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Synthesis of 1-(4-chlorobenzoyl)-4-(dimethylamino) pyridin-1-ium chloride and determination of cytotoxicity and induction of apoptotic protein in human colon cancer cells

Abstract

Background:!In this experiment, we synthesized a new compound 1-(4-chlorobenzoyl)-4-(dimethylamino) pyridin-1-ium chloride (SM-9) and examined its toxicity and anticancer activity on human colon cancer (HCT-116) cells.&Methods:!We uncovered the underlying mechanism of cell toxicity and apoptosis in HCT-116 cells due to SM-9 compound exposure via MTT assay and protein profiling array and gene expression through the RT-PCR.&Results: [19]Our data showed that the SM-9 compound activated caspase-3, caspase-8, p21, p27, p53 proteins involved in apoptosis in HCT-116, thereby inducing cytotoxicity, the formation of reactive oxygen species, and apoptosis. [19]The results of this study showed that the SM-9 compound has advantageous qualities and need to be taken as an anticancer medication. [19]This research concluded that through inducing apoptotic pathways in human colon cancer (HCT-116) cells, the SM-9 compound possesses anti-cancer capabilities.

Keywords: Cytotoxicity; Protein profiling array; Oxidative stress; HCT-116 cells; [5]Caspase-3/8, p21, p53 gene expression

Introduction

Over the past ten years, ionic liquids have drawn a lot of attention due to their special qualities that may be useful for cutting-edge technologies and procedures. Ionic liquids include, among others, corrosion inhibitors (El-Hajjaji et al., 2019), antimicrobials (Albalawi et al., 2018, Titi et al., 2021), and antiviral and anticancer medicines (Titi et al., 2020).[10] In this work, we created an ionic liquid (1-(4-chlorobenzoyl)-4-(dimethylamino) pyridin-1-ium chloride, SM-9 compound, SM-9 compound) and tested its cytotoxicity and antioxidant qualities on human colon cancer (HCT116) cells for a duration of 24 hours.[10] The toxicity of SM-9 compounds is caused by a number of processes, one of which is the increased generation of ROS in stressed live cells. Mitochondria are the main source of ROS generation in tissue, and the most of ROS produced in the chain of electron transport system (Hansford et al., 1997).[14] The electron release from the chain of electron transport system directly to oxygen, and inducing tiny-lived free radicals such as singlet oxygen and superoxide anion etc (Hansford et al., 1997).[7] In this experiment we have synthesized new chemical compound and investigate the toxic effects of synthesized drug namely SM-9 compound on human colon cancer cells.[6] Ali et al.[6], (2023) has reported that biosynthesize cobalt nanoparticles with garlic and onion peel inhibit the growth of bacteria.[7] In addition, our findings will be supportive in determining the safety purpose of synthesized compound and it will be helpful as a raw material for manufacturing of anticancer drug. 4-(Dimethylamino)pyridine is a cheap and ingenious chemical induced skin toxicity.[6] ROS creates cellular oxidative stress and it leads colon damages and colon cancer (Bardelčíková et al.,[5] 2023), but at cellular level colon's natural defense system gets damaged in response to chemical compound.[5] ROS,

which seriously deteriorates bio-membranes, biomolecules, and induces the expression of matrix metalloproteinases (Tu and Quan, 2016).

Drug toxicity includes various mechanisms, mainly the generation of extra reactive oxygen species (ROS). Schumacker et al., (2014) and Almutairi et al., (2021) have reported that the damage of the mitochondrial is due to overproduction of ROS in cells. Toxicity induced by contaminant and drug compound in target cells is due to production of ROS, apoptotic and inflammatory process (El-Sayed et al., 2005). Oxidative stress has mainly resulted from the generation of ROS. Extra production of ROS induce imbalance of antioxidant mechanism in cells and as a consequence it leads to peroxidation of lipid molecules and other oxidative related stress enzymes (Zhuang et al., 2018). This is the first study that reported the adverse effects of synthesized drug SM-9 compound on human colon cancer cells. In this study we are determining the safely effects of SM-9 compound on human colon cancer cells.

