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Effect of 5'-fluoro-2'-deoxycytidine and sodium butyrate on the genes of the intrinsic apoptotic pathway, p21, p53, cell viability, and apoptosis in human hepatocellular carcinoma cell lines

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Abstract

Backgrounds: Epigenetic regulation such as DNA methylation plays a major role in chromatin organization and gene transcription. Additionally, histone modification is an epigenetic regulator of chromatin structure and influences chromatin organization and gene expression. The relationship between DNA methyltransferase (DNMTs) expression and promoter methylation of the tumor suppressor genes (TSGs) has been reported in various cancers. Previously, the effect of 5-aza-2'-deoxycytidine (5-AZA-CdR), trichostatin A (TSA), and valproic acid (VPA) was shown on various cancers. This study aimed to investigate the effect of 5'-fluoro-2'-deoxycytidine (FdCyd) and sodium butyrate on the genes of the intrinsic apoptotic pathway, p21, p53, cell viability, and apoptosis in human hepatocellular carcinoma SNU449, SNU475, and SNU368 cell lines**.**

Materials and Methods: In this lab trial study, the SNU449, SNU475, and SNU368 cells were cultured and treated with 5'-fluoro-2'-deoxycytidine and sodium butyrate. To determine cell viability, cell apoptosis, and the relative gene expression level, MTT assay, flow cytometry assay, and qRT-PCR were done respectively.

Results: 5'-fluoro-2'-deoxycytidine and sodium butyrate changed the expression level of the BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, and p53 gene (P<0.0001) by which induced cell apoptosis and inhibit cell growth in all three cell lines, SNU449, SNU475, and SNU368.

Conclusion: Both compounds played their roles through the intrinsic apoptotic pathway to induce cell apoptosis.

Keywords: Carcinoma, Hepatocellular, Methylation, P21, P53

Introduction

In mammalian cells, the cell cycle is controlled by cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs) which regulate critical checkpoints. The cell cycle progression is controlled by CDKs and CKIs, whose activity can be upregulated or down-regulated by a wide range of molecular mechanisms. CKIs are being developed as potential cancer therapeutics. CKIs are frequently silenced in human cancer because of DNA hypermethylation and histone deacetylation (1). They are divided into two families comprising INK4 (p15, p16, p18, and p19) and CIP/KIP (p21, p27, and p57) (2). Therefore, CKIs are regarded as a therapeutic target for cancer therapy. Epigenetic regulation such as DNA methylation plays a major role in chromatin organization and gene transcription. In the mammalian genome, DNA methylation, transfer of a methyl group onto the C5 position of the cytosine, is the mechanism by which gene transcription is activated or inactivated in the cells. This change can silence genes through a process that leads to the alteration of chromatin structure and chromatin compaction (3). The DNA methylation of mammalian genomic is catalyzed by a group of enzymes, DNA methyltransferases (DNMTs), that can be divided into DNMT1, DNMT2, DNMT3A, and DNMT3B (4). The relationship between DNMTs expression and promoter methylation of the tumor

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suppressor genes (TSGs) has been reported in various cancers such as DNMT1, and DNMT3B in breast cancer cell line MCF-7, DNMT3B in T24 bladder cancer cells, DNMT1, and DNMT3B in human colon cancer HCT116 cells and ovarian cancer cell lines HeyA8, HeyC2, SKOV-3 and PA-1, and DNMT1 in prostate cancer cell line LNCaP (5). DNA methyltransferase inhibitors (DNMTIs) have shown substantial potency in reactivating epigenetically silenced TSGs in numerous cancers (6). These compounds can induce their apoptotic roles through various molecular mechanisms. Previously, the effect of DNMTI 5-aza-2'-deoxycytidine (5-AZA-CdR) on DNMTs gene expression was shown in hepatocellular carcinoma (HCC) LCL-PI 11 cell line (7), p15INK4, p16INK4, p18INK4, and p19INK4 in HCC PLC/PRF/5 cell line (8), p16INK4a, p14ARF, and p15INK4b gene expression in pancreatic cancer MIA Paca-2 cell line (9), and CIP/KIP family (p21, p27, and p57) gene expression in colon cancer SW480 cell line (10). Several experimental studies have shown other apoptotic pathways for DNMTIs. It has been shown that DNMTIs such as decitabine and zebularine activates the intrinsic apoptotic pathway by up-regulation of the intrinsic apoptotic genes such as Bak, loss of mitochondrial transmembrane potential, and the generation of reactive oxygen species (ROS) (11). Besides, these agents can induce apoptosis through DR/extrinsic apoptotic pathway (12). In addition to DNA methylation, histone modification is an epigenetic regulator of chromatin structure and influences chromatin organization and gene expression. The balance between histone acetylation and deacetylation is important for gene transcription. Recent studies showing that histone acetyltransferase (HAT) and deacetylase (HDAC) activities play a major role in the regulation of transcription, they regulate the acetylation of histone proteins and transcription factors. Therefore, histone acetylation is catalyzed by HATs, whereas the reverse reaction, histone deacetylation, is performed by HDACs (13). A structurally diverse group of compounds has been demonstrated that can inhibit HDACs activity leads to cell growth inhibition, differentiation, and apoptosis induction of cancer cells (14). Previously, the effect of histone deacetylase inhibitors trichostatin A (TSA) and valproic acid was reported on HCC (15, 16). It has been reported that HDACIs can induce apoptosis via the intrinsic/mitochondrial and the death receptor (DR)/extrinsic pathways. The intrinsic pathway is activated via the upregulation of several pro-apoptotic BH3-only Bcl-2 family genes such as Bid, Bim, and Bmf. Further, HDACIs can activate the extrinsic pathway through the upregulation of DR expression and their ligands such as TRAIL (17). This study aimed to investigate the effect of 5'-fluoro-2'-deoxycytidine (FdCyd) and sodium butyrate, as a histone deacetylase inhibitor, on the genes of intrinsic apoptotic pathway (BAX, BAK and APAF1, Bcl-2, and Bcl-xL), p21, p53, cell viability, and apoptosis in human hepatocellular carcinoma SNU449, SNU475, and SNU368 cell lines.

