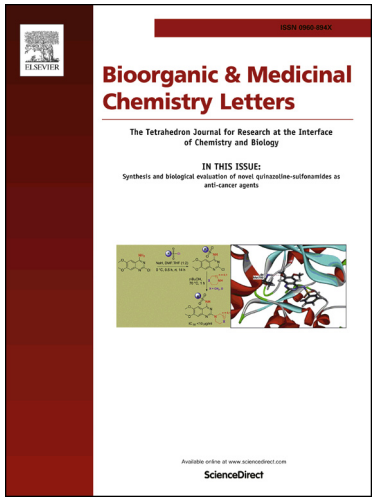
# Accepted Manuscript

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# Design, synthesis and anticancer activity of novel nopinone-based thiosemicarbazone derivatives

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**Abstract**

A series of new nopinone-based thiosemicarbazone derivatives were designed and synthesized as potent anticancer agents. All these compounds were identified by $^{1}$H-NMR, $^{13}$C-NMR, HR-MS spectra analyses. In the *in vitro* anticancer activity, most derivatives showed considerable cytotoxic activity against three human cancer cell lines (MDA-MB-231, SMMC-7721 and Hela). Among them, compound **4i** exhibited most potent antitumor activity against three cancer cell lines with the $IC_{50}$ values of 2.79 ± 0.38, 2.64 ± 0.17 and 3.64 ± 0.13 μM, respectively. Furthermore, the cell cycle analysis indicated that compound **4i** caused cell cycle arrest of MDA-MB-231 cells at G2/M phase. The Annexin V-FITC/7-AAD dual staining assay also revealed that compound **4i** induced the early apoptosis of MDA-MB-231 cells.

*Keywords*: Nopinone; Thiosemicarbazone; Anticancer; Apoptosis

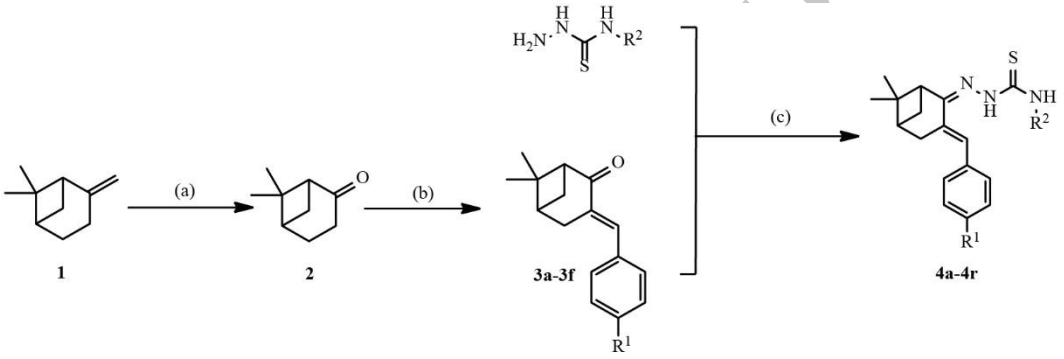
Malignant tumor is a kind of disease that causes tremendous threat to human life and health. There is increasing number of people died because of cancer in recent years[1]. Nowadays, chemotherapy is still the main method in the treatment of cancer. In spite of new anticancer agents developed, accumulation of toxicity has limited its application as antitumor drugs[2,3]. Thus, the investigations of new anticancer drugs with high efficacy and low side effects are still an urgent demand for medicinal chemists.

Thiosemicarbazone, containing thiourea based functional nucleus, is a vital chelator of metal ions and many of them are seriously significant with biological activities, especially in anticancer activity[4,5]. Thus, thiosemicarbazone derivatives were often considered as a tumor inhibitor via their capability of chelation with iron and copper ions in cancer cells[6]. Recently, a lot of thiosemicarbazone derivatives have been synthesized and their antitumor activities were also reported[7-9]. For example, 3-AP is a thiosemicarbazone derivative with potent anticancer activity and ribonucleotide reductase inhibitory activity, which has been advanced into clinical trials[10, 11]. In addition, some other thiosemicarbazones such as DpT, DpC, Dp44Mt also showed potent anticancer activity[12]. Therefore, thiosemicarbazone have proved to be a promising resource for the discovery of new anticancer agents.

As natural products have complex and diverse structures, it plays an important role in new drug discovery. As a major component of turpentine, $\beta$-pinene is an important natural terpene resource and is widely used for producing natural perfume. $\beta$-pinene possess an extensive spectrum of biological activities such as anticancer, antibacterial, antiviral and antidepressant activities[13-16], but the application of $\beta$-pinene in pharmaceuticals is less than its application in spices. $\beta$-pinene is easily oxidized to nopinone. From the literature, nopinone was converted to a variety of derivatives and it

has experienced considerable attention for its amazing applications as drugs, fluorescent materials, spices, etc[17,18, 21,22].