2. Materials and methods

2.1. Chemical and reagents

Trypsin, antibiotic/antimitotic solution (100×), stabilized, 2′,7′- I chlorodihydrofluorescein diacetate (H2DCFDA), MTT dye, and sodium chloride were purchased from Sigma-Aldrich (St.Louis, Missouri, United States). Human Apoptosis Array C1(Code: AAH-APO-1-2) Ray Biotech, United States. Fetal bovine serum (FBS), and Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM), and from Gibco company USA. All other chemicals such as 4-chlorobenzoyl chloride (1.1 eq), DMSO and ethyl alcohol etc. were purchase de from local market Riyadh Saudi Arabia.

2.2. Synthesis new chemical material

2.2.1. Synthesis of new chemical compound 1-(4-chlorobenzoyl)-4-(dimethylamino) pyridin-1-ium chloride (SM-9)

4-chlorobenzoyl chloride (1.1 eq) was added to a solutionof4-dimethylaminopyridine(1 eq) in toluene, which was followed by stirring at 80 ◦C for 24 h. The separation of viscous liquid from the initially obtained clear and homogeneous mixture of4-Dimethylaminopyridine and 4-chlorobenzoyl chloride in toluene marked the completion of the reaction. Extraction was used to separate the product from the unreacted starting materials and solvent. Finally, all volatile organic compounds were removed from the residue by drying it under pressure (Fig.1 a, b).

2.2.2. Characterization of SM-9 compound

1-(4-chlorobenzoyl)-4-(dimethylamino) pyridin-1-ium chloride: MP 83–84 °C, 1H NMR (DMSO, 400 MHz,) δ 8.17 (d, 2H), 7.91 (d, 2H), 7.54 (d, 2H), 6.94 (d, 2H), 3.14 (s, 6H). ^{13}C NMR (DMSO, 100 MHz,) δ 166.9, 157.4, 139.6, 138.3, 131.6, 130.1, 129.2, 107.4, 40.); IR (vmax cm−1) 3129 (C–H, sp2), 1559–1469 (C=C), 1163 (C–N), 1080 (C–O) (Fig.1 a, b).

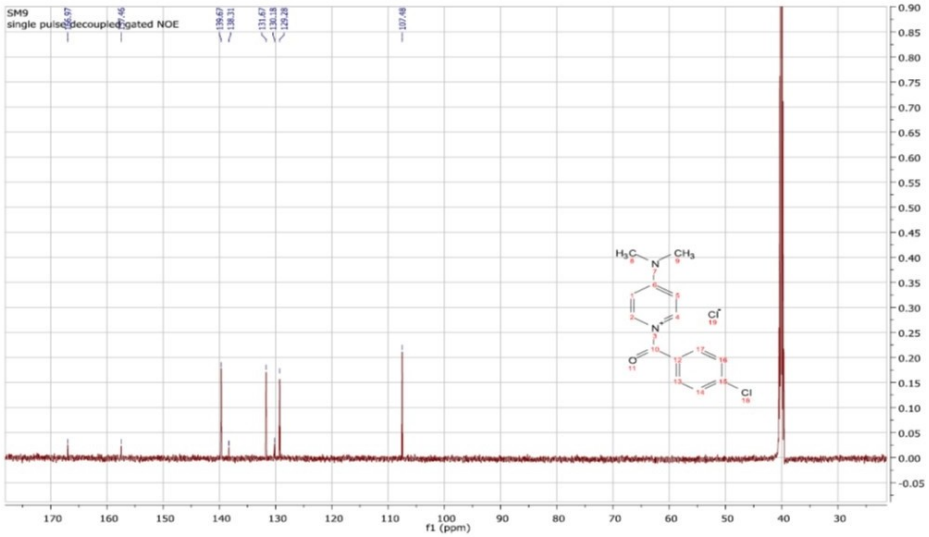

Fig.1 a ^{13}C NMR spectrum of 1-(4-chlorobenzoyl)-4-(dimethylamino) pyridin-1-ium chloride (SM-9)