Materials and Methods

Materials

Human hepatocellular carcinoma SNU449, SNU475, and SNU368 cell lines were purchased from the National Cell Bank of Iran-Pasteur Institute. The 5'-fluoro-2'-deoxycytidine, sodium butyrate, and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Sigma (St. Louis, MO, USA). The compounds, 5'-fluoro-2'-deoxycytidine and sodium butyrate, were dissolved in dimethyl sulfoxide (DMSO) and sterile water respectively to make a work stock solution. Further concentrations of the compounds, 5'-fluoro-2'-deoxycytidine, and sodium, were obtained by diluting the provided stock solution. Other necessary materials and kits were purchased as provided for previous works (18, 19). The SNU449, SNU475, and SNU368 cells were maintained in DMEM supplemented with fetal bovine

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serum 10% and antibiotics in a humidified atmosphere of 5% CO_2 in air at 37°C.

Cell culture and cell viability

The SNU449, SNU475, and SNU368 cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in 5% CO_2 overnight, and then the cells were seeded into 96-well plates (3×10^5 cells per well). After 24 h, the culture medium was replaced with a medium containing various doses of Fd Cyd (0, 0.5, 1, 2.5, 5, and 10 μM) and sodium butyrate (0, 1, 2.5, 5, 10, and 25 μM), the control groups were exposed to an equivalent volume of solvents. After 24 of treatment, the treated and untreated cells were investigated by MTT assay according to Standard protocols to determine cell viability, the MTT assay was achieved as we described previously (20, 21). The investigators did not combine treatment in the current study.

Cell apoptosis assay

To determine SNU449, SNU475, and SNU368 cell apoptosis, the cells were cultured at a density of 3×10^5 cells/well and treated with compounds (FdCyd and sodium butyrate), based on IC 50 values indicated in table Ⅰ, for 24 h, the control groups were exposed to an equivalent volume of solvents. Then, the SNU449, SNU475, and SNU368 cells were harvested by trypsinization, washed with cold PBS, and resuspended in Binding buffer (1x). Finally, 5 μL of Annexin V-FITC solution and 10 μL of PI solution were used according to the protocol, the cells were incubated for 15 minutes at room temperature in the dark and measured with a Becton Dickinson FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). Each expriment was performed in triplicate.

Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

To determine the relative expression level of the BAX, BAK, APAF1, Bcl-2, Bcl-xL), p21, and p53 gene qRT-PCR were done. The SNU449, SNU475, and SNU368 cells (at a density of 3×10^5 cells/well) were treated with FdCyd and sodium butyrate, based on IC 50 value, for 24 h, the control groups were exposed to an equivalent volume of solvents. Then qRT-PCR was done as previously reported works (22, 23). The primer sequences are shown in Table Ⅱ (24-31). The primers of the selected genes were obtained from Previously published articles and the specificity of the primers is carried out using A Basic Local Alignment Search Tool (BLAST) to avoid secondary binding sites. The result showed that the selected primers specifically recognize the related genes.

Ethical consideration

This work is a lab trial study that was approved by the Ethics Committee of Jahrom University of Medical science with a code number of IR. JUMS.REC. 1399.120.

Results

Result of cell viability by the MTT assay

The cell viability of the SNU449, SNU475, and SNU368 cells treated with various doses of FdCyd (0, 0.5, 1, 2.5, 5, and 10 μM) and sodium butyrate (0, 1, 2.5, 5, 10, and 25 μM) was investigated by MTT assay. As shown in figure 1, FdCyd and sodium butyrate induced significant cell growth inhibition ($P< 0.001$). The IC50 value was calculated by Graph pad prism 8 as indicated in Table I.

Result of determination of cell apoptosis

To determine cell apoptosis, the cells were treated with the compounds (as mentioned in the materials and method section) and then stained using annexin-V-(FITC) and PI to determine apoptotic cells in the early and late apoptosis stages. As indicated in figures 2-4, both compounds induced cell apoptosis significantly ($P<0.001$).

