Anticancer Properties of Fluorinated Aminophenylhydrazines on A549 Lung Carcinoma Cell Line

Zafer Hasan Ali SAK¹, Faruk SÜZERGÖZ², Veli Tarık KASUMOV³, *Ali Osman GÜROL⁴,⁵

¹. Department of Chest Disease, Medical Faculty, Harran University, Sanliurfa, Turkey
². Department of Biology, Science Art Faculty, Harran University, Sanliurfa, Turkey
³. Department of Chemistry, Science Art Faculty, Harran University, Sanliurfa, Turkey
⁴. Department of Immunology, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
⁵. Department of Medical Pharmacology, Istanbul Medicine Faculty, Istanbul University, Istanbul, Turkey

*Corresponding Author: Email: ogurol@yahoo.com
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Abstract
Background: Non-small cell lung cancer (NSCLC) is responsible for up to 85% of deaths associated with lung cancer. Chemotherapy is still an important treatment method on the treatment of inoperable cases. In this study, the anticancer properties of a series of Schiff bases were tested on the A549 cell line representing NSCLC.

Methods: Fluorinated Schiff bases (compounds 1-6) were synthesized based on 2-amino phenylhydrazines and benzaldehydes containing fluorine were used. The cytotoxic effects of the compounds on the A549 cell line were determined by colorimetric MTT assay and the antiproliferative effects of the compounds on the A549 cell line by the CFSE method. To demonstrate the development of apoptosis, cleaved caspase-3 expression in cells was tested using the immunofluorescence method. Morphological changes indicating apoptosis in cells were determined by histopathological staining methods (H & E, giemsa, PAP).

Results: The strongest cytotoxic effect on A549 lung cancer cells was obtained with compound 6 (IC50: 0.64 μM) containing 5 fluorine atoms. The strongest antiproliferative effect on A549 cells was achieved with compound 5 (PI: 4.95) carrying 2 fluorine atoms. Apoptosis induction was effective in cell death. In addition to cleaved caspase-3 expression, chromatin condensation, marginalization, and apoptotic bodies were observed in the cells.

Conclusion: Some of the compounds tested showed high cytotoxic and antiproliferative effects, indicating that these compounds could be potential chemotherapeutic agent candidates for lung cancer. The result of immunofluorescence and immunohistochemical analysis showing that the cytotoxic effects have been induced by apoptosis is an important advantage.

Keywords: Schiff bases; IC50; A549; Lung cancer; Apoptosis

Introduction

Scientists predict that lung cancer will continue being the most common form of cancer over the next decades. There would be twice as many people living with lung cancer in the following decades than there are now. The main cause for the
rise is attributed to longer lifespan (1-3). Lung cancer is caused by gene mutations in a lung cell making the cell unable to repair DNA damage and unable to program cell death. Mutations can occur because of different causes (4-7). Most lung cancers occur because of inhaling carcinogenic. Lung cancer is caused by gene mutations in a lung cell making the cell unable to repair DNA damage and unable to program cell death. Mutations can occur because of different causes (4-7). Most lung cancers occur because of inhaling carcinogenic. Lung cancer can be classified histopathologically into two main types—non-small cell lung cancer (NSCLC) and small cell lung cancer. NSCLC is the most common type of lung cancer accounting for 80% to 85% of all lung cancer diagnoses, while small cell lung cancer accounts for the remaining 20% (8, 9).

Schiff base compounds play a significant role in the development of modern coordination chemistry, in catalysis, new photochromic materials and elements for constructing of optical switches or optical memory devices (10). Some of the Schiff base compounds are also used in the medicinal, pharmaceutical and agrochemical fields (11).

Fluorine is a small highly electronegative atom and the incorporation of fluorine in organic molecules could enhance their metabolic and chemical stability, increase lipophilicity, membrane permeability as well as the binding affinity of the drug to the molecular targets (12, 13). Because of the desirable properties of organofluorine compounds, fluorine has become very important in the design and development of new drugs (13-18). “The dramatic effect of fluorine on the biological activity of numerous herbicides, insecticides, and fungicides has earned fluorine a unique place in the toolbox of agrochemical chemists” (12, 19). The introduction of fluorine into an organic molecule can bring about many changes in physical properties including increased chemical and metabolic stability, enhanced lipophilicity, conformational changes, and changes in polarity and chemical reactivity (18, 19). In this study, the anticancer properties of a series of fluorinated Schiff bases were tested on the A549 cell line, representing NSCLC.