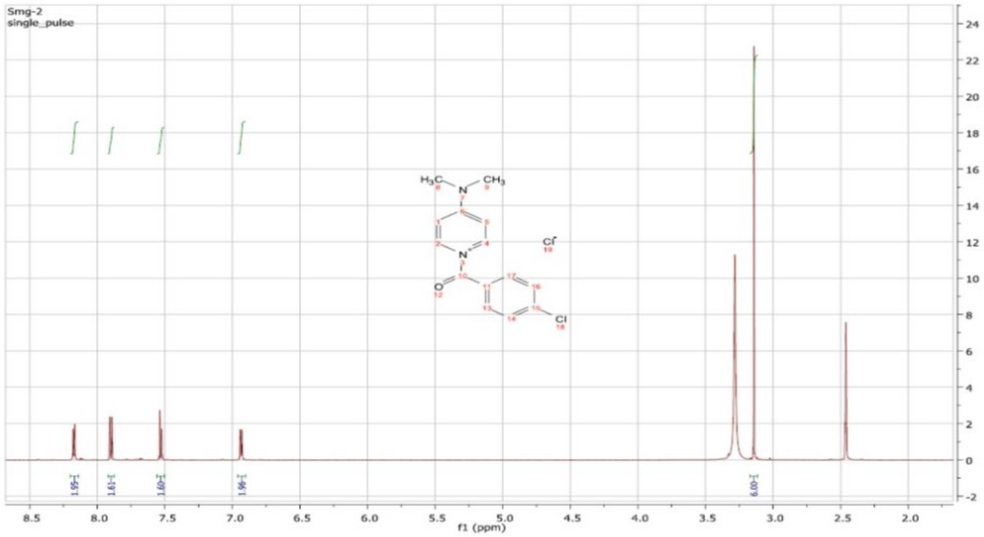

Fig.1 b ^{1}H NMR spectrum of 1-(4-chlorobenzoyl)-4-(dimethylamino) pyridin-1-ium chloride (SM-9)

2.3. Cell culture

The colon cancer (HCT 116) cell lines was bought from ATCC&(American Type Culture Collection) Manassas Virginia USA. The cells were maintained in DMEM with

fetal bovine serum (10%) and antibiotics&penicillin and streptomycin (10000U/ml) in CO2 incubator (5%) at 37°C.

2.4. Exposure of SM-9 compound

HCT-116 cells were sub-cultured for 24 h before exposure to SM-9 compound. Stock suspension of SM-9 compound (50 mg /ml DMSO) was prepared in slightly warm DMSO. The stock suspension of drug was diluted as per experimental concentration. In this experiment we applied different concentrations of compound (control, solvent control, 50, 100, 200, 300,400 and 500 µg/ml) to determine the toxicity of compound. The untreated cells were considered as the control.

2.5. MTT assay

Cell viability of HCT-116 cells were determined by using by MTT test (Alarifi et al., 2015) after exposure to various concentration of compound (control, solvent control, 50, 100, 200, 300,400 and 500 µg/ml) over 24 hrs. After adding, MTT dye(0.05 mg/ml) to each well the plate was incubated for 4 hrs. The formazan crystal was solubilized in organic solvent and after 15-minute incubation at room temperature. The optical density of culture palate was measured at 570 nm by using a microplate reader (BioTek Instruments, Winooski, VT, USA) with Gen5 software (version 1.09).

The half-maximal inhibitory concentration (IC_{50}) 24 hr of SM-9 compound was determined on the basis of MTT test result. Based on IC_{50} 24 hr of SM-9 compound, we have fixed 3 sub-lethal concentrations for further experiment (Table 1).

