ANTICANCER PROPERTIES OF FLUORINATED HYDRAZINE DERIVATIVES ON FOUR TYPES OF PANCREATIC CANCER

KOYUNCU, M.[1] – SUZERGOZ, F.[1] – BOYRAZ, M. U.[2] – OKSAK, N.[3] – SHEKHANY, B.[4*] – KASIM, V.[5] – GUROL, A. O.[6,7]

[1]*Department of Biology, Science Art Faculty, Harran University, Sanliurfa, Turkey*

[2]*Department of Histology and Embryology, Veterinary Faculty, Harran University, Sanliurfa, Turkey*

[3]*Department of Physiology, Health Science Faculty, Harran University, Sanliurfa, Turkey*

[4]*Department of Medical Laboratory, Technology Soran Technical College, Erbil Polytechnic University, Erbil 44001, Iraq*

[5]*Department of Chemistry, Science Art Faculty, Harran University, Sanliurfa, Turkey*

[6]*Department of Immunology, Aziz Sancar Institute of Experimental Medicine, University of Istanbul, Istanbul, Turkey*

[7]*Department of Medical Pharmacology, Istanbul Medical Faculty, University of Istanbul, Istanbul, Turkey*

**Corresponding author*
e-mail: bestoon.hamed@epu.edu.iq; phone: +96-475-0414-4509

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Abstract. Pancreatic cancers are asymptomatic until the disease reaches an advanced stage and are almost always fatal. Chemotherapy is the main treatment option, especially for metastatic pancreatic cancer, while adjuvant chemotherapy remains essential for resected pancreatic cancer. Fluorinated phenylhydrazine Schiff base derivatives (C1-6) were synthesized and tested for their anticancer activities in normal prostate (PNT1a) and pancreatic cancer cell lines (Capan-1, MIA PaCa-2, Panc-1 and TGP52). Cytotoxicity was determined by IC_{50} analysis using adenosine triphosphate (ATP) assay. Apoptosis induction was determined by annexin-V, cleaved caspase-3 expression and mitochondrial membrane potential ($\Delta\Psi m$) analysis. Multidrug resistance (MDR) profile was analyzed using anti-P-glycoprotein (anti-P-gp) in flow cytometry. In terms of cytotoxic effects, the best results were observed on MIA PaCa-2 and TGP52 (C6 and C1, respectively), while the strongest antiproliferative effect was observed on TGP52 with C1. The lowest P-gp expression was observed in MIA PaCa-2 and TGP52 (C1 and C5, respectively). Activation of the apoptotic pathway was revealed by the expression of annexin-V, cleaved caspase-3, and decreases in $\Delta\Psi m$ in cells. The cytotoxic and antiproliferative effects of the compounds on pancreatic cancer types were prominent, especially in di-tert-butylsalicylaldehydes. These effects occurred via the induction of apoptosis which is the most preferred cell death pathway in cancer cells.
Keywords: *cancer, cytotoxicity, apoptozis, schiff base, fluorine*

Introduction

Pancreatic cancer is one of the most lethal and aggressive type of cancer among all major cancers (Siegel et al., 2023) Since the symptoms of this disease are often vague, early diagnosis and effective treatment are challenging (Farhangnia et al., 2024).

The only treatment method that provides a potential cure for pancreatic cancer is surgical resection. It has been shown that survival rates improve with the addition of

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adjuvant chemotherapy. Further evidence shows that survival rates improve with neoadjuvant chemoradiotherapy (McGuigan et al., 2018). However, most patients show poor response to treatment and developed advanced or spreading cancer, and in this case, treatments such as chemotherapy and radiotherapy can only extend the survival of patients by a few months (Farhangnia et al., 2024). Another important problem in cancer treatment is the development of drug resistance (Dembic, 2020). Ongoing research worldwide aims to identify effective chemotherapeutic agents to combat the difficulties encountered in the treatment of pancreatic cancer and the poor prognosis.

Schiff bases are compounds obtained from the condensation of aldehydes or ketones with primary amines, first synthesized by the German chemist Schiff, and are called "imine" or "azomethine" compounds because they contain C = N in their structures. In modern chemistry, Schiff bases and their derivatives have an important place in biomedical applications and materials science as catalysts (Deswal et al., 2022). Schiff bases attract attention in terms of their use as pigments, dyes, catalysts, intermediates in organic syntheses and polymer stabilizers, as well as their wide range of biological activities (Menati et al., 2013). Many studies are ongoing on the use of these compounds as antibacterial, antifungal, antitumor, and anticarcinogenic agents in the field of health (Nagesh et al., 2015).

Fluorine is a small and highly electronegative atom, and the incorporation of fluorine in organic molecules could enhance their metabolic and chemical stability and membrane permeability, as well as the binding affinity of the drug to the molecular targets (Amit et al., 2001). As a consequence of the desirable properties of organofluorine compounds, fluorine has become very important in the design and development of new drugs (Chan and O'Hagan, 2012). 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 (Dolbier, 2000; Gesto et al., 2012).

