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Original Article

# Anticancer activities and cell death mechanisms of 1H-indole-2,3-dione 3-[N-(4 sulfamoylphenyl) thiosemicarbazone] derivatives

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**ABSTRACT**

In this study, the cytotoxic effects of 1*H*-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazone derivatives namely, **4a-d** were evaluated using cell kinetic parameters including the cell index, mitotic index, labelling index and apoptotic index on HeLa cells taken from a human cervix carcinoma. All compounds were evaluated using cell index parameters at 5, 10, 20, 40, 80, 100 and 160 µM concentrations. As a result of this, it was seen that all **4a-d** compounds were effective in different concentrations. Different cell death mechanisms were proposed for **4a-d**. When all the parameters were examined, it was found that the bromine substituted **4c** was the most potent antiproliferative compound in the tested compounds. The difference was significant between the control and experimental groups ($p<0.01$). In addition, a statistically significant difference was noted among all experimental groups ($p<0.01$).

**Keywords:** 1*H*-indole-2,3-dione, thiosemicarbazone, antiproliferative effect, HeLa cells

## INTRODUCTION

Cancer is caused by abnormal cell division and growth of certain body cells and the invasion of surrounding tissues (WHO 2018). Even though many influential chemotherapeutic agents have been approved to treat cancer, their uses are limited due to serious side effects and toxicity. Therefore, researchers have been trying to develop effective but also less deleterious new anticancer agents (Sarkar and Li 2006; Remesh 2012).

1*H*-indole-2,3-dione (isatin) is a versatile moiety and compounds bearing the isatin chemical scaffold demonstrate diverse pharmacological or biological properties (Patel et al. 2006; Zhou et al. 2006; Karalı et al. 2007; Pakravan et al. 2013; Liu et al. 2014). Furthermore, various structure activity relationship (SAR) studies have demonstrated that the presence of electron-withdrawing groups (fluor, chlor, trifluoromethoxy, nitro etc.) at the position 5 of the isatin ring scaffold enhances anticancer activity (Hall et al. 2009; Vine et al. 2009; Lv et al. 2011; Gabr et al. 2017). After approval of the 2-indolinone derivatives (unitinib, as a multi-targeted receptor, tyrosine kinase inhibitor for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor by FDA), 2-indolinone analogs have aroused interest because of their anticancer activities (Gan et al. 2009; Vine et al. 2009; Eldehna et al. 2015).

A series of 1*H*-indole-2,3-dione 3-thiosemicarbazones obtained from the condensation of substituted isatin with thiosemicarbazides has been reported as active against ovarian carcinoma, cervix carcinoma and uterine sarcomacell lines (Pape et al. 2016; Singh et al. 2017). The inhibitory effects of 1*H*-indole-2,3-dione 3-thiosemicarbazones bearing a 4-sulfamoyl phenyl moiety were investigated using human carbonic anhydrase (hCA) I, II, IX and XII isoenzymes. The tested compounds displayed selectivity against hCA IX and XII. $K_I$ values of the compounds were found to be at low nanomolar levels (Karalı et al. 2017).

In the present study, 1*H*-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazone] derivatives were evaluated for their anticancer activities and different cell death mechanisms using cell kinetic parameters including the cell index, mitotic index, labelling index and apoptotic index.

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## MATERIALS AND METHODS

### Cytotoxicity

### Cell Culture

The HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with FBS (Foetal Bovine Serum), streptomycin (100 µg/mL), and penicillin (100 IU/mL; Gibco).

### Compound Concentrations

Stock solutions of each of **4a-d** were freshly prepared in DMSO. Seven different working solutions of each of **4a-d** (5, 10, 20, 40, 80, 100 and 160 µM) were prepared.

### Real Time Cell Analyzer (RTCA): Cell Index

For this purpose, the real time cell analyzer (RTCA, xCELLigence, Roche) is used for label-free and real-time monitoring of cell properties. This system is impedance-based technology and uses specially designed microtiter plates containing interdigitated gold microelectrodes to non-invasively monitor the viability of cultured cells using electrical impedance as the measure.