Result of determination of genes expression in FdCyd **treated cell lines**

- SNU449, SNU475, and SNU368 cell lines

The effect of FdCyd on BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, and p53 gene expression was evaluated by quantitative real-time RT-PCR analysis. The result demonstrated that this compound up-regulated the BAX, BAK, APAF1, p21, and p53 and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in all three cell lines, SNU449, SNU475, and SNU368, as indicated in figure 5. As indicated in this Figure, this compound up-regulated the BAX, BAK, APAF1, p21, and p53 and down-regulated Bcl-2, and Bcl-xL gene expression significantly after 24 h of treatment in Hep3B, and SMMC-7721 cell lines. Additionally, this compound up-regulated the BAX, BAK, and APAF1, p21, and p27 and down-regulated Bcl-2, and Bcl-

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xL significantly after 24 h of treatment in all three cell lines, SNU449, SNU475, and SNU368, as indicated in figure 5.

Result of determination of genes expression in sodium butyrate-treated cell lines

- SNU449 cell line

The effect of sodium butyrate on BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, and p53 gene expression was evaluated by quantitative real-time RT-PCR analysis. The result demonstrated that this compound up-regulated the BAX, BAK, APAF1, p21, and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in the SNU449 cell line as indicated in figure 6. It had no significant effect on p53 expression.

- SNU475, and SNU368 cell lines

The effect of sodium butyrate on BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, and p53 gene expression was evaluated by quantitative real-time RT-PCR analysis. The result demonstrated that this compound up-regulated the BAX, BAK, APAF1, p21, and p53 and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in SNU475, and SNU368 cell lines as indicated in figure 6. The result demonstrated that this compound up-regulated the BAX, BAK, APAF1, p21, and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in the SNU449 cell line as indicated in figure 4. It had no significant effect on p53 expression. Besides, this agent up-regulated the BAX, BAK, APAF1, p21, and p53 and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in the SNU475, and SNU368 cell lines.

Table I: IC50 values of FdCyd and Sodium butyrate. µM: micromole; IC50: half maximal inhibitory concentration

Cell line	Drug/ µM	Duration/Hour	IC50	LogIC50	R squared
SNU449	FdCyd	24	2.398	0.3798	0.7753
SNU475	FdCyd	24	2.890	0.4609	0.7291
SNU368	FdCyd	24	2.043	0.3104	0.7680
SNU449	Sodium butyrate	24	6.976	0.8436	0.9553
SNU475	Sodium butyrate	24	4.175	0.6207	0.9388
SNU368	Sodium butyrate	24	4.550	0.6580	0.9780

Table II: The Primer Sequences of BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, p53, and GAPDH.

Primer	Primer sequences (5' to 3')	Product length	Reference
BAX **Forward** **Reverse**	 AGTAACATGGAGCTGCAGAGGAT GCTGCCACTCGGAAAAAGAC	77 bp	24
BAK **Forward** **Reverse**	CCTGCCCTCTGCTTCTGA CTGCTGATGGCGGTAAAAA	82 bp	25
APAF1 **Forward** **Reverse**	 TGCGCTGCTCTGCCTTCT CCATGGGTAGCAGCTCCTTCT	142 bp	26
Bcl-2 **Forward** **Reverse**	 TGGCCAGGGTCAGAGTTAAA TGGCCTCTCTTGCGGAGTA	147 bp	27
Bcl-xL **Forward** **Reverse**	 TCCTTGTCTACGCTTTCCACG GGTCGCATTGTGGCCTTT	62 bp	28
p21 **Forward** **Reverse**	 CTGGAGACTCTCAGGGTCGAA GGATTAGGGCTTCCTCTTGGA	197 bp	29
p53 **Forward** **Reverse**	 ATGTTTTGCCAACTGGCCAAG TGAGCAGCGCTCATGGTG	153 bp	30
GAPDH **Forward** **Reverse**	 TGTTGCCATCAATGACCCCTT CTCCACGACGTACTCAGCG	148 bp	31