In our previous study, many applications of nopinone have been investigated such as anticancer[19,20], antimicrobial[20], insecticidal activities[21] and fluorescent dyes[22,23]. Nevertheless, there are few literatures reporting the synthesis of thiosemicarbazone derivatives with nopinone moiety. The aforementioned findings give us an impetus to design and synthesize thiosemicarbazone derivatives of nopinone and explore their potential values as anticancer agents.




**3a**: $R^1$=H
**3b**: $R^1$=$CH_3$
**3c**: $R^1$=$OCH_3$
**3d**: $R^1$=F
**3e**: $R^1$=Cl
**3f**: $R^1$=$NO_2$

**4a**: $R^1$= H, $R^2$= H
**4b**: $R^1$= $CH_3$, $R^2$= H
**4c**: $R^1$=$OCH_3$ , $R^2$= H
**4d**: $R^1$=F , $R^2$= H
**4e**: $R^1$= Cl, $R^2$= H
**4f**: $R^1$= $NO_2$, $R^2$= H
**4g**: $R^1$= H, $R^2$= Ph
**4h**: $R^1$= $CH_3$, $R^2$= Ph
**4i**: $R^1$= $OCH_3$, $R^2$= Ph

**4j**: $R^1$= F, $R^2$= Ph
**4k**: $R^1$= Cl, $R^2$= Ph
**4l**: $R^1$=$NO_2$ , $R^2$= Ph
**4m**: $R^1$=H , $R^2$= $CH_3$
**4n**: $R^1$= $CH_3$, $R^2$= $CH_3$
**4o**: $R^1$= $OCH_3$, $R^2$= $CH_3$
**4p**: $R^1$= F, $R^2$= $CH_3$
**4q**: $R^1$= Cl, $R^2$= $CH_3$
**4r**: $R^1$= $NO_2$, $R^2$= $CH_3$

**Scheme 1.** Synthetic route for the final compounds **4a**-**4i** from *β*-pinene. Reagents and conditions: (a) $KMnO_4$, $H_2SO_4$, acetone, room temperature, 6h; (b) substituted benzaldehyde, sodium ethoxide, ethyl alcohol, reflux, 8h; (c) hydrochloric acid, ethyl alcohol, reflux, 4-6h.

The target compounds were synthesized via a 3-step synthetic route from *β*-pinene (**1**) as shown in Scheme 1. *β*-Pinene (**1**) was easily oxidized to nopinone (**2**) by potassium permanganate. The key intermediates, *α*, *β*-unsaturated ketones (**3a**-**3f**) of nopinone were synthesized by reacting compound **2** with substituted benzaldehyde in the presence of absolute ethyl alcohol and sodium ethoxide according to our earlier work[24]. At last, the ketones **3a**-**3f** were further reacted with different substituted thiosemicarbazides to obtain target products **4a**-**4r**. All the synthesized compounds were

purified by recrystallization or silica gel column chromatography, and their structures were characterized by HR-MS, $^{1}$H-NMR and $^{13}$C-NMR spectra analyses.

**Table 1** The anticancer activities of compounds **4a**-**4r**

| compound | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | MBA-MD-231 | SMMC-7721 | Hela | Hlf-1 |
| **4a** | 4.57±0.21 | 9.53±0.10 | 12.79±0.01 | >40 |
| **4b** | 3.98±0.16 | 9.04±0.04 | 14.35±0.01 | >40 |
| **4c** | 6.21±0.14 | 8.30±0.14 | 10.63±0.02 | >40 |
| **4d** | 3.38±0.59 | 16.90±0.08 | 10.93±1.10 | >40 |
| **4e** | 4.09±0.44 | 12.36±0.45 | 10.63±0.09 | >40 |
| **4f** | 13.88±0.14 | 14.64±0.24 | 24.55±0.21 | >40 |
| **4g** | > 40 | >40 | 23.32±0.03 | >40 |
| **4h** | 8.66±0.17 | 3.94±0.18 | 7.11±0.04 | >40 |
| **4i** | 2.79±0.38 | 2.64±0.17 | 3.64±0.13 | >40 |
| **4j** | > 40 | >40 | 6.97±0.08 | >40 |
| **4k** | > 40 | >40 | 20.42±0.03 | >40 |
| **4l** | > 40 | >40 | 18.98±0.10 | >40 |
| **4m** | > 40 | 7.46±0.11 | 24.84±0.09 | >40 |
| **4n** | > 40 | 19.26±0.09 | 21.94±0.11 | >40 |
| **4o** | > 40 | >40 | 31.15±0.10 | >40 |
| **4p** | > 40 | >40 | >40 | >40 |
| **4q** | > 40 | 16.99±0.16 | 8.81±0.12 | >40 |
| **4r** | > 40 | >40 | 9.97±0.13 | >40 |
| Etoposide | 29.27±0.45 | 40.44±0.17 | 7.89±0.79 | >40 |

The designed 18 compounds were evaluated for cytotoxicity against human breast cancer cell line

(MDA-MB-231), human cervical carcinoma cell line (Hela) and human hepatocarcinoma cell line (SMMC-7721) and human normal embryonic lung fibroblast (Hlf-1) using MTT assay. Etoposide was selected as positive control and the $IC_{50}$ values for inhibition of proliferation were illustrated in Table 1.