In the experimental process, 100 µL of cell culture medium was added to each well for the impedance background measurement. After adding 6000 cells for each well, the final volume was 200 µL. The E-Plates (16 E-Plate) were incubated at 37°C with 5% $CO_2$ and monitored on the RTCA system at 15-minute time intervals for up to 24 hours without treatment of compounds and following 72 hours with treatment of compounds (Cetin and Topcul 2017).

### Mitotic Index

HeLa cells were plated on coverslips and treated with either the control or experimental group for 0-72 h. The cells were then fixed using Carnoy fixative (ethanol:acetic acid, 3:1) and stained using the Feulgen method. The number of cells in the mitotic phases (including the late prophase, metaphase, anaphase and telophase; n) per total cells (3,000-3,500; C) was determined under light microscope. The MI (%) was scorred using the following formula: MI=(n/C)x100 (Topcul et al. 2013).

### $^3$H-Thymidine Labelling Index

For $^3$H-thymidine labelling index analysis (which determines cells in the S phase), HeLa cells were seeded into round coverslips which were in 24-well plates at a density of $2x10^4$ cells per well and incubated for 24 hrs. Then the cells were treated with the **4a-d** experimental concentrations. At the end of the experimental period, cells were treated with medium containing 1 µCi/mL $^3$H-thymidine for 20 min to evaluate the labelling index (Cetin and Topcul 2017).

### Autoradiography

After exposure for 3 days at 4°C, autoradiograms were developed with a D-19 developer solution (Kodak, New York, USA) and fixed with Fixaj B (Kodak, New York, USA). The coverslips were evaluated after being stained with Giemsa for 3 min. The labeling index was determined by counting at least 3000 cells/coverslip. The index is expressed as percentage labeled nuclei (Cetin and Topcul 2017).

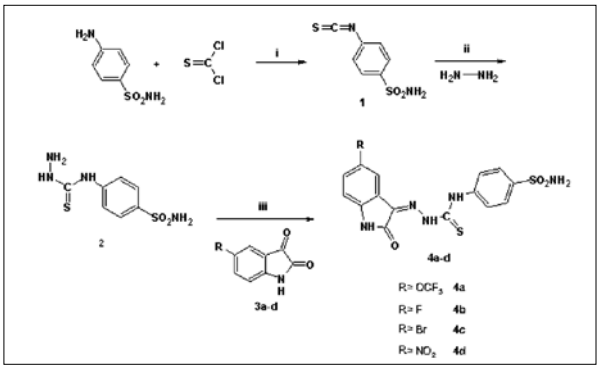
### Apoptotic index (AI)

HeLa cells were collected and then fixed with methanol:Phosphate Buffered Saline (PBS) (1:1) and methanol. Fixed cells mounted on slides, stained with 0.5 mg/mL4',6-diamidino-2-phenylindole (DAPI) for 30 min and washed with PBS. Nuclear morphology of the cells was visualized using an Olympus fluorescence microscope. For evaluation of the AI, at least 250 cells were counted for control and each of the experimental groups (Cetin and Topcul 2017).

## RESULTS AND DISCUSSION

### Chemistry

(4-Sulfamoylphenyl)isothiocyanate **1** was prepared by reacting of sulfanilamide with thiophosgene in water containing concentrated hydrochloric acid. Hydrazine hydrate was reacted with **1** in ethanol to give N-(4-sulfamoylphenyl)thiosemicarbazide **2** 1*H*-indole-2,3-dione 3-[N-(4-sulfamoylphenyl)thiosemicarbazones] **4a-d** were synthesized by reacting **2** with 5-substituted 1*H*-indole-2,3-dione **3a-d** in ethanol containing a catalytic amount of sulphuric acid (Karalı et al. 2017) (Scheme 1).




**Scheme 1**. Synthesis of **4a-d**. Reagents and conditions: i) $H_2O$, HCl, stirred ii) EtOH, stirred, cooled iii) EtOH, $H_2SO_4$, reflux.

### Anticancer Activity

### Cell Index

The cell index values obtained from the real-time cell analysis system were examined following the application of **4a-d** to HeLa cells. 5, 10, 20, 40, 80, 100 and 160 µM concentrations were used for all compounds. Figures 1-4 show the curves of the most effective concentrations of these compounds.