Compounds with different mechanisms of action can be used alone or in combination with existing chemotherapeutic agents to create synergistic effects and to minimize drug resistance and increase the effectiveness of cancer cell treatment. It is reported that Schiff bases inhibit topoisomerase 2, bind to microtubules and prevent their function, and cause apoptosis by arresting cells in the G2/M phase of mitosis during cell division. The fact that Schiff bases have an important place in cancer research is revealed by these interactions (Acharya et al., 2019). From these perspectives, the cytotoxic, antiproliferative, and apoptotic effects of fluorinated hydrazine-derived Schiff bases on pancreatic cancer cell lines were investigated with the hope of opening new horizons in chemotherapy in this cancer type, which is almost always fatal.

Materials and methods

Structural analysis of the compounds

Six fluorinated phenylhydrazine Schiff bases were synthesized based on fluorine-containing phenylhydrazines and salicylaldehydes (*Table 1*). Briefly, condensation reactions of fluorinated anilines with salicylaldehyde derivatives were carried out by mixing 2 and 3 fluorine containing anilines with 3-4 drops of formic acid catalyst in equimolar ratios; absolute ethanol solutions of fluorinated anilines with salicylaldehyde (SAL), 3,5-di-tert-butylsalicylaldehyde (3-5TBS) in two-necked flasks and boiling under reflux for 10-15 h. After crystallization, they were filtered through vacuum glass

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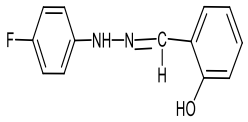
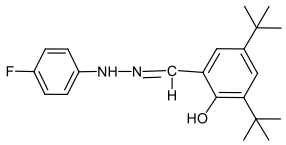
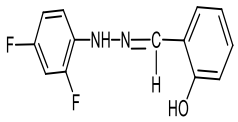
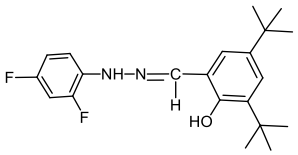
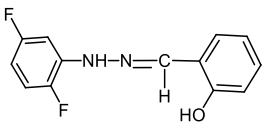
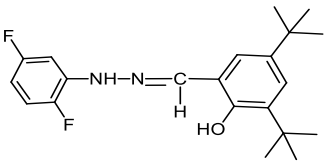
filters and washed with ethanol and dried at room temperature. If necessary, the products were purified by column chromatography. The yields of the products were determined by the melting points of the products (Stuart Scientific Melting Point Apparatus) (*Table 2*).

Table 1. *Chemical properties of compounds*

#	Compounds	Formula		mw* (gr/mol)
		Chemical	Open	
C1	4-FphHz-SAL	$C_{13}H_{10}NOF_1$		230
C2	4-FphHz-3,5DTBS	$C_{21}H_{23}N_2OF_1$		378
C3	2,4-F_2phHz-SAL	$C_{13}H_{10}N_2OF_2$		285
C4	2,4-F_2phHz-3,5DTBS	$C_{21}H_{26}N_2OF_2$		396
C5	2,5-F_2phHz-SAL	$C_{13}H_{10}N_2OF_2$		248
C6	2,5-F_2phHz-3,5DTBS	$C_{21}H_{26}N_2OF_2$		360

*molecular weight

Electronic spectra analysis data of the compounds

Samples of the synthesized compounds were prepared in absolute ethanol and scanned in the UV/Vis spectrometer in the range of 200-600 nm. It was observed that the absorption coefficients of the spectra were generally in the range of 200-400 nm and 20000-80000 M^{-1} cm^{-1} (*Table 3*).

IR spectra and elemental analysis data of the compounds

Elemental analyses were performed on the device (CHNS-932 LECO). The data obtained were compared with the theoretical values of the atoms (C, H, N) in the compounds, and the purity levels were determined (*Table 2*). FT-IR spectra and NMR spectra were used in the structural analysis of the compounds. The compounds were

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dissolved in potassium bromide, and their spectra were taken on a spectrophotometer device (Perkin Elmer Spectrum RXI FT-IR Spectrometer). For nuclear magnetic resonance spectra, the compounds were dissolved in chloroform-d and prepared for analysis. 1H NMR spectra were taken on Agilent-NMR-vnmrs 400 MHz and BRUKER-600 MHz spectrometers and in CDCl3. 19F NMR spectra were scanned at 376 MHz (Agilent-NMR-vnmrs-400 MHz) and 564 MHz (BRUKER-600 MHz) (*Table 4*).

Table 2. *Melting points, IR spectra, and elemental analysis of the compounds*

#	Name	*Mp (°C)	**IR (cm^{-1})		Elemental analysis		
					C (%)	H (%)	N (%)
C1	4-FpHhidrazin-SAL	169.3	νCH = N:1618 νC = C:1596-1567, νC-H: 848-2947, νO-H…N:2670, νN-H:3318	Theoric;	67.82	4.82	12.17
				Experimental;	67.23	4.831	11.73
C2	4-FpHhidrazin-3,5DTBS	124	νCH = N:1639, νC = C:1609-5180, νC(CH3)3: νC-H:2847-2957	Theoric;	73.65	7.95	8.18
				Experimental;	73.17	7.636	7.871
C3	2,4-F2pHhidrazin-SAL	151	νC = C:1595-1568, νO-H-N:2700, νC-H:2848-2975	Theoric;	62.9	4.06	11.29
				Experimental;	63.09	4.19	10.86
C4	2,4-F2Hhidrazin-3,5DTBS	123	νCH = N:1615 νC = C:1595, 1600, 1581 νC(CH3)3: νC-H: 2849-2959, νO-H…N:1700, νN-H:3337	Theoric;	69.98	7.27	7.77
				Experimental;	69.24	6.935	7.62
C5	2,5-F2pHhidrazin-SAL	153	νCH = N:1638,1622 νC = C:1596, 1573, 1550, νC-H:2843-3096, νN-H:3307, νO-H..N: 2760	Theoric;	62.9	4.06	11.29
				Experimental;	58.89	3.743	10.54
				Theoric;			
C6	2,5-F2pHhidrazin-3,5DTBS	144	νCH = N:1640-1649, νC = C:1615,1598, 1562, νC-H:2848-2953, νC(CH3)3:2848-3054 νO-H..N:2650, νN-H:3378	Experimental;	69.46	7.045	7.567
				Theoric;	69.98	7.27	7.77