Table 1. The half-maximal inhibitory concentration (IC_{50}) and concentrations for SM-9 compound was used on the HCT-116 cells.

IC50 -24 h = 400 µg/ml SM-9 compound	
Percentage %	Concentrations
1/8 of IC_{50}	50 µg/ml
½ of IC_{50}	100 µg/ml
2/3 of IC_{50}	266 µg/ml

2.6. Evaluation of ROS generation

Due to exposure of SM -9 compound (50, 100, 266 µg/ml) to HCT-116 cells the production of ROS levels was evaluated as per Ali et al., (2021) method.[11] The cells ($3x10^4$) was sub cultured in a black bottom culture plate (96 well) and kept in a CO_2 incubator at 37°C for 24 h.[0] Later treatment to compound the culture plates were incubated for 24 hrs.[0] Later 24 h, DCFH-DA (10µM) was mixed/ well for 35 minute at 37°C.[77] Later incubation, plate was wash away with chilled PBS and fluorescence intensity was evaluated at 485 nm excitation and 520 nm emissions using by a microplate reader with Gen5 software (version 1.09)[34] (Bio-Tek Instruments, Winooski, VT, USA). Obtained results was expressed in percent of fluorescence intensity with compare with control.[6][23] A separate set of experiments were carried out to assess for generation of ROS through qualitative analysis method (Ali et al., 2021).

2.6. Protein array for detecting apoptosis

To find out the mechanism of apoptosis induced by SM-9 compound, a protein array (Human Apoptosis Array C1(Code: AAH-APO-1-2) Ray Biotech, United States) was applied for finding the part of proteins related to apoptosis. HCT-116 cells was exposed with the SM-9 compound (0, 50, 100 and 266 µg/ml) for 24h. Extracted proteins (200 µg) from control and exposed cells was incubated 5 h with the human apoptosis antibody array.[0] The protein The apoptosis array membranes was scanned in a Biorad Chemi Doc

XRS+ Imaging System (Tide Mill Road, Units 3, 4, USA). The obtained data was analyzed by using Image Lab software (Biorad Chemi Doc XRS+), and the protein signal intensity of all samples were assessed according to the kit's ((Code: AAH-APO-1-2) Ray Biotech, USA) instructions.

2.7. Gene expression

2.7.1. RNA isolation and cDNA synthesis

The fresh cell lysate of control and SM-9 compound treated HCT cells were prepared. As per manufactures protocol of RNeasy Mini Kit (Qiagen, Germany), we have isolated total RNA from the above cells. The quality of RNA was determined by means of NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). We have synthesized cDNAs by using cDNA Synthesis Kit (BioRad, USA) as per kit protocol.

2.7.2. Reverse transcription (RT-PCR)

The apoptotic genes such as Bax, Bcl2, and Tp53 expression in HCT cells were analyzed by using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) and Light cycler 480 (Roche, Basel, Switzerland) as per kit information. We have done all experiment in duplicate. The following primers for apoptotic genes expression in SM-9 compound treated HCT cells was used as in Table 2. GAPDH used as a housekeeping gene, and the fold change in relative quantification were determined by using this formula $2^{-\Delta\Delta Ct}$.

Table 2. List of primer sequences of apoptotic genes.

Gene	Primer F sequence	Primer R sequence
Bcl2	ATGTGTGTGGAGAGCGTCAA	GGGCCGTACAGTTCCACAAA
Bax	TGAAGCGACTGATGTCCCTG	GGGCCGTACAGTTCCACAAA
TP53	TGAAGCGACTGATGTCCCTG	CAAAGATGGTCACGGTCTGC
GAPDH	GGGAAGCTTGTCATCAATGG	GAGATGATGACCCTTTTGGC

2.8. Statistical analysis

Results were analyzed by SPSS 26.0 software (IBM) and expressed as mean ± standard deviation (SD). *p value 0.05, **p value 0.01 were considered statistically significant.