**Materials and Methods**

**Chemicals**

The 6 fluorinated phenylhydrazine benzaldehyde Schiff bases were synthesized on the basis of amino phenylhydrazines and fluorinated benzaldehydes (Table 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical name</th>
<th>Molecular formula</th>
<th>Structural formula</th>
<th>mw</th>
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</thead>
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<td>6</td>
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<td><img src="image" alt="Structural formula" /></td>
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</tr>
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</table>

**Cell lines**

The anticancer properties of the compounds were tested on A549 lung carcinoma cell line purchased from the American Type Culture Collection. A549 cells exhibited epithelial-like adherent cell morphology. For serial passages of the A549 cell line 10% FBS containing Ham’s F12K media was used. Serial passages were carried out at 37 °C in 5% CO$_2$. Available at: http://ijph.tums.ac.ir
with humidified atmospheres.

**Application of the compounds**

Cells at a concentration of $10^5$ cells/ml were seeded in triplicate in 96 well plates and incubated at 37 °C in 5% CO$_2$ with humidified atmospheres until the cells exhibited 80% confluence. Medium was replaced with fresh medium containing 1, 10, 100 and 1000 μM of the compounds and incubated for 72 h at the same culture conditions.

**IC$_{50}$ determinations**

In vitro anti-cancer evaluation of the synthesized compounds on A549 cell line was determined using an ATP assay. For the ATP assay, the reagents were prepared according to the manufacturer's instructions (Sigma) and used for the assay. Addition of reagents to plate wells resulted in cell lysis, ATP release, luciferin-luciferase reaction with ATP catalysis and chemiluminescence. The resulting chemiluminescence amount was measured using a microplate luminometer (Spectramax-M5, Molecular Device, USA) to determine the OD values indicative of the amount of cellular ATP. OD values from ATP analysis were obtained using Grafpad Analyze Software (v.demo) to calculate IC$_{50}$ values for each compound.

**Cell proliferation index**

Cell proliferation was assessed using 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) dye, which was half-shared by two daughter cells during replication. The cell suspension was incubated with CFSE dye (Sigma) at 15 μM concentration for 20 min in 5% CO$_2$ air at 37 °C under dark light conditions and the resulting green fluorescence intensity of the cells were measured using a flow cytometer (FXC 500, Coulter, USA) immediately after the incubation. CFSE stained cells were incubated with the synthesized compounds at IC$_{50}$ concentrations for 72 h at 37 °C in 5% CO$_2$ and analyzed in the flow cytometer using a green fluorescein channel listing mode data which was used to calculate Proliferative index values (PI) of A549 cells using a FCS Express 5 flow (v.demo) cytometry software program.

**Detection of apoptosis**

The apoptosis induction of the synthesized compounds in the cells were analyzed by immunofluorescent and histopathological staining methods. After the incubation of cells with the compounds at IC$_{50}$ concentrations at 37 °C in 5% CO$_2$ for 72 h, cleaved caspase-3 and gienza, hematoxilin eosin (H&E) and Papanicoleau (PAP) analyses were performed. Cleaved caspase-3 expression was analyzed using an indirect immunofluorescent staining method. Cells were incubated for 30 min with mouse antihuman, anti-cleaved caspase-3 MoAb (Cell Signaling Technology) and then incubated with FITC-labeled goat anti-mouse MoAb (Santa Cruz Biotechnology) for 20 min. Slides were examined using a fluorescent microscope (x200 mag) to observe the expression of cleaved caspase-3 on the cells. Histopathological analyses were performed using standard giemza, H&E and PAP staining protocols. Slides were examined using light microscope (x200 mag.) to observe chromatin condensation and marginalization, nuclear deterioration and apoptotic bodies.

**Results**

**Cytotoxic activities of the compounds**

IC$_{50}$ values were determined using luminometric ATP methods. Synthesized fluorinated aminophenylhydrazines were screened for their IC$_{50}$ activity on A549 cell lines. Results are presented in Fig. 1. As is evident from the data, the screened compounds especially compound 6 showed remarkably significant antitumor activity on A549 lung cancer cell line (IC$_{50}$: 0.64 μM).

**Antiproliferative effects of compounds**

The inhibition of cell proliferation by the action of the administered compounds were determined by flow cytometry using CFSE method and the proliferative indices (PI) are shown in Fig. 2.
Compound 5 showed the strongest antiproliferative effect on A549 cells (PI: 4.95).

**Expression of apoptosis-related molecules in cells**

Immunofluorescent properties of apoptosis in A549 lung cancer cell lines were studied following aminophenylhydrazines treatment (compounds 1-6). Results are shown in Fig. 3. The antibody specific for cleaved caspase-3, selectively stained the cytoplasm of cells, indicated production of apoptosis-related molecules in cells.