*Melting point, ** Infrared spectroscopy

Table 3. *UV/VIS electronic spectra of the compounds*

#	Name	Solvent	UV/Vis Electronic Spectrum λ (nm)
C1	4-FpHhidrazin -SAL	CH3CH2OH	202, 216*, 243, 312, 350
C2	4-FpHhidrazin-3,5DTBS	CH3CH2OH	202, 214, 227*, 243, 309, 352
C3	2,4-F2pHhidrazin -SAL	CH3CH2OH	208, 239, 298, 346
C4	2,4-F2Hhidrazin -3,5DTBS	CH3CH2OH	205, 214, 242, 300, 323*,348
C5	2,5-F2pHhidrazin-SAL	CH3CH2OH	208*, 218, 243, 280*, 288, 324, 349
C6	2,5-F2pHhidrazin-3,5DTBS	CH3CH2OH	207, 242, 305, 344

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Table 4. *1H NMR and 19F NMR analysis data of the compounds*

#	Name	OH	CH = N	Ar-H; 19F-NMR	CH (Alifatic)	N-H
C1	4-FpHhidrazin-SAL	11.64 (s,1H)	9.86 (s,1H)	7.89(s,1H), 7.22 (s,1H) 7.13 (d,1H), 7.00 (t,1H) 6.93-6.88 (m,4H), 1.58 (s,1H) -123.30 (s,1F).		7.40, (s,1H)
C2	4-FpHhidrazin-3,5DTBS	11.64 (s,1H)	7.85 (s,1H)	7.59 (d,4H), 7.34 (d,1H) 7.31 (d,1H), 7.03-6.98 (m,4H) 6.96-6.93 (m,2H) -123.82 (s,1F)	1.45 (s,9H) 1.32 (s,9H)	7.89 (s,1H)
C3	2,4-F2pHhidrazin – SAL	10.65 (s,1H)	7.93 (s,1H)	7.45 (s,1H) 7.26-7.19 (m,3H) 7.15 (d,1H) 7.00 (d,4H) 6.92-6.89 (t,1H) 6.87-6.83 (m,3H) -111.53– (-)112.45(m,2F)		7.45 (s,1H)
C4	2,4-F2Hhidrazin - 3,5DTBS	10.94, (s,1H)	7.99 (s,1H)	7.33 (d,2H) 7.29-7.25 (m,2H) 6.91-6.84 (m,2H) -113.37– (-)113.48(m,2F)	1.47 (s,9H) 1.31 (s,9H)	7.41 (s,1H)
C5	2,5-F2pHhidrazin-SAL	10.49 (s,1H)	7.97 (s,1H)	7.29 – 7.26 (m,1H) 7.18 (t,1H) 7.02-6.69 (m,4H) 6.93-6.92 (t,1H) 6.53-6.48 (m, 1H) -114.17– (-)115.22(m, 2F)		7.65 (s,1H)
C6	2,5-F2pHhidrazin-3,5DTBS	10.77 (s,1H)	7.99 (s,1H)	7.04-6.69 (m,3H), 6.51-6.47 (m,1H) -109.543– (-)109.89(m,2F)	1.48 (s,9H) 1.31 (s,9H)	7.57 (s,1H)

Preparation of compounds for analysis

The chemical formulas and names of the fluorinated phenylhydrazine-derived Schiff bases (C1-6) used in the study are given in *Table 1*. The stock molar solutions of each compound were dissolved in alcohol to a concentration of 1 mM and sterilized by passing through an injector-type filter (20 μm, Millipore). The compounds were stored in cryotubes at -20°C until analysis.

Cell lines

Cell lines, one normal healthy and four pancreatic cancer cell lines, were obtained from ATCC. Normal epithelial (PNT1a) cell line was grown in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma), (Kowalska et al., 2022). The pancreatic adenosarcoma (Capan-1) cell line was grown in IMDM (Gibco) supplemented with 20% FBS (Lee et al., 2012). The pancreatic carcinoma (MIA PaCa-2) cell line was grown in DMEM (Gibco) containing 10% FBS and 2.5% horse serum (Sigma), (Ware et all., 2016). The pancreatic epithelioid carcinoma (Panc-1) cell line was grown in DMEM (Gibco) containing 10% FBS (El Wadei et al., 2012). The pancreatic islet cell tumor

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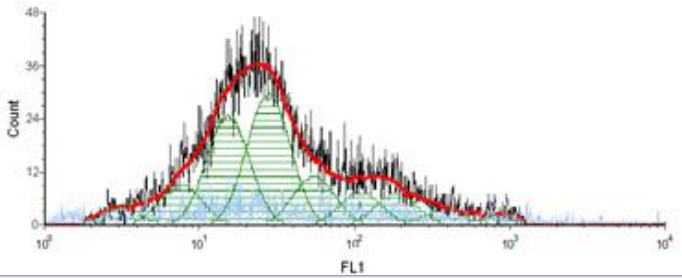
(insulinoma) cell line (TGP52) was grown in a mixture of DMEM (Dulbecco's Modified Eagle's Medium, Gibco) and Ham's F12 medium (Sigma) containing 10% FBS (Turbitt et al., 2024). All cell lines were cultured in a humidified incubator (Heal Force, HF 212 UV) at 37°C and 5% CO_2. Cells in culture flasks were harvested by trypsinization when they were 80% confluent to obtain sufficient cells to be used in experiments.