3. Results

3.1. Determination IC_{50} value of SM-9 compound

We have calculated the median inhibitory concentration (IC_{50} for 24 h) of SM-9 compound on HCT-116 cells by using MTT test result through software OrigenPro 8.5 program. The IC_{50} value of SM-9 compound on HCT-116 cells was 400 µg/ml (Figure 2, Table 1).

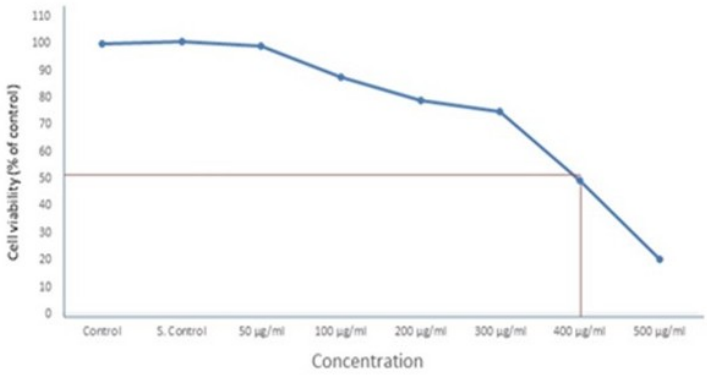

Figure. 2. Determination of IC50 24 h for SM-9 drug in HCT-116 cells Each value represents the mean ±SE of five experiments. n= 3, *$p < 0.05$ * *$p < 0.01$ vs. control.

3.2. Cytotoxicity

[0] Cytotoxicity of SM-9 compound was determined in HCT cells by using MTT assay[0]. SM-9 compound induced toxicity in a dose-dependent manner. SM-9 compound induced high cell death in HCT cells than untreated cells. A significant toxic effect of SM-9 compound was seen in HCT cells at 500 µg/ml of SM-9 compound (Figure 3).

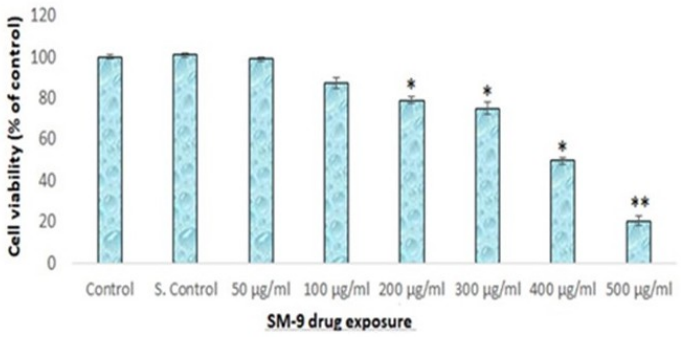

Figure. 3. Cytotoxicity of SM-9 drug in HCT-116 cells over 24 h, as evaluated by MTT assays. Each value represents the mean ±SE of three experiments. n= 3, $*p < 0.05$ $**p < 0.01$ vs. control.

3.3. Intracellular reactive oxygen species (ROS)[44]

The production of ROS was increased as exposure of compound was increased and we have observed high intensity of green fluorescence at 266 µg/ml of SM-9 compound (Figure 4A, E)[7]. In this study we have determined the production of intracellular ROS by measuring DCF intensities in SM-9 compound exposed cells (Figure 4)[44]. The maximum production of ROS was found at concentration of SM-9 compound 266 µg/ml (Figure 4 A).