When the cell index values obtained following application of **4a-d** to HeLa cells were compared with the standard curve, it was shown that there were different effects for different concentrations for all compounds. The bromine substituted **4c** was tested at 5, 80 and 100 µM concentrations using the control group. **4c** showed significant DNA damage on HeLa cells at 80 and 100 µM concentrations. The trifluoromethoxy substituted **4a** and the nitro substituted **4d** were examined using the control group on HeLa cells. Both compounds had cytostatic effects as a distinct cell death type at 160 µM. The fluorine substituted **4b** showed a cytostatic effect with uncertainty at 100µM concentration to HeLa cells.

### Mitotic Index

After administration of **4a** at 160 µM, **4b** at 100 µM, **4c** at 80 µM and **4d** at 60 µM for 0-72 h on HeLa cells, 3000 cells were counted for both the control and experimental groups. The mitotic index values belonging to **4a-d** are shown in Table 1.

The mitotic index is a scale for the proliferation case of a cell population. When the mitotic index values were examined, it was seen that while the bromine substituted **4c** had the most antiproliferative activity at 80 µM, the fluorine substituted **4b** had no significant effect compared with the other substances. The trifluoromethoxy substituted **4a** and the nitro substituted **4d** showed effects at 160 µM. The difference was significant between the control and experimental groups ($p<0.01$). In addition, a statistically significant difference was noted among all experimental groups ($p<0.01$).

### Labelling Index

Labelling index parameters were applied at 80, 100 and 160 µM concentrations on HeLa cells for **4a-d**. 3000 cells were counted for both the control and experimental groups. Labelling index values of **4a-d** are shown in Table 2.

The effects of **4a-d** on the S phase of HeLa cells are similar to those of mitotic index values. **4c** saw the most greatly reduced DNA synthesis of HeLa cells among **4a-d**. The bromine substituted **4c** produced significant inhibition at 80 µM, while the trifluoromethoxy substituted **4a** and the nitro substituted **4d** showed an inhibitory effect at 160 µM for DNA synthesis. The difference was significant between the control and experimental groups ($p<0.01$). In addition, a statistically significant difference was noted among all experimental groups ($p<0.01$).

### Apoptotic Index

In the apoptotic index parameter, 80, 100, and 160 µM concentrations were applied to **4a-d** respectively. 250 cells were counted for both the control and experimental groups. Apoptotic index values of **4a-d** are shown in Table 3.

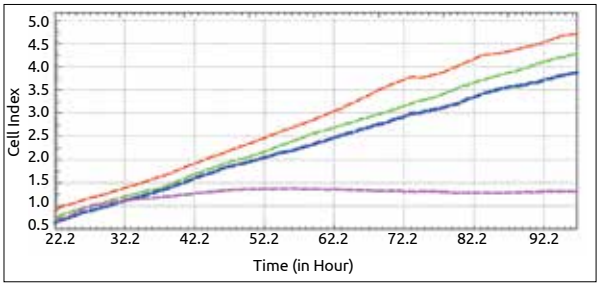



**Figure 1**. Cell index values of HeLa cells treated with 10, 80 and 160 µM concentrations of **4a** (--Control, -- 10µM, -- 80µM and-- 160 µM).

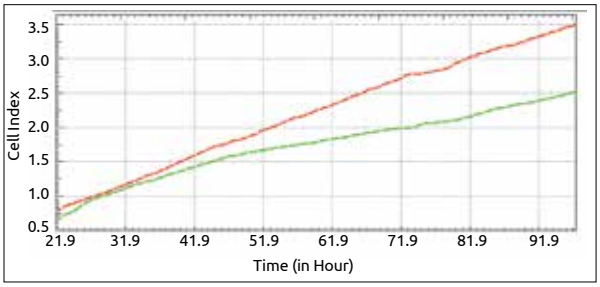



**Figure 2**. Cell index values of HeLa cells treated with 100 µM concentration of **4b** (--Control and -- 100µM).

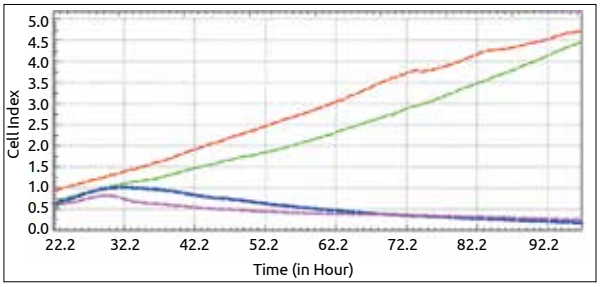



**Figure 3**. Cell index values of HeLa cells treated with 5, 80 and 100 µM concentrations of **4c** (--Control, -- 5µM, -- 80 µM and-- 100 µM).