Cytotoxicity analysis

Stock solutions of compounds were seeded in triplicate into 96-well culture plates at doses of 1, 10, 100, and 1000 µM, with a volume of 10 µl per well. Only 10 µl of solvent was added to the control wells. Cells were added to the wells at 10^4 cells/ml per well. Culture plates were cultured in a humidified atmosphere at 37°C and 5% CO_2 for 72 h. ATP test reactants (ATP Cell Viability Luciferase Assay, Sigma) were added to the culture wells according to the manufacturer's leaflet and then read in a bioluminescence device (Spectramax-M5 Molecular Devices) within 5-10 min. The IC_{50} value of each compound was calculated using optical density (OD) values in the GraphPad® analysis program.

Cell proliferation analysis

Antiproliferative effects of the compounds were assessed by CFSE (Carboxyfluorescein succinimidyl ester, Sigma). Cells were labeled by incubating with 15 µM CFSE for 20 min at 37°C in a humidified environment containing 5% CO_2. The fluorescence levels of CFSE-labeled cells were examined with a fluorescence microscope (Juli, NanoEnTek), and the maximum fluorescence levels shown by the flow cytometry device (Coulter) were determined. Each compound was added to the U-bottom 96-well culture flasks at the IC_{50} doses. CFSE-labeled cells were seeded into the wells at a concentration of 10^4 cells/ml per well. Culture plates were cultured in a humidified atmosphere at 37°C and 5% CO_2 for 96 h. At the end of this period, cells were analyzed with a flow cytometry (Beckman coulter, USA), and LISTMOD data were obtained. The analysis of proliferative index (PI) of the cells was performed using LISTMOD data in the FCS Express 5 analysis software (*Fig. 1*).



Division Index	Proliferation Index	% Divided	Peak Ratio	# of Fitted Cells	# of Original Cells	RMS Error	R-Chi Square	Deg of freedom	Starting Generation Mean	Background Noise
25,2	25,2	100,0	0,5	8509	328,6	17,9	1,1	992	1486,8	0,0

Figure 1. *CFSE cell proliferation analysis. CFSE measurement in flow cytometry after cells were treated with compounds for 72 h. PI were calculated using a green fluorescent amount of each generation, which half shared the CFSE by daughter cells (FCS Express 5 analysis software). The black peak indicates the fluorescent signals. Blue peaks indicate background noise. Green lines indicate cell generations. Red peak indicates the fluorescent level of CFSE retained in cells treated with the chemicals*

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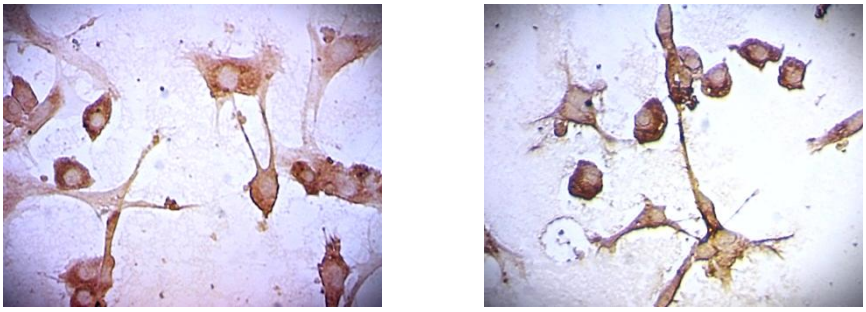
Immunohistochemical analysis

Anti-caspase-3 (Cell Signaling Technology) and anti-annexin-V (ExbioPharma) MoAbs were used for immunohistochemical analysis. Flat-bottom culture flasks were used to perform these analyses. Each compound was added to the culture well at the IC_{50} dose at which it showed the strongest cytotoxicity. Cells were seeded into the wells at 10^4 cells/ml per well. Culture plates were cultured in a humidified atmosphere at 37°C and 5% CO_2 for 24 h. Immunohistochemical staining was performed according to the manufacturer's instructions. Briefly, the wells were washed with distilled water, incubated with peroxidase blocking solution (Scy Tek Laboratories) for 15 min, and then protein blocking solution was added and incubated for 5 min. After washing, the MoAbs was added and incubated for 1 h. Then, polyvalent antibody solution (Scy Tek Laboratories) was treated for 10 min, HRP (Horse radish peroxidase, Scy Tek Laboratories) for 10 min, and DAB (Scy Tek Laboratories) for 15 min, respectively. At the end of these steps, three washes were performed with PBS. Background staining was performed using hematoxylin (Merck). Cells were examined under a light microscope (x100 mag. *Fig. 2*).