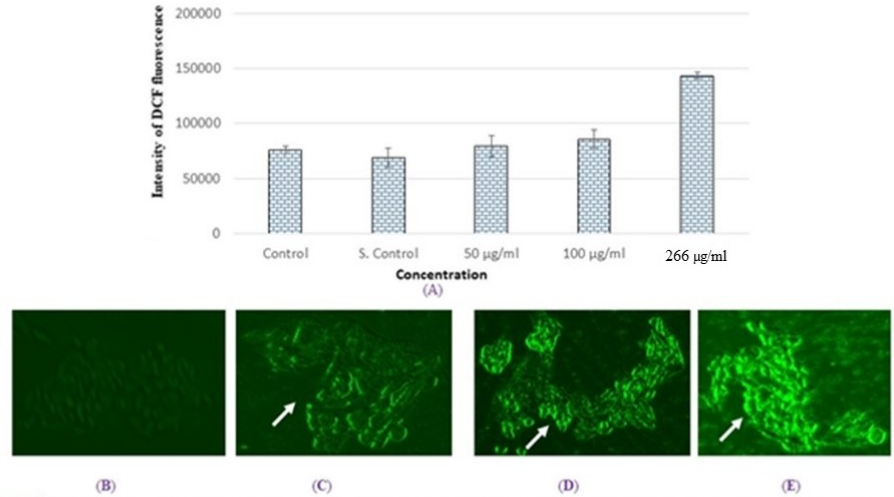

Figure. 4 . Production of intracellular ROS in HCT cells for 24 h due to SM-9 drugs exposure (A). Percent of DCF fluorescence intensity and generation of green fluorescence in HCT cells for 24 h (B). Control HCT cells (C). HCT cells at 50 µg/ml (D). HCT cells at 100 µg/ml (E). HCT cells at 266 µg/ml. Each value represents the mean ±SE of three experiments. *$p < 0.05$ vs. control. Arrow indicate green fluorescent intensity in cell as marker of ROS generation.

181

182 3.4. Apoptotic protein expression

183 Due to treatment of various concentration of SM-9 compound to HCT cells for 24 h, our
184 team member analyzed specific proteins related apoptosis and toxicity using Human
185 apoptosis protein array (Fig. 5a,b,c).[11] As indicated in Fig. 5, we found changes in those
186 apoptotic proteins.[82] Many proteins were down regulated including Bad, Bcl2, CD40, and
187 upregulated Bax, Caspse-3, Caspase-8, CD40L, cytochrome C, Fas, Fas, ligand, Hsp70,
188 HTRA, IGF-1sr, IGF-2, IGFBP5, IGFBP6, livin, P21, P27, P53, survivin, TNF-β, TNFR-
189 II, TRAIL-1, TRAIL-2, and TRAIL-3, depending on its role in apoptosis pathway (Fig. 5[24]
190 a, b, c).[0]

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
2	Pos	Pos	Neg	Neg	Blank	Blank	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
3	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	Blank	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
6	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
7	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos
8	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Blank	Blank	Neg	Neg	Neg	Pos

Blank: Blank spot

Neg: Negative control spot

Pos: Positive control spot

(a)

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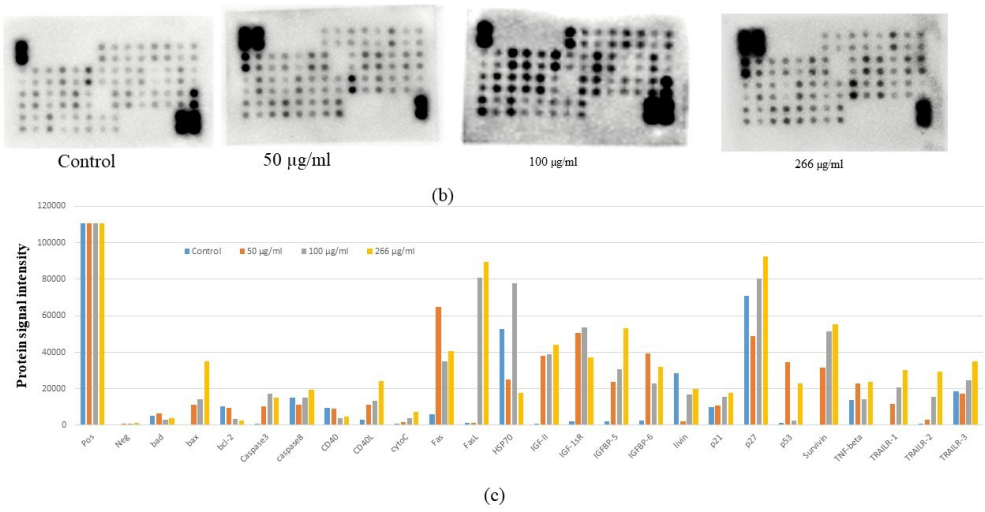

