Original Article

Effect of 5'-Fluoro-2'-Deoxycytidine and Sodium Butyrate on the Gene Expression of the Intrinsic Apoptotic Pathway, p21, p27, and p53 Genes Expression, Cell Viability, and Apoptosis in Human Hepatocellular Carcinoma Cell Lines

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Abstract

Background: Epigenetic mechanisms play an important role in the regulation of gene expression and genetic information. DNA methyltransferases are a family of enzymes that methylate DNA at the promoter region of the gene which can significantly contribute to gene silencing and carcinogenesis. In addition, histone deacetylation leads to gene silencing and tumorigenesis. Our previous work indicated that histone deacetylase (HDAC) inhibitors can induce its apoptotic role through down-regulation of HDACs. This study aimed to investigate the effect of 5'-fluoro-2'-deoxycytidine (FdCyd) and sodium butyrate on the genes of intrinsic apoptotic pathway (BAX, BAK and APAF1, Bcl-2, and Bcl-xL), p21, p27, and p53 gene expression, cell viability, and apoptosis in human hepatocellular carcinoma Hep3B, SMMC-7721, and HA22T/VGH cell lines.

Materials and Methods: The Hep3B, SMMC-7721, and HA22T/VGH cells were cultured and treated with FdCyd and sodium butyrate. To determine cell viability, cell apoptosis, and the relative gene expression level, MTT assay, flow cytometry assay, and quantitative real-time polymerase chain reaction were done, respectively.

Results: Both compounds induced significant cell growth inhibition and cell apoptosis significantly ($P < 0.0001$). Sodium butyrate up-regulated the BAX, BAK, APAF1, p21, p27, and p53 and down-regulated Bcl-2, and Bcl-xL significantly in all three cell lines. Similar results were observed in the Hep3B, and SMMC-7721 cell lines treated with FdCyd. It has no significant effect on p53 gene expression in HA22T/VGH. The expression of the other genes in this cell line was similar to other cell lines.

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

Keywords: Acetylation, carcinoma, hepatocellular, methylation

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Introduction

Epigenetic mechanisms play an important role in the regulation of gene expression and genetic information. Depending on the epigenetic modification pattern, a gene can be silenced and expressed. These mechanisms include epigenetic modifications of DNA (such as methylation) and histones (e.g., histone modification) that are stable and reversible. DNA methyltransferases (DNMTs)

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are a family of enzymes that methylate DNA at the promoter region of the gene, which can significantly contribute to gene silencing and carcinogenesis.[1] The progressive CpG island hypermethylation of tumor suppressor genes (TSGs) leads to carcinogenesis.[2] Re-expression of silenced TSGs and restoration of their normal function can be achieved through the use of DNMT inhibitors (DNMTIs)[3] which are divided into three groups: (a) nucleoside inhibitors (e. g. 5-azacytidine [azacitidine, 5AC]); (b) nonnucleoside inhibitors (such as epigallocatechin-3-gallate [EGCG]); and (c) rationally designed inhibitors.[4-6] DNMTIs can play their apoptotic roles through various mechanisms. Previously, we demonstrated that DNMTI 5-Aza-CdR can induce apoptosis through down-regulation of DNMT1, DNMT3a, DNMT3b gene expression in hepatocellular carcinoma (HCC) LCL-PI 11 cell line.[7] Besides, we reported that DNMTI zebularine can induce apoptosis through DNMT1, DNMT3a, and DNMT3b down-regulation and up-regulation of p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2 gene expression in colon cancer LS 174T,[8] and LS 180 cell lines.[9] Further, we reported that DNMTI 5-Aza-CdR can induce apoptosis through up-regulation of p15INK4, p16INK4, p18INK4, and p19INK4 genes in the HCC PLC/PRF/5 cell line.[10] Several studies have demonstrated that DNMTIs induce apoptosis via mitochondrial/intrinsic apoptotic pathway, proapoptotic genes (such as Bax) up-regulation, and antiapoptotic genes (e.g., Bcl-2) down-regulation.[11] This effect could be p53-dependent and independent pathways.[12,13] As mentioned, histone deacetylation leads to gene silencing and tumorigenesis. Histone acetylation and deacetylation are controlled by the action of two groups of enzymes including histone acetyltransferases and histone deacetylase (HDACs), respectively.

HDAC inhibitors (HDACIs) are novel anticancer agents that induce cell apoptosis, cell differentiation, and cell cycle arrest.[14] These compounds can be divided into different structural groups, including hydroxamic acids (such as vorinostat, and trichostatin A [TSA]), benzamides, cyclic peptides, and short-chain fatty acids (e.g., valproic acid [VPA] and sodium butyrate).[14] Our previous work indicated that HDACI TSA can induce can induce its apoptotic role through up-regulation of the p16INK4a, p14ARF, p15INK4b genes in Colon Cancer Caco-2 Cell Line.[15] In ddition, we demonstrated that HDACI VPA can downregulate Bcl-2, Bcl-xL, and Mcl-1 and upregulate p21, p53, Bax, Bak, and Bim resulting in apoptosis induction HCC HepG2 cell line.[16] Further, we reported that HDACI VPA induces apoptosis via up-regulation of CIP/KIP family (p21, p27, and p57) genes expression in colon cancer SW480 cell line.[17] Several studies have been shown that HDACIs play their apoptosis role through extrinsic (death receptor upregulation) and intrinsic (BH3-only Bcl-2 family up-regulation) pathways. Additionally, the activation of the intrinsic apoptotic pathway is the predominant molecular mechanism and the apoptotic pathway of HDACIs.[18] This study aimed to investigate the effect of 5'-fluoro-2'-deoxycytidine (FdCyd) and sodium butyrate on the genes of intrinsic apoptotic pathway (BAX, BAK and APAF1, Bcl-2, and Bcl-xL), p21, p27, and p53 gene expression, cell viability, and apoptosis in human HCC Hep3B, SMMC-7721, and HA22T/VGH cell lines. In fact, we decided to determine whether the FdCyd and sodium butyrate would reactivate TSGs silenced by methylation and deacetylation.