A B

Figure 2. *Apoptosis analysis by annexin-V and cleaved caspase-3 expression. Immunohistochemical analyses of early and late apoptotic markers in cells. Expression of annexin-V, an early marker of apoptosis, in cells incubated with compounds for 24 h (A). Expression of cleaved caspase-3, an indicator of advanced apoptosis, in cells incubated with compounds for 72 h (B)*

Mitochondrial membrane potential (ΔΨm) analysis

Changes in ΔΨm of cells due to the effects of compounds were performed by flow cytometry using rhodamine 123. Cells were seeded in a U-bottom 96-well culture plate at IC_{50} doses of each compound at a concentration of 10^4 cells/ml. Following 72 h of incubation in culture, 10 µl of rhodamine 123 (1 mg/ml, Sigma) was applied to the cells and incubated for another 60 min at 37°C. After incubation, cells were analyzed by flow cytometry (Beckman coulter, USA). The autofluorescence was determined using unstained cells, and the fluorescence values read in the FL1 histogram revealed the accumulation levels of rhodamine 123 in the cells (*Fig. 3*).

MDR analysis

The expression of P-gp in cells due to the effects of compounds was performed by flow cytometry using anti-P-gp MoAb. The cells were seeded at a dose of 10^4 in the wells of U-bottomed 96-well culture plates at IC_{50} doses of each compound and incubated for

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72 h under culture conditions. 10 µl of anti-P-gp (Santa Cruz Biotechnology) was applied to the cells and incubated at 37°C for 60 min in the dark. After incubation, the cells were analyzed by flow cytometry (Beckman coulter, USA). The nonspecific staining limit was determined with isotype control MoAb, and the fluorescence values read in the FL1 histogram revealed the P-gp expression levels in the cells (*Fig. 4*).

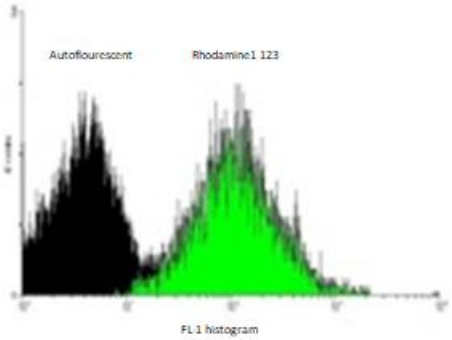

Figure 3. *Mitochondrial membrane potential analysis by rhodamine 123 accumulation. Rhodamine 123 measurement in flow cytometry after cells were treated with compounds for 72 h. The black peak indicates the level of autofluorescence exhibited by the cells, and the green peak indicates the level of rhodamine 123 retained in cells treated with the chemicals*

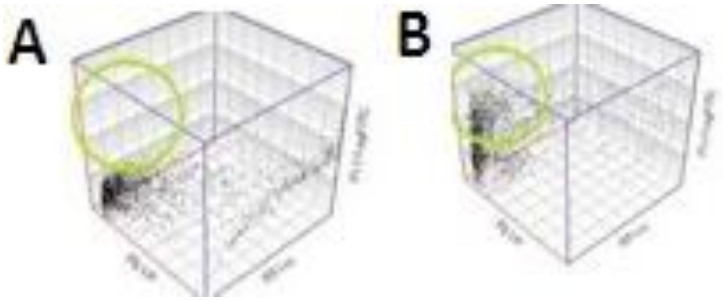

Figure 4. *MDR profile analysis by P-gp expression. Measurement of P-gp expression after 72 h treatment of cells with compounds. A) Non-specific binding level of isotype control moAbs in cells. B) Binding level of anti-P-gp moAbs in cells treated with chemicals*

Results

Synthesis of the chemicals

In the IR spectra of the compounds synthesized based on fluorinated anilines and salicylaldehyde derivatives, the observation of peaks at 1650-1660 cm-1 originating from the carbonyl groups (CH = O) of salicylaldehyde derivatives and the observation of peaks at 1605-1630 cm-1 belonging to the vibration frequencies of the -CH = N- azomethine group indicate that the synthesis was successful.

The characteristic IR values observed in the IR spectra of fluorinated hydrazine Schiff bases are shown in *Table 1*. The peaks that should be observed in the IR spectra of these compounds are expected to occur due to the symmetric and asymmetric vibrations of the νCH = N, νC = C, νOH, νN-H and νC-H functional groups in the C(CH3)3 group.

The characteristic peaks in the structures of fluorinated hydrazines are N-H, C = C, and CH = N groups in the benzene ring. The characteristic peaks originating from these

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groups in the spectra were observed in the ranges of νN-H: 3357-3370 cm^{-1}, νC = C: 1603-1566 cm^{-1}, νCH = N:1608-1638 cm^{-1}, νC-H:2815-3008 cm^{-1}, respectively.

Cytotoxicity

Dose-dependent cytotoxic effects of six fluorinated Schiff bases were evaluated in normal and pancreatic cancer cell lines using the ATP luminescence test (*Table 5*). The IC_{50} values of the compounds on the PNT1a cell line, which represents normal cells, were determined at high levels in other compounds except C4 (6.5 µM). This is a favorable result, indicating that the compounds have a lower cytotoxic effect on normal healthy cells than on cancer cells. Despite this positive result, the strongest cytotoxic effects for Capan-1 and Panc-1 were obtained by C4, which was not a very desirable result. Although C1 (9.3 µM) seems to be an alternative for Panc-1, an IC_{50} dose below 10 µm could not be obtained for Capan-1. It is a great advantage that the compounds with the most potent IC_{50} values for MIA PaCa-2 and TGP52 (C6: 6.1 µM and C1: 3.5 µM, respectively) also have high IC_{50} values for the normal healthy cell line PNT1a.