Fig. 5. Analysis of apoptosis-related proteins in HCT-116 cells due to SM-9 compound exposure **(a)** Template showing the location of apoptosis-related antibodies spotted on the Human Apoptosis Array C1 kit. **(b)** Equal amounts of protein extracts from control and treated HCT-116 cells were analyzed using the antibody array. The chemiluminescent intensities were quantified by densitometry. A positive control was used to normalize the results from different membranes. **(c)** Representative bar graph of the apoptotic upregulated and downregulated proteins.

[0]

192

[9]

1933.5. Apoptotic gene expression in HCT-116 cells

194 To confirm the mechanism of toxicity induced by SM-9 compound in HCT-116 cells we

195have determined the expression some specific gene such Bcl2, Bax and Tp53. [9] RT-PCR

196analysis was performed to determine the expression level of the apoptotic genes e.g.

197Bcl2, Bax and p53 in HCT cells. [76] The higher expression of gene expression was observed

198at 200 µg/ml of SM-9 compound after 24 h of exposure (Figure 6 A, B.C). The p53 gene

199was downregulated at 100 µg/ml of SM-9 compound but upregulated at 50 µg/ml and

200266 µg/ml of SM-9 compound in cells (Figure 6 C). [0] Other apoptotic genes, such as Bcl2

201and Bax were expressed in HCT cells (Figure 6 A, B).

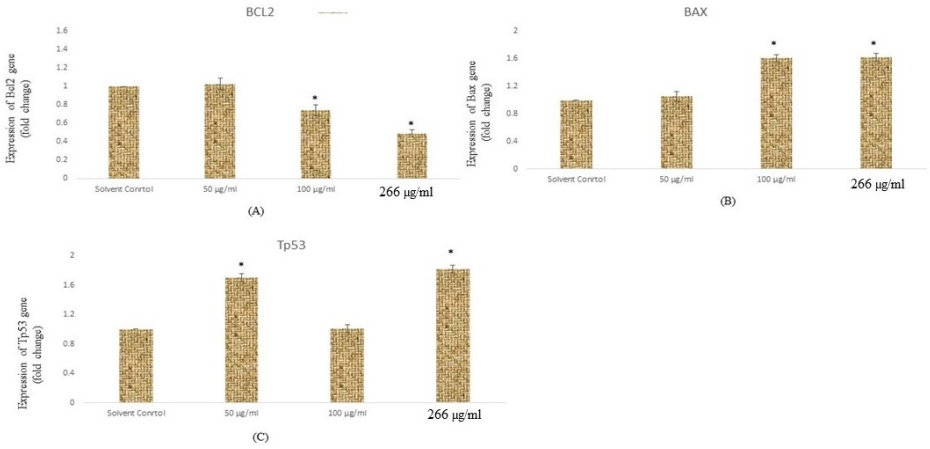

Figure 5. Expression of apoptotic genes in HCT cells exposed to SM-9 drug for 24 h. Expression of (A) Bcl2 (B) Bax, (C) p53, gene in HCT cells for 24 h. Results are expressed in average ± SD of triplicate experiments. *p < 0.05 vs. solvent control