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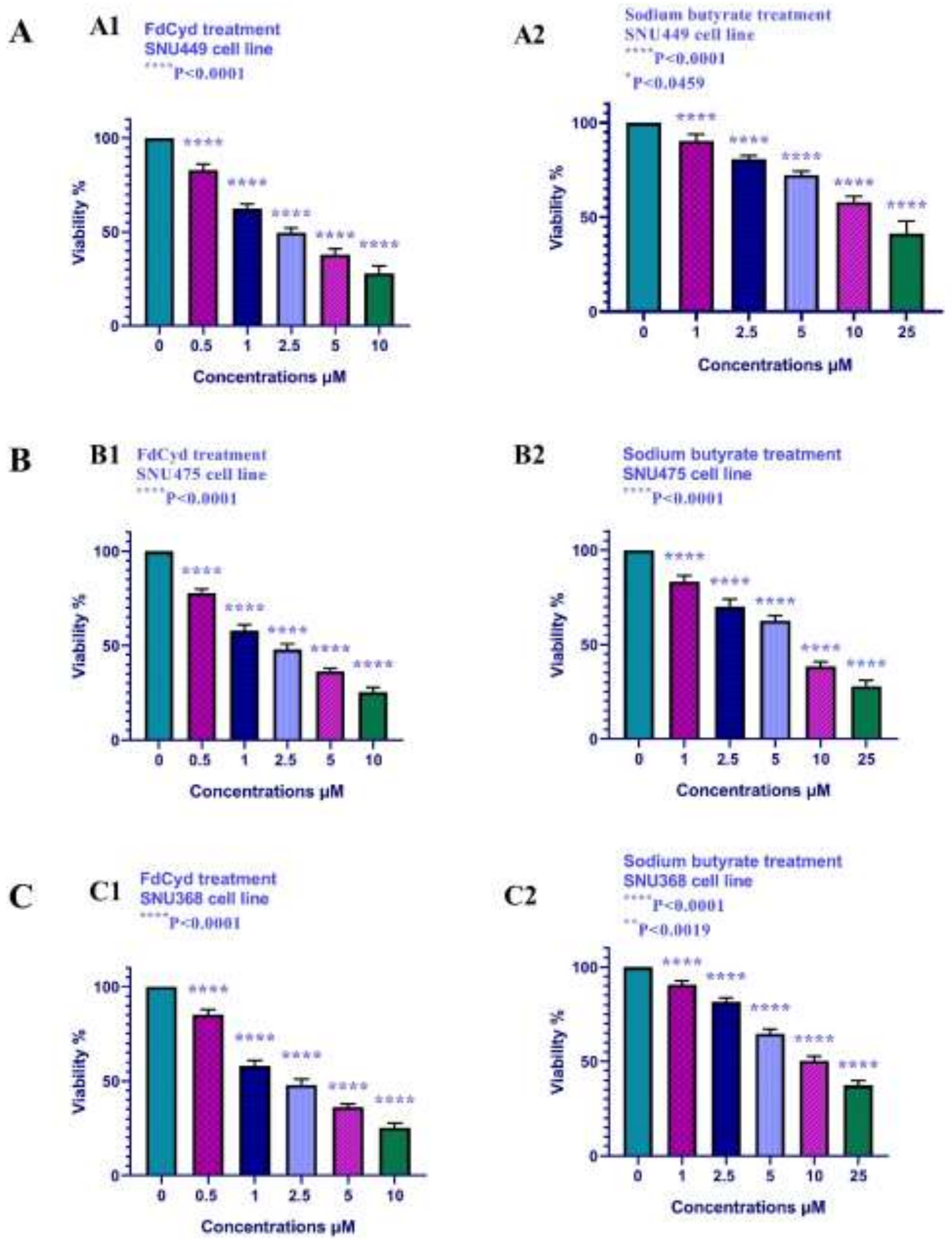

Figure1. In vitro effects of FdCyd (0, 0.5, 1, 2.5, 5, and 10 µM) and sodium butyrate (0, 1, 2.5, 5, 10, and 25 µM) on SNU449, SNU475, and SNU368 cell viability determined by MTT Assay at 24 h. Both compounds inhibited the growth of all three cell lines significantly in a dose-dependent manner.

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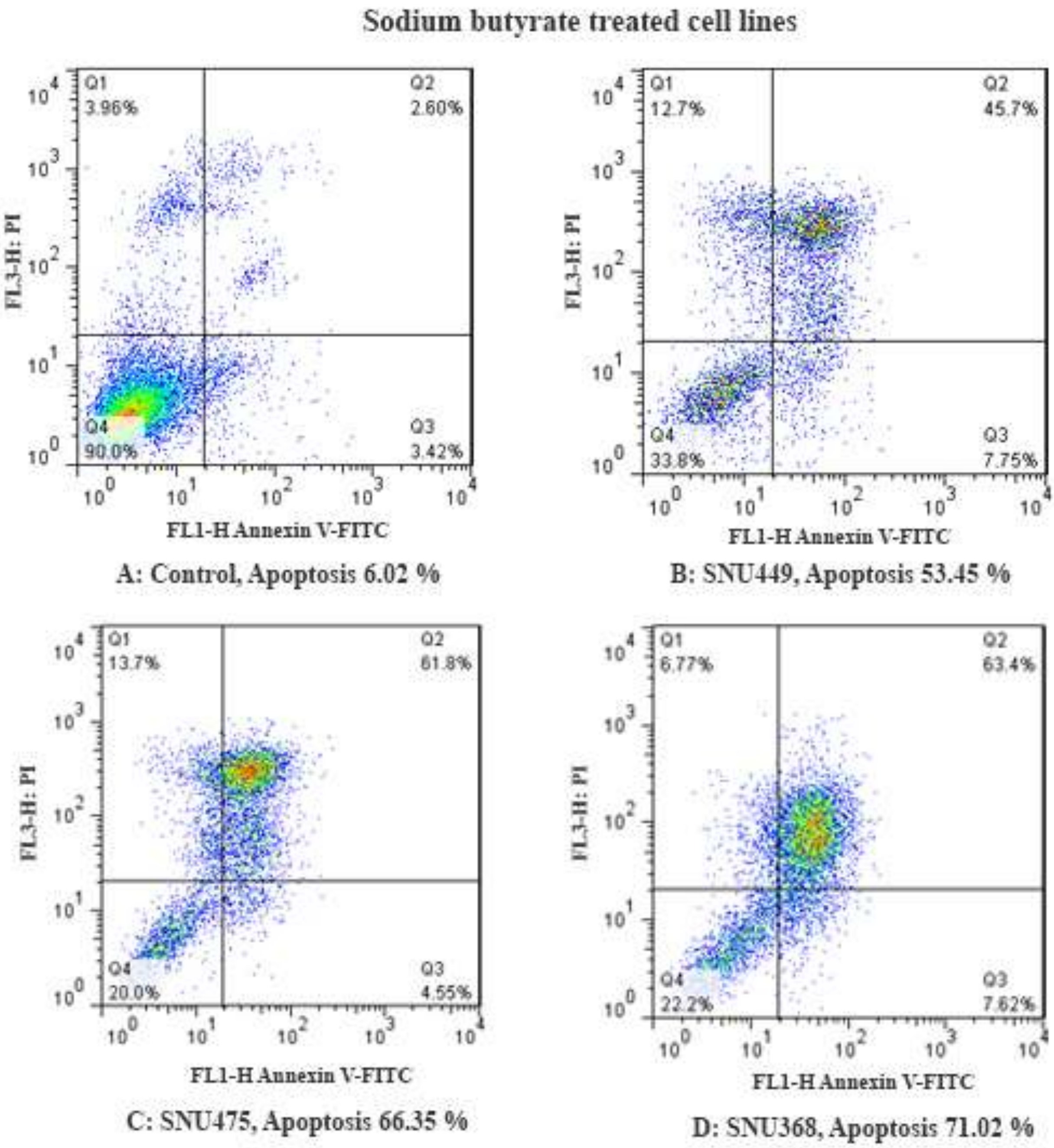