In cytotoxic assay, eight compounds (**4a**-**4f**, **4h**, **4i**) showed strong antineoplastic activity against three tested cancer cell lines (MDA-MB-231, SMMC-7721 and Hela cells) *in vitro*，especially compound **4i** showed the most potent activity with $IC_{50}$ values of 2.79 μM (for MDA-MB-231 cells), 2.65 μM (for SMMC-7721 cells) and 3.64 μM (for Hela cells), stronger than those of positive control etoposide. In addition, compounds **4m**, **4n** and **4q** exhibited good antitumor activity against two tested cancer cell lines (SMMC-7721 and Hela cells) and compounds **4g**, **4j**-**4l**, **4o**, **4r** were only active to one tumor cell (HeLa cells). However, compound **4p** had no significant anticancer activity for all three tumor cell lines. It is important to note that this class of compounds did not show notable cytotoxicity against human embryonic lung fibrobiast (HLF-1) with $IC_{50} > 40$ μM.

From structure-activity relationships of all compounds, these nopinone-based thiosemicarbazide derivatives **4a**-**4f** with no substitution at terminal amino group ($R^2$ = H) showed more potent anticancer activities. When hydrogen atoms of the active amino group were replaced with a methyl group (**4m**-**4r**, $R^2 = CH_3$), the activity of thiosemicarbazide derivatives decreased significantly. In addition, it can be found that compounds (**4a**-**4f**, **4g**-**4l** or **4m**-**4r**) with the *p*-electron-donating group ($CH_3$, $OCH_3$) on phenyl ring conferred good antitumor activity than their analogs. Whereas, the electron-withdrawing (Cl, F, $NO_2$) group have greatly reduced the activity of the corresponding derivatives. Interestingly, when phenyl-substitution at amino position ($R^2$ = Ph) and *p*-electron-donating group ($R^1 = CH_3$, $OCH_3$) appeared in the same molecule, the compounds (**4h**, **4i**)

obtained prominent antitumor activity.

Then, to investigate whether compound **4i** induced cell apoptosis, compound **4i**-treated MDA-MB-231 cells were stained with Annexin V-FITC/7-AAD and then analyzed by flow cytometry. As shown in Fig. 1A, compound **4i** induced cell apoptosis in a dose-dependent manner. The percentage of early apoptotic cells (Fig. 1A) significantly increased from 1.61% to 42.9% after treatment with different concentrations of compound **4i** (0, 2.5, 5, 10 μM). These results suggested that compound **4i** could efficiently induce apoptosis of MDA-MB-231 cells in a dose-dependent manner.