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204 [39]4. Discussion

205 The production of novel chemical compounds has increased significantly in recent years
206 on a global scale, and it is fascinating to use this technology to the pharmaceutical
207 industry for the benefit of human health.[39] However, a significant and concurrent
208 consequence of these growing chemical-based uses could be detrimental to the
209 ecosystem, as people may not be aware of the risk of drug exposure or the different ways
210 that chemicals can infiltrate biological systems (Yang et al., 2012). This study looked at
211 the biological reactions that occurred when the SM-9 compound was exposed to human
212 colon cancer (HCT-116) cells. We found that the SM-9 compound caused dose-
213 dependent toxicity, reactive oxygen species, and apoptosis in HCT-116 cells. The
214 finding that HCT-116 cells are susceptible to the SM-9 chemical is more significant.[11] The
215 bioactivity of SM-9 compound may be due to presence of 1-(4-chlorobenzoyl)-4-
216 (dimethylamino) group is worthy for further analysis.[0] The production level of ROS was
217 higher in HCT-116 cells at maximum concertation of SM-9 compound exposure

demonstrate that higher dose of this drug exposure will be more effective to minimize the growth of cancerous cells.[11] ROS has a major role in various cellular mechanisms, such as cell cycle, cell proliferation, and gene expression, and ultimately the mechanism of cell growth was stopped or cell death occurred (Almutairi et al., 2020).[6] Our investigation showed that the SM-9 chemical was advantageous for usage as an anti-cancer medication and sensitive to HCT-116 cells.[6] By using RT-PCR analysis to assess the apoptotic potential of the SM-9 compound in these cells, it was found that higher compound concentrations had a greater apoptotic effect.[25] Due to the correlation between the induction of cytotoxicity, oxidative stress, and apoptotic gene expression, it was evident that exposure to a more dose of SM-9 chemical was more effective. In HCT-116 cells, all of these results proved the SM-9 compound's anticancer capabilities.[25] The untreated HCT-116 cells showed decreased protein signal fluorescence intensity of various proteins such as bad, bax, bcl2, p53, p21, Hsp70, p27, TRAIL-1, TRAIL-2, TRAIL-3 and TNF β with comparison to the SM-9 compound treated HCT-116 cells.[25] The current finding indicated the effect of SM-9 compound to cause inhibition of the bad, bcl2 signaling pathway in the HCT-116 cells.[6] These findings suggest that bad, bcl2 regulation could be chunked via SM-9 compound.[6] The results revealed that both types of apoptotic pathways may be involved in the induction of the apoptosis in HCT-116 cells by upregulating caspase-8 and p53 and reducing bad, bcl2 into the cytoplasm and nucleus.[10] The concerns of the mitochondrial-intrinsic actions indicated by mRNA and the upregulation of p53, bax, and cytochrome c could elicit mitochondrial damage and dysfunction by enhancing the levels of caspase-8.

[0] Protein&arrays&are& useful&because&it& can&provide&a&map& of&known&cell&apoptotic and

signaling&proteins.&[12]

Protein&arrays&have&shown&a&unique&ability&to&analyze&signaling&pathways&using&small&nu

mbers&of&cultured&cells&or&cells&(Grubb&et&al.,&2003).&Using&this&approach,&untreated&and&tr

eated&HCT-116&cells&of&protein&lysate&is&arrayed&onto&nitrocellulose-coated&slides.&Main&te

chnological&components&of&this&method&offer&unique&advantages&over&to&detect&multi&prot

ein&expression.

In this study a useful methodology for the synthesis of a novel chemical compound was described.[7] The results showed that SM-9 compound has high efficacy and should be given particular consideration in anticancer activity.[7] Furthermore, this finding demonstrated that SM-9 compound killed the colon cancer cells and induced apoptosis through various activated proteins, gene such as caspase-8, p53, NF-β, and bax.[23] The results established that SM-9 compound may be applied as a medicinal drug alone or in combination with other chemotherapeutics for considering different types of cancer cells. In the future, we will investigate about the mechanism of toxicity due to SM-9 compound in vivo experiments[35]

Declaration of conflicting interests

None

Data Availability Statement

The original contributions presented in the study are included in the article.

Funding Statement[0]

None

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Supplementary Material

Associated with this article arethe1H and 13C NMR spectra of SM-9 compound.

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