Figure 2. The apoptotic effect of sodium butyrate on SNU449, SNU475, and SNU368 cells versus control groups at 24 h. The sodium butyrate induced significant apoptosis. The results were obtained from three independent experiments. Maximal apoptosis was seen in the Hep3B cell line after 24 h.

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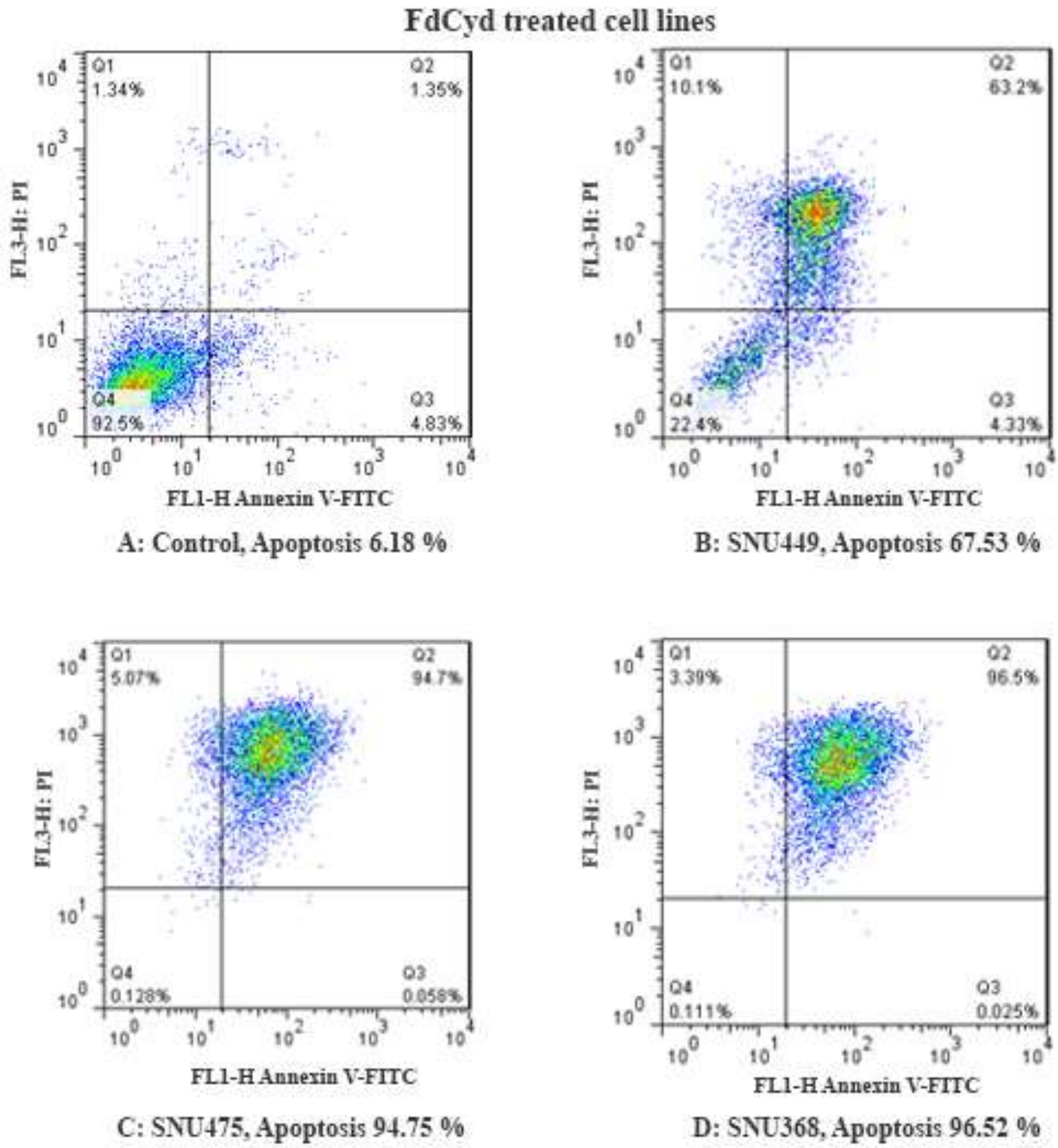