Table 5. *IC_{50} values of compounds in normal and pancreatic cancer cells.*

	PNT1a		Capan-1		MIA PaCa-2		Panc-1		TGP52	
	IC_{50} (µM)	95% CI	IC_{50} (µM)	95% CI	IC_{50} (µM)	95% CI	IC_{50} (µM)	95% CI	IC_{50} (µM)	95% CI
C1	59.7	19.7-84.5	26.8	17.3-43.7	125.9	93.9-141.1	9.3	7.1-16.2	20.1	16.1-36.9
C2	155.7	98.5-284.7	14.2	10.7-22.8	44.5	32.3-54.2	74.1	27.8-102.5	3.9	2.3-7.1
C3	67.4	27.8-102.5	35.2	25.3-42.6	13.5	7.8-27.5	78.2	27.8-102.5	19.9	15.3-38.7
C4	6.5	4.7-15.3	6.1	5.1-8.9	22.5	16.3-33.7	4.0	2.9-7.8	16.2	12.1-24.8
C5	36.2	15.3-44.8	91.1	68.6-143.2	107.1	68.7-136.2	43.5	25.7-58.1	35.1	27.3-41.6
C6	117.0	78.9-146.2	74.4	65.7-88.7	7.0	4.9-16.3	42.2	29.2-56.3	140.2	103.9-229.1

ATP analysis were carried out in triplicate order to determine IC_{50} value of each compound

Antiproliferative effect

The CFSE analysis results (*Table 6*), which show the ability of the compounds to prevent the proliferation of normal healthy and cancer cells, draw attention to the fact that the most affected cells are PNT1a cells. In these cells, three compounds (C1, C2, and C5) showed strong antiproliferative effects. The most pleasing effect was observed in our target cancer cells, C1 (PI: 3.3) and TGP52 cells. This means that these cells were able to undergo approximately 3 divisions during 96 h. An antiproliferative effect of the same strength was also observed on MIA PaCa-2 with C5 (PI: 6.1).

Table 6. *PI values of compounds in normal and pancreatic cancer cells*

Compounds	Proliferative index (PI)				
	PNT1a	Capan-1	MIA PaCa-2	Panc-1	TGP52
C1	5.6	23.6	11.5	22.9	3.3
C2	3.4	33.7	52.8	7.0	53.5
C3	10.3	38.6	36.2	19.3	10.9
C4	19.1	11.2	32.8	9.6	6.1
C5	4.7	12.4	6.1	10.9	20.3
C6	10.3	23.8	31.1	10.0	20.0

Proliferative index was determined for each compound using the CFSE staining in flow cytometry

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Apoptosis induction

The expressions of annexin-V, which indicate early apoptosis development, and cleaved caspase-3, which is an important effector of apoptosis because it can disintegrate the cell, were observed in the cells after the application of the compounds. Annexin-V analysis results, which show early signs of apoptosis induction in normal healthy and pancreatic cancer cells, show that compounds have a role in apoptosis stimulation in cell death. Cleaved caspase-3 analysis results, which were performed to further investigate the role of apoptosis in cell death, proved that cell death occurs through apoptosis (*Fig. 2*).

ΔΨm changes are of great importance in the development of apoptosis in cells. Among the compounds used in the study, it was observed that 3,5 di-tert-butylsalicylicdehyde derivatives were more effective on ΔΨm, C2 had a negative effect on ΔΨm and reduced rhodamine 123 accumulation effectively in Capan-1, MIA PaCa-2 and Panc-1 (30.8%, 27.3% and 57.1%, respectively). It was also observed that C4 had the strongest effect on MIA PaCa-2. In this case, it is noteworthy that the effect of the Di-tert butyl structure is more prominent than the fluorine atoms in the molecules. However, C4 also caused low rhodamine 123 accumulation in the normal cell line PNT1a (*Table 7*).

Table 7. *Rhodamine 123 accumulation under the effects of compounds in normal and pancreatic cancer cells*

Compounds	Rhodamine 123 accumulation (%)				
	PNT1a	Capan-1	MIA PaCa-2	Panc-1	TGP52
C1	40.4	32.5	28.2	19.2	41.6
C2	38.3	30.8	15.7	27.3	57.1
C3	26.2	26.2	30.0	16.1	30.3
C4	9.5	20.9	36.9	12.7	20.2
C5	68.6	28.3	22.8	22.5	32.8
C6	26.2	13.9	21.9	22.8	19.0

Mitochondrial membrane potential analyses were determined for each compound using rhodamine 123 staining in flow cytometry

MDR profile

The power of triggering P-gp expression, which is one of the mechanisms leading to the development of multidrug resistance, was most favorable in MIA PaCa-2 and TGP52 cells with C1 and C5 (5.2% and 3.2%, respectively). P-gp expression was observed in a wide range between 4.4% and 30.3%, depending on the compounds in the normal cell line PNT1a (C5 and C1, respectively). C1 is more specific among the compounds as it causes the highest P-gp expression in PNT1a, as a protective the normal cells, and the lowest in Capan1 and MIA PaCa-2 (*Table 8*).