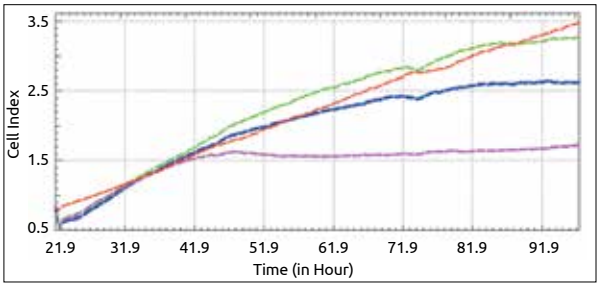



**Figure 4**. Cell index values of HeLa cells treated with 80, 100 and 160 µM concentrations of **4d** (--Control, -- 80 µM, -- 100 µM and-- 160 µM).

**Table 1. Mitotic index (%) values of 4a-d on HeLa cells**

| | | Mitotic Index (%) | | | |
|---|---|---|---|---|---|
| | | 4a | 4b | 4c | 4d |
| Time (Hours) | Control | 160 µM* | 100 µM* | 80 µM* | 160 µM* |
| 24 | 6.24±0.11$^{SD}$ | 3.56±0.03 | 4.15±0.07 | 2.94±0.04 | 5.18±0.06 |
| 48 | 8.27±0.07 | 3.18±0.01 | 4.72±0.05 | 1.18±0.01 | 4.15±0.04 |
| 72 | 8.96±0.05 | 2.96±0.03 | 5.14±0.02 | 0.38±0.02 | 4.13±0.05 |

* Significantly different $p<0.01$
SD: Standard deviation

**Table 2. Labelling index (%) values of 4a-d on HeLa cells**

| | | Labelling Index (%) | | | |
|---|---|---|---|---|---|
| | | 4a | 4b | 4c | 4d |
| Time (Hours) | Control | 160 µM* | 100 µM* | 80 µM* | 160 µM* |
| 24 | 5.13±0.06$^{SD}$ | 2.24±0.02 | 3.21±0.03 | 1.97±0.03 | 3.95±0.04 |
| 48 | 6.17±0.03 | 2.11±0.03 | 3.12±0.02 | 1.16±0.01 | 3.17±0.04 |
| 72 | 6.96±0.07 | 1.03±0.01 | 4.14±0.03 | 0.21±0.02 | 2.96±0.02 |

* Significantly different p<0.01
SD: Standard deviation

**Table 3. Apoptotic index (%) values of 4a-d on HeLa cells**

| | | Apoptotic Index (%) | | | |
|---|---|---|---|---|---|
| | | 4a | 4b | 4c | 4d |
| Time (Hours) | Control | 160 µM* | 100 µM* | 80 µM* | 160 µM* |
| 24 | 6.17±0.06$^{SD}$ | 12.15±0.02 | 8.16±0.03 | 18.19±0.11 | 10.23±0.06 |
| 48 | 7.21±0.05 | 12.19±0.03 | 9.18±0.04 | 19.01±0.08 | 14.06±0.03 |
| 72 | 7.96 ±0.09 | 14.33±0.01 | 10.12±0.03 | 22.15±0.03 | 15.04±0.07 |

* Significantly different p<0.01
SD: Standard deviation

As in other parameters, the apoptotic index values of the bromine substituted **4c** were shown to significantly increase apoptotic cell ratio at 80 µM. The trifluoromethoxy substituted **4a** and the nitro substituted **4d** were found to increase the apoptotic index values at 160 µM. The fluorine substituted **4b** had no significant apoptotic effect compared with the other substances. The difference was significant between the control and experimental groups (p<0.01). In addition, a statistically significant difference was noted among all experimental groups (p<0.01).

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**Conflict of Interest:** The authors have no conflict of interest to declare.

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