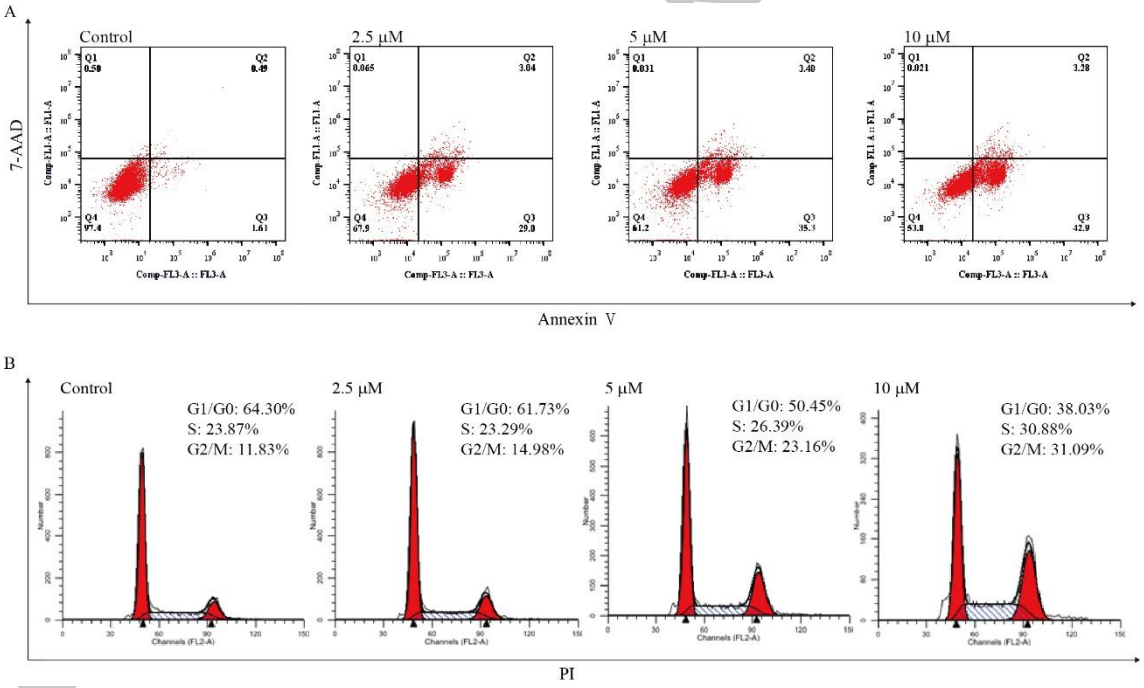



**Figure 1.** (A)Annexin V-FITC/7-AAD dual staining assay. MDA-MB-231 cells were treated with different concentrations of compound **4i** (0, 2.5, 5, 10 μM) and stained with Annexin V-FITC/7-AAD and analyzed for apoptosis using flow cytometer. (B) Cell cycle assay. MDA-MB-231 cells were treated with different concentrations of compound **4i** (0, 2.5, 5, 10 μM) and stained with propidium iodide and analyzed for cell cycle using flow cytometer.

Finally, the cell cycle analysis was carried out in the study the mechanism of cytotoxicity toward MDA-MB-231 cells. MDA-MB-231 cells were treated with 0, 2.5, 5 and 10 μM of **4i** for 48h. As shown in Fig. 1B, compound **4i** led to a dose-dependent induction of cell cycle arrest in the G2/M

phase. When compared to untreated cell group, the percentage of **4i**-treated cells in G2/M phase increased from 14.98% to 31.09% and the percentage of **4i**-treated cells in G0/G1 phase decreased from 61.73% to 38.03%. These phenomena proved that compound **4i** induced MDA-MB-231 cell cycle arrest in G2/M phase.

Thiosemicarbazones are a structurally diverse family of compounds that have been extensively studied because of their broad spectrum of pharmacological applications[25]. Many compounds belonging to the thiosemicarbazone family have been examined for cytotoxic activity against several cancer types[26, 27]. Although thiosemicarbazones with antiproliferative activity exhibit a wide structural diversity, most of them exhibit mechanisms of action including ribonucleotide reductase and topoisomerase IIα inhibition[28], reactive oxygen species generation[29], DNA damage[30], transition metal chelators[31] and induce redox intracellular imbalance[32]. In case of our study, compound **4i** can significantly arrest the cell cycle at G2/M phase and induce the apoptosis of cancer cells, which are important characteristics of numerous tubulin polymerization inhibitors previously reported[33-36]. Therefore, we deduce that this class of compounds may probably be antimitotic agents by inhibiting tubulin polymerization and thus induce the apoptosis of cancer cells.

In conclusion, 18 new nopinone-based thiosemicarbazone derivatives (**4a**-**4r**) have been synthesized. It is noteworthy that some compounds exhibited strong growth inhibitory activity against MDA-MB-231, Hela and SMMC-7721 cancer cells compared with positive control etoposide. Among these thiosemicarbazone derivatives, compound **4i** exhibited obvious anti-proliferative activity against all tested cancer cell lines and low cytotoxicity on human normal embryo lung fibroblasts (Hlf-1). These findings highlighted the potential of this class of derivatives as new anticancer agents. More research will be carried out to explore the in-depth structure-activity

relationships and the antitumor mechanisms, especially tubulin polymerization activity of these derivatives.

**Acknowledgements**

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**Supplementary data**

Supplementary data associated with this article can be found, in the online version, at

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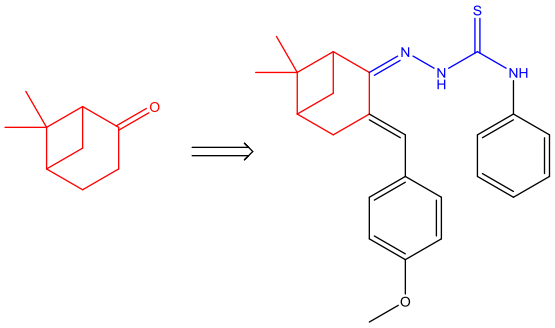
**Graphic Abstract**




**4i**

| | |
|---|---|
| $IC_{50}$ for MDA-MB-231 | 2.79 μM |
| $IC_{50}$ for SMMC-7721 | 2.64 μM |
| $IC_{50}$ for Hela | 3.64 μM |
| Toxicity for Hlf-1 | >40 μM |