Discussion

Pancreatic cancer, one of the most lethal and aggressive cancer types among all cancers (Siegel et al., 2023), continues to be a problem due to unusual (unusual) symptoms, rapid progression, lack of reliable early diagnosis biomarkers, and lack of effective treatment options (Shakerian et al., 2025). Chemotherapy treatments show minimal benefit in survival due to drug resistance, poor penetration, and tumor recurrence (Gupta and

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Murtaza, 2025). Therefore, developing effective chemotherapeutics has become an important need to achieve therapeutic efficacy in pancreatic cancer treatment. For this purpose, six phenylhydrazine-based fluorinated Schiff bases were synthesized, and the anticancer properties of the compounds were tested on both normal and pancreatic cancer cells.

Table 8. *P-gp expression effects of compounds in normal and pancreatic cancer cells*

Compounds	P-gp expression (%)				
	PNT1a	Capan-1	MIA PaCa-2	Panc-1	TGP52
C1	30.3	10.5	5.2	22.5	25.1
C2	15.6	29.4	8.8	25.4	37.3
C3	11.2	18.8	5.9	52.1	4.3
C4	28.4	35.9	7.3	15.2	18.3
C5	4.4	18.9	15.2	33.4	3.2
C6	7.3	14.9	9.1	21.8	7.2

MDR analyses were determined for each compound using anti-P-gp MoAb labeling in flow cytometry

Schiff bases are considered valuable compounds in medicine and the pharmaceutical industry due to their roles in biological processes. Fluorinated Schiff base derivatives can be effective against cancer cells by triggering DNA division, as well as their antibacterial and antifungal properties. These compounds continue to be used in the search for alternative chemotherapeutic agent candidates, especially for use in cancer treatment, by synthesizing new derivatives (Han et al., 2021).

Some studies have shown that some Schiff base derivatives have anticancer activities in pancreatic cancer cells. Copper conjugates of nimesulide Schiff base derivatives are reported to have cytotoxic effects in BxPC-3 (COX-2 positive) and MiaPaCa (COX-2 negative) pancreatic cancer cells (Ambike et al., 2007). Yusof et al. (2019) studied the cytotoxic effects of o-vanillin derivative Schiff bases and organotin (IV) compounds in different cancer types and reported that these compounds inhibited growth in the MIA pancreatic cancer cell line. In another study, isatin-based Schiff bases targeting VEGFR-2 inhibition were synthesized and some of the compounds were reported to exhibit significant and moderate antiproliferative properties against MCF7 (breast), HCT116 (colon) and PaCa2 (pancreatic) cancer cell lines, compared to reference drugs 5-fluorouracil (5-FU) and sunitinib (Seliem et al., 2022). In our study, the anticancer effects of fluorinated hydrazine derivative Schiff bases on Capan-1, MIA PACA-2, Panc-1, and TGP52 pancreatic cancer cell lines were tested, and it was determined that these compounds showed varying levels of cytotoxic effects against pancreatic cancer cells. The cytotoxic activity of C4 was observed to be stronger on Panc-1 and Capan-1, while C2 was found to be effective on TGP52 and C6 on MIA PaCa-2. The mechanism or mechanisms by which the 3-5 ditertbutyl adducts in the compounds contribute to the cytotoxic power of the compounds is a matter of curiosity. This situation has the potential to open a new investigation area for molecular cellular research.

The capacity of a chemotherapeutic agent to prevent cell proliferation is an important issue for its anticancer activity, together with its cytotoxic effect. These two concepts are often presented as the same thing. MTT test results are usually expressed as antiproliferative effect. However, the direct killing of cells by the agent used in the

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treatment, which we call cytotoxicity, and the prevention of cell proliferation are not the same thing. In our study, the CFSE cell proliferation test, which quantitatively demonstrates the power of compounds to truly prevent cell proliferation, was used. In short, with this test, we monitor the sharing of the fluorescent CFSE dye given to the cells by half during cell division in flow cytometry. In this way, we can determine how many divisions the cells have undergone during the culture process, i.e., how many times the fluorescent dye is halved. A few anticancer studies are using this method on A549 (lung cancer) and K562 (leukemia) cell lines (Sak et al., 2021; Shekhany et al., 2023). In our study, the most potent antiproliferative effects of the compounds were observed by salicylaldehydes on TGP52 and MIA PaCa-2 (C1 and C5, respectively) and by ditertbutyl salicylaldehydes on Capan-1 and Panc-1 (C2 and C4, respectively).