Figure 3. The apoptotic effect of FdCyd on SNU449, SNU475, and SNU368 cells versus control groups at 24 h. The FdCyd induced significant apoptosis. The results were obtained from three independent experiments. Maximal apoptosis was seen in the Hep3B cell line after 24 h.

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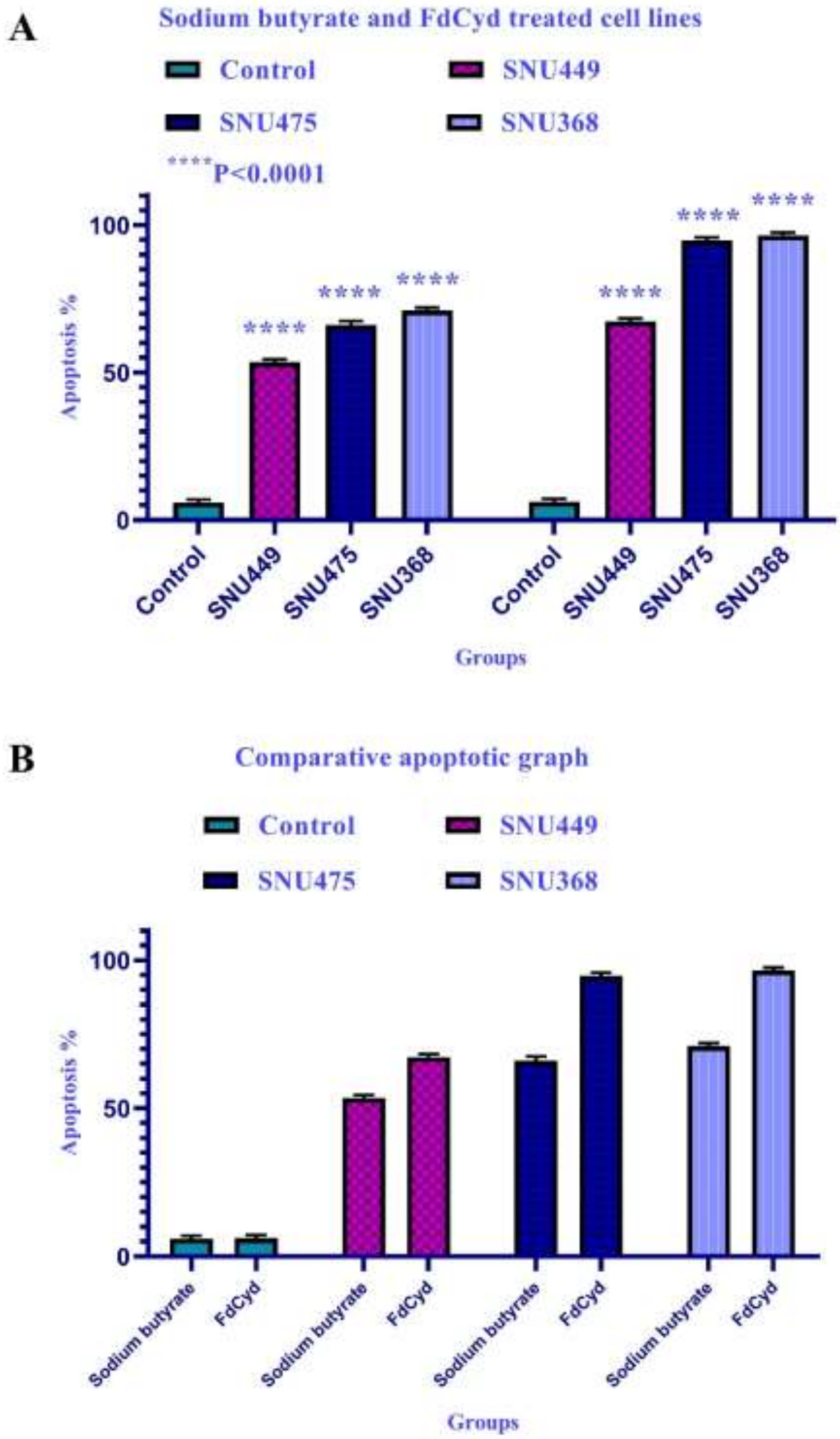

Figure 4. The comparative apoptotic effects of sodium butyrate in comparison to FdCyd on SNU449, SNU475, and SNU368 cells (A-C). Asterisks (*) indicate significant differences between the treated and untreated control groups. As demonstrated above, FdCyd had a more significant apoptotic effect in comparison to sodium butyrate.

