N}-\text{NH}$ ).

$^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$  (ppm): 102.38 (C), 106.67 (CH), 112.77 (CH), 115.41 (CH), 116.89 (C), 118.79 (C), 120.28 (CH), 123.74 (2CH, d,  $J = 8.4$  Hz), 129.85 (CH), 132.13 (CH, d,  $J = 13.7$  Hz), 132.45 (2CH), 133.42 (CH), 142.94 (CH), 146.92 (C), 148.33 (C), 150.41 (C), 174.78 (C).

HRMS (ESI) (m/z):  $[\text{M}+\text{H}]^+$  calcd. for  $\text{C}_{19}\text{H}_{12}\text{ClN}_5\text{O}_3\text{S}$ : 426.0422, found: 426.0424.

## 4.2. Biochemistry

### 4.2.1. Cell culture and drug treatment

Cell lines were obtained from American Type Culture Collection (ATCC). A549 human lung adenocarcinoma and L929 mouse fibroblast cells were cultured and drug treatments were carried out as previously described [22].

### 4.2.2. MTT assay

MTT assay was performed as previously described in the literature [22] with small modifications [20]. Cisplatin was used as a positive control. The SI values were also calculated according to the formula [23] below:

$$\text{SI} = \text{IC}_{50} \text{ for normal cell line} / \text{IC}_{50} \text{ for cancerous cell line}$$

### 4.2.3. Flow cytometric analyses of apoptosis

After the incubation of A549 cells with compounds **1**, **5**, **6**, **7** and cisplatin at  $\text{IC}_{50}$  concentrations, phosphatidylserine externalization, which indicates early apoptosis, was detected using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) on a BD FACSAria flow cytometer for 24 h. Annexin V staining protocol was applied according to the manufacturer's instructions (BD Pharmingen, San Jose, CA, USA) and analyzed by a BD FACSAria flow cytometer using FACSDiva version 6.1.1 software (BD Biosciences, San Jose, CA, USA).

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