Alterations in apoptotic cell rates are responsible not only for tumor development and progression but also for tumor resistance to treatment. While most chemotherapeutics use intact apoptotic signaling pathways to trigger cancer cell lysis, defects in death pathways can lead to drug resistance and treatment failure (Pistritto et al., 2016). Apoptosis has a complex mechanism involving many signaling pathways and can be induced by extrinsic or intrinsic pathways, particularly caspase-mediated pathways in the cell. Intrinsic and extrinsic stimuli that combine to activate apoptotic caspases lead to cellular morphological and biochemical changes that signal apoptosis (Wong, 2011). In our study, molecular markers indicating apoptosis and cellular morphological changes were observed to understand the mechanism of the lethal effects of fluorinated Schiff base derivatives on pancreatic cancer cells. In cleaved caspase-3 analyses, the expression of this molecule in pancreatic cancer cell lines was monitored immunohistochemically, and the expression of this molecule was detected in all cell lines treated with the compounds. Cleaved caspase-3 in cells is an important cellular protein structure that indicates apoptosis (Hua et al., 2025; Shaaban et al., 2024). Many studies have shown that cleaved caspase-3 expression is an indicator of the development of apoptosis in cells (Li et al., 2020; Üremiş et al., 2024). It has been shown that the antitumor activity of copper (II) complex Schiff bases on MCF-7 cells is due to increased BAX expression and also increased caspase-3 and caspase-9 activity (Ma et al., 2012). It is reported that copper (II) complex Schiff bases act like synthetic nucleases and anticancer drugs on MDA-MB-231 cells and drive the cells to apoptosis through caspase-3 activation (Zhou et al., 2016). Annexin-V is expressed in the early stages of apoptosis, together with other cell death signals in cell death (Koopman et al., 1994; Vermes et al., 1995). The observation that the gold (III) complex of Schiff bases on pancreatic cancer cell lines leads to annexin-V expression as well as apoptosis-associated morphological changes indicates that the expression of this molecule is important in the development of apoptotic processes (Hassan et al., 2015). Şenkuytu et al. (2024) showed that Schiff base compounds induced apoptosis in A549 lung cancer cells and led to caspase-3, caspase-7, caspase-8, caspase-9, and annexin V expressions in these cells compared to untreated controls. In our study, Schiff base compounds caused the expression of annexin-V, an early apoptosis marker, in pancreatic cancer cell lines in short-term cultures. The observation of both cleaved caspase-3 and annexin-V expression in pancreatic cancer cells suggests that the compounds use apoptosis as a cell death mechanism (*Fig. 2*).

Rhodamine 123 is a delocalized lipophilic cation compound that targets mitochondria and accumulates in the mitochondrial matrix against the concentration gradient due to the negative membrane potential of the inner mitochondrial membrane and provides information about changes in membrane potential (Battogtokh et al., 2018; Zielonka et

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al., 2017) The expression of molecules belonging to the caspase family plays a central role in the induction of mitochondria-dependent cell apoptosis, and in stress situations, including the involvement of chemotherapeutic agents, oligomerization of some proapoptotic proteins in the outer mitochondrial membrane, leading to the release of cytochrome C, induces apoptosis in the cell through caspase-9/caspase-3 cascade reactions (Zhang et al., 2019). In this study, the contribution of the compounds to cell death via apoptosis in pancreatic cancer cells was also supported by the accumulation of rhodamine 123 in the $\Delta\Psi m$, which is another analysis indicating the development of apoptosis in cells. Di-tert-butylsalicylaldehyde derivatives; C2 in MIA PaCa-2, C4 in Panc-1 and C6 in Capan-1 cells have apoptotic effects, demonstrated by low levels of rhodamine 123 accumulation, annexin-V and cleaved caspase-3 expressions, revealing that apoptosis is effective as a death pathway in cells.

P-gp is a protein expressed in the cell membrane and is responsible for pumping some foreign substances, including drugs, out of the cell. The most studied efflux transporter of chemotherapeutics in cells is P-glycoprotein (P-gp) encoded by the MDR1 gene (Morau et al., 2024). P-gp efflux activity is one of the most important mechanisms of the development of multidrug resistance (MDR) in cancer, as its overexpression reduces intracellular concentrations of chemotherapeutics below therapeutic levels (Breier et al., 2012; Sui et al., 2012). It has been reported that MDR1 expression is increased in other cancers, including pancreatic cancer, and this contributes to the development of multidrug resistance (Zhan et al., 2015). The role of P-gp in pumping chemotherapeutic agents out of tumor cells, thereby preventing their accumulation, may be illustrated by the overexpression of MDR1 in gemcitabine-induced resistance in pancreatic cancer (Yang et al., 2024). Considering all these, it is an important advantage that among the Schiff bases used in our study, there are compounds that cause very low levels of P-gp expression, especially on MIA PaCa-2 and TGP52. Detailed consideration of the mechanism by which these compounds cause such low levels of P-gp expression may lead us to important clues. The fact that PNT1a is a normal cell and that P-gp expression is also a mechanism that protects cells from death suggests that high P-gp expression can be considered an advantage.

Conclusions

As a conclusion, it was observed that the cytotoxic and antiproliferative effects of hydrazine derivative fluorinated Schiff base derivative compounds on four cell lines representing pancreatic cancer types were remarkably effective, and it was noted that those carrying ditertbutyl groups were more active than salicylaldehydes that did not carry these groups. Another advantageous aspect of the compounds is that apoptosis, which is the most desired cell death pathway, was activated.

Author contributions. Meryem KOYUNCU, Faruk SUZERGOZ, Mustafa Unal BOYRAZ, Nilgun OKSAK, Bestoon SHEKHANY, Veli KASIM, Ali Osman GUROL, contributed to the study conception and design. Material preparation and analysis were performed by Meryem KOYUNCU and Faruk SUZERGOZ. The first draft of the manuscript was written by Meryem KOYUNCU, and all authors commented on the previous versions of the manuscript. The other authors would like to thank Dr. Nilgun OKSAK for constructive criticism, language editing and proofreading of the manuscript. All authors read and approved the final manuscript.

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