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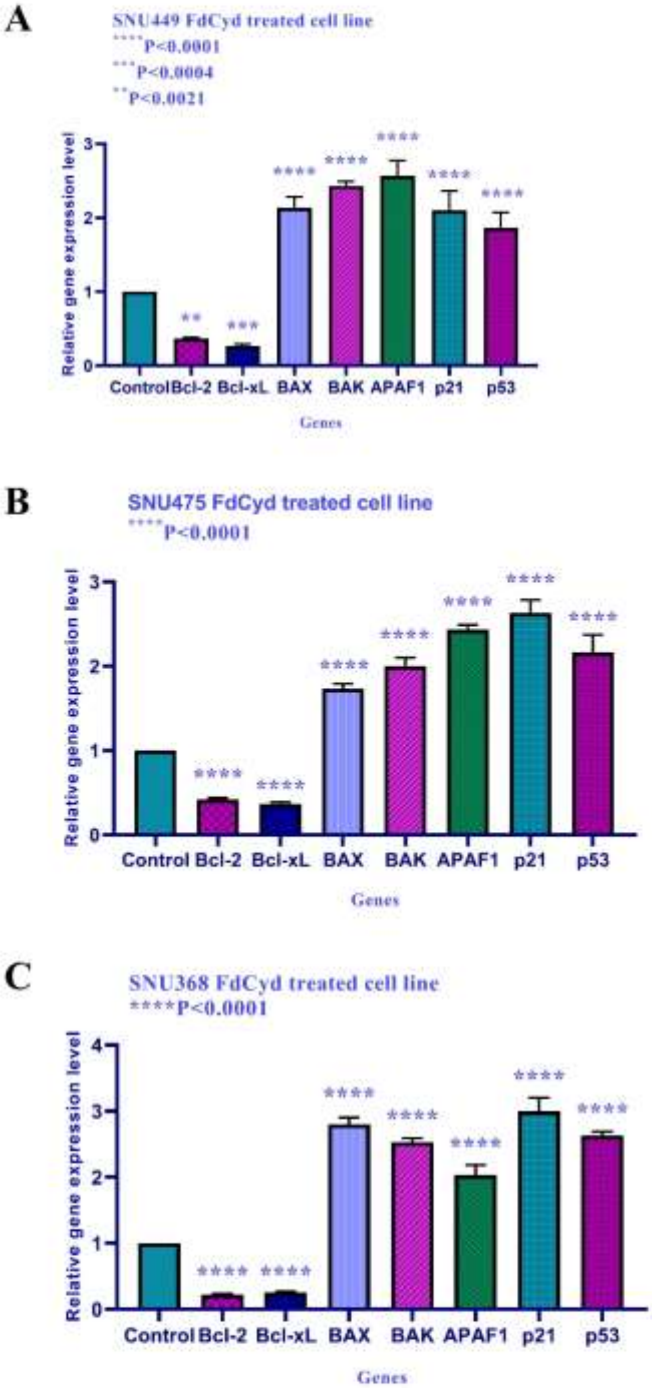

Figure 5. The relative expression level of BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, and p53 in the SNU449, SNU475, and SNU368 cell line treated with FdCyd versus untreated control groups at 24 h.

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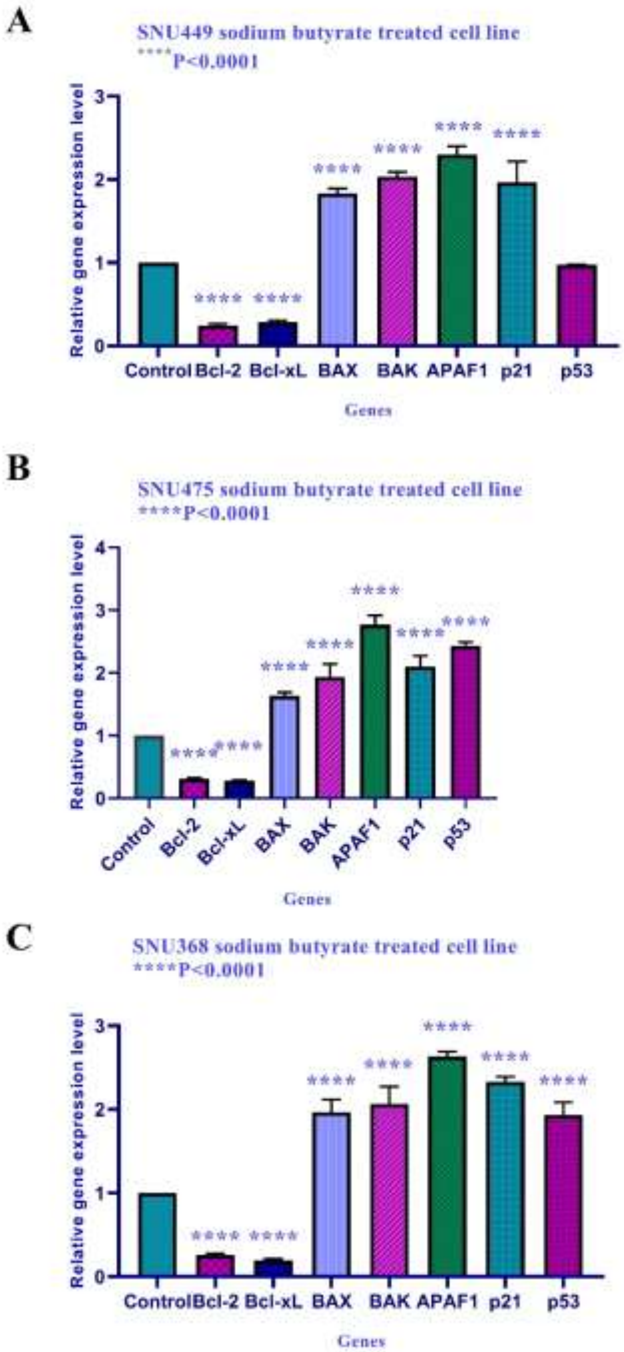