**Changes in cell morphology**

Specific morphological changes indicative of apoptosis were identified in A549 lung carcinoma cell lines after the administration of compounds 1-6 treatment presented in Fig. 4a-c. All cell lines exhibited apoptotic cell morphology because of treatment with the compounds. Normal cell morphology was observed in the untreated cells, while...
apoptosis-indicating cellular changes such as chromatin condensation, marginalization and apoptotic bodies were observed in the cells with compounds.

Fig. 4: Morphologic changes on A549 lung carcinoma cells treated with aminophenylhydrazines. Cells were stained with giemza (a), H&E (b) and PAP (c) after 48 h incubation with synthesized fluorinated aminophenylhydrazines and then examined under light microscope (Mag. x200)

Discussion

Lung cancer is a deadly form of cancer with a high mortality rate and is becoming increasingly widespread. Despite the availability of some chemotherapeutic agents that have relatively favorable effects in some types of the cancer, an effective chemotherapeutic agent has not yet been discovered in the treatment of lung cancer. Since lung cancer is generally asymptomatic at early stages, the diagnosis is usually administered at advanced stages (20). In advanced stages of lung cancer, some chemotherapy protocols are standard chemotherapy regimens (21).

The in vitro anti-cancer properties of compounds derived from plant and chemical sources for the discovery of new chemotherapeutic agents in cancer treatment are the subject of continuous interest in research. Schiff bases, along with other biological organisms, are among the important compounds that are often the subject of anticancer research. Some Schiff bases and their metal complexes have been reported to have strong anticancer effects on many cancer cell lines (22). Some Schiff compounds have remarkable properties as anticancer compound candidates. The introduction of fluorine into an organic molecule can bring about many changes in physical properties such as increased chemical and metabolic stability, enhanced lipophilicity, conformational changes, and changes in polarity and chemical reactivity. We investigated the cytotoxic antiproliferative and morphological effects of Schiff bases with fluorine compounds on A549 cells in vitro. Among the six compounds [1-6] we have synthesized, compounds 2 and 5 showed very strong cytotoxic and antiproliferative effects and apoptotic changes in cell morphology.

Our results indicated that these powerful effects increased with the number of fluorine atoms such that a stronger cytotoxic effect was observed in the five-fluorinated compounds compared to the two-fluorinated compounds. Compound 5 has stronger potency in inhibiting proliferations of A549 cells when the proliferative index values are taken into account. Although a strong cytotoxic effect was exhibited by compound 6 on A549 cells the preliminary power of cell proliferation remains at the lower level of the two fluorous analogs.

Combinations of compounds 5 and 6 have shown strong cytotoxic and anti-proliferative effects. This combination can be compounded in the lower dose due to the low IC 50 value of compound 6.
There are currently very few studies on the anti-proliferative effects of Schiff bases on cancer cells. In a study comparing the antiproliferative effects of fluorinated Schiff bases and doxorubicin on cancer cells, we found that fluorinated Schiff base molecules, had antiproliferative effects on the K562 cells, but less than doxorubicin (23). Ruthenium (III) complexes of heterocyclic tridentate Schiff bases have antiproliferative effects on kidney cancer (TK10), melanoma (UACC-62) and breast cancer (MCF-7) cell lines (24).

In our study, we used the CFSE method to examine the antiproliferative effects of Schiff bases on cancer cells. CFSE is the most common used method to determine cell proliferation. By using CFSE with flow cytometry, proliferation index values could be obtained quantitatively and the antiproliferative effects of the compounds on the cells could be evaluated precisely compared to other methods.

From the cleaved-caspase 3 expression and cell morphology analysis, it is concluded that the cytotoxic effects of the compounds on the A549 cells are mediated by apoptosis stimulation in the cells. It is preferable to induce apoptosis in cells rather than the necrosis effect of the ideal chemotherapeutic agent thereby eliminating the presence of cell debris in the environment and there will be no reactions that adversely affect the organism.

Conclusion

Some of the compounds tested showed high cytotoxic and antiproliferative effects, indicating that these compounds can be potential chemotherapeutic agents for lung cancer. Immunohistochemical and morphological results indicated that cell death is caused by apoptosis stimulation and that compounds have the ability to induce apoptosis in cells at various levels, depending on the structure of the compound and the cell line.

The presence of cytotoxic effects that have been induced by apoptosis is an important advantage in cancer treatment.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

References