Figure 6. The relative expression level of BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, and p53 in the SNU449, SNU475, and SNU368 cell line treated with sodium butyrate versus untreated control groups at 24 h.

Discussion

DNMTIs and HDACIs have been applied to the treatment of solid and hematological malignancies. Treatment with these compounds leads to apoptosis induction. Apoptosis, programmed cell death, shares common cell death machinery, comprising extrinsic/death receptor and

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mitochondrial/intrinsic dependent pathways. It can be initiated by many different pathological and physiological stimuli (32). DNMTIs and HDACIs can induce apoptosis through various mechanisms such as intrinsic and extrinsic pathways (33, 34), and also the reactivation of cyclin-dependent kinase inhibitors reactivation (35, 36). Recent findings indicated that FdCyd and sodium butyrate can induce apoptosis in human hepatocellular carcinoma SNU449, SNU475, and SNU368 cell lines via the intrinsic apoptotic pathway, the up-regulation of BAX, BAK, APAF1, and down-regulation of Bcl-2, and Bcl-xL gene expression. Besides, they reactivated cyclin-dependent kinase inhibitor p21 gene expression. Both compounds induced significant apoptosis in all three cell lines, whereas sodium butyrate could not induce significant up-regulation of p53 in the SNU449 cell line. Further, minimal cell apoptosis was observed in the SNU449 cell line treated with sodium butyrate.

Similarly, it has been shown that DNMTIs and HDACIs induce apoptosis through the intrinsic apoptotic pathway (37, 38). This pathway is one of the major apoptosis pathways in mammalian cells. It is defined by the release of mitochondrial cytochrome c into the cytosol where it binds to Apaf-1 and then assembles into an oligomeric apoptosome complex. The complex recruits caspase-9, caspase-9 activates effector caspases (e.g. caspases-3 and -7), which cleave various cellular proteins, leading to cell death. It has been shown that DNA methyltransferase inhibitor zebularine induces apoptosis by a mitochondrial-mediated pathway in gastric cancer, Bax upregulation, and Bcl-2 down-regulation (39). A similar result has been reported in AML, HL60 and KG1 cells treated with 5-aza-2′ deoxycytidine (decitabine) (40, 41).

As reported in this work, it has been shown that sodium butyrate induces cell cycle arrest associated with mitochondria-mediated apoptosis accompanied by a decrease in Bcl-2 expression in colon carcinoma HCT116 and SW480 cells (42). Another study has demonstrated that sodium butyrate induces the loss of mitochondrial membrane potential, release of cytochrome c, activation of caspase 9 and caspase 3, and apoptosis induction in human hepatoma HuH-6 and HepG2 cells (43). Several studies have shown that sodium butyrate treatment increases the up-regulation in pro-apoptotic Bax expression, and down-regulation of anti-apoptotic Bcl-2 and Bcl-XL in U937 human leukemic cells (44).

Furthermore, it was found that FdCyd and sodium butyrate up-regulated p21 gene expression significantly in SNU449, SNU475, and SNU368 cell lines. Consistent with this, it has been indicated that sodium butyrate and trichostatin A increase the expression levels of p21, Bad, and decreases the expression of Bcl-2, Bcl-xL, Bax human glioma T98G, U251MG, and U87MG cells (45). Further evaluation demonstrated that FdCyd increased cyclin-dependent kinase inhibitor p21 in all three cell lines. Inconsistent with the current study, other researchers have shown that DNMTIs can up-regulate p21 gene expression in pancreatic cancer cell line CFPAC-1 cells (46).

As demonstrated in the current study, several experimental works indicated that DNMTIS and HDACIs induce P53-dependent and -independent up-regulation of p21 gene expression in AML cells (47, 48). Finally, the mentioned apoptotic pathways are not the only molecular mechanism of the DNMTIS and HDACIs. The extrinsic/death receptor apoptotic pathway is another mechanism of these compounds (49, 50). This mechanism has been not evaluated in this work. Therefore, this assessment is recommended.

Conclusion

In conclusion, the result of this study indicated that 5'-fluoro-2'-deoxycytidine and sodium butyrate can induce their apoptotic effects through extrinsic

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apoptotic pathways in hepatocellular carcinoma SNU449, SNU475, and SNU368 cell lines in a p53-dependent and –independent manner.

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Conflict of interest

The authors declare no conflict of interest.

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