Materials and Methods

Materials

Human HCC Hep3B, SMMC-7721, and HA22T/VGH cell lines were purchased from the National Cell Bank of Iran-Pasteur Institute. The FdCyd, sodium butyrate, and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma (St. Louis, MO, USA). The compounds, FdCyd and sodium butyrate, were dissolved in dimethyl sulfoxide (DMSO) and sterile water, respectively to make a work stock solution. Further concentrations of these agents were obtained by diluting the provided stock solution. Other necessary materials and kits were purchased as provided for our previous works.[19,20] The Hep3B, SMMC-7721, and HA22T/VGH cells were maintained in DMEM supplemented with fetal bovine serum 10% and antibiotics in a humidified atmosphere of 5% CO2 in air at 37°C. This work is a lab trial study approved by the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC. 1399.122.

Cell culture and cell viability

The Hep3B, SMMC-7721, and HA22T/VGH cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in 5% CO2 overnight, and then the cells seeded into 96-well plates (3×10^5 cells per well). After 24 h, the culture medium was replaced with a medium containing various concentrations of FdCyd (0, 1, 2.5, 5, 10, and 25 μM), and sodium butyrate (0, 1, 2.5, 5, 10, 25, and 50 μM), the control groups were exposed to an equivalent volume of solvents. After 24 h 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.[21,22]

Cell apoptosis assay

To determine Hep3B, SMMC-7721, and HA22T/VGH cell apoptosis, the cells were cultured at a density of 3×10^5 cells/well and treated with FdCyd and sodium butyrate, based on IC 50 values indicated in Table 1, for 24 h, the control groups were exposed to an equivalent volume of solvents. Then, the Hep3B, SMMC-7721, and HA22T/VGH 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 min at room temperature in the dark and measured with a Becton Dickinson FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).[23] Annexin V-FITC allows the direct evaluation of early apoptotic cells, and PI distinguishes membrane permeabilized and late apoptotic cells.

Each experiment was performed in triplicate.

Real-time quantitative reverse transcription-polymerase Chain reaction (quantitative real-time polymerase chain reaction)

To determine the relative expression level of the BAX, BAK, and APAF1, Bcl-2, and Bcl-xL, p21, p27, and p53 gene quantitative real-time polymerase chain reaction (qRT-PCR) was done. The Hep3B, SMMC-7721, and HA22T/VGH cells (at a density of 3×10^5 cells/well) were treated with FdCyd and sodium butyrate, based on IC 50 values, for 24 h, the control groups were exposed to an equivalent volume of solvents. Then qRT-PCR was done as our previous works.[24,25] Total RNA (100 ng) was reverse transcribed to cDNA by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Real-time RT-PCR was performed by the Maxima SYBR Green RoxqPCR master mix kit (Fermentas). Real-time PCR reactions were performed using the Steponeplus (Applied Biosystem). Thermal cycling conditions were: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 15 seconds, and extension at 72°C for 15 s. Data were analyzed using the comparative Ct (ΔΔct) method. A melting curve was used to determine the melting temperature of specific amplification products and primer dimmers. The primer sequences are shown in Table 2.[26-34]

Table 1: IC50 values of FdCyd and sodium butyrate

Cell line	Drug/μM	Duration/h	IC50	Log IC50	R^2
Hep3B	FdCyd	24	1.867	0.2711	0.9239
SMMC-7721	FdCyd	24	1.920	0.2833	0.9633
HA22T/VGH	FdCyd	24	3.856	0.5861	0.9427
Hep3B	Sodium butyrate	24	5.423	0.7342	0.9688
SMMC-7721	Sodium butyrate	24	4.134	0.6163	0.9505
HA22T/VGH	Sodium butyrate	24	4.050	0.6074	0.9373

These values were obtained after 24 h of treatment, the treated and untreated glioblastoma and neuroblastoma cells were investigated by MTT assay and the data were analyzed by Graph Pad Prism 8.0.

Statistical analysis

The database was set up with the SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA) and Graph Pad Prism 8.0 for data analysis. Results are expressed as mean ±standard deviation (SD) for n = 3 independent experiments. Statistical comparisons between groups were performed with ANOVA (one-way ANOVA). A significant difference was considered as $P < 0.05$.

Results

Cell viability

The cell viability of the Hep3B, SMMC-7721, and HA22T/VGH cells treated with various doses of FdCyd (0, 1, 2.5, 5,

Table 2: The primer sequences of BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, p27, p53, and GAPDH

Primer	Primer sequences (5' to 3')	Product length (bp)	References
BAX		77	[26]
Forward	AGTAACATGGAGCTGCAGAGGAT		
Reverse	GCTGCCACTCGGAAAAAGAC		
BAK		82	[27]
Forward	CCTGCCCTCTGCTTCTGA		
Reverse	CTGCTGATGGCGGTAAAAA		
APAF1		142	[28]
Forward	TGCGCTGCTCTGCCTTCT		
Reverse	CCATGGGTAGCAGCTCCTTCT		
Bcl-2		147	[29]
Forward	TGGCCAGGGTCAGAGTTAAA		
Reverse	TGGCCTCTCTTGCGGAGTA		
Bcl-xL		62	[30]
Forward	TCCTTGTCTACGCTTTCCACG		
Reverse	GGTCGCATTGTGGCCTTT		
p21		197	[31]
Forward	CTGGAGACTCTCAGGGTCGAA		
Reverse	GGATTAGGGCTTCCTCTTGGA		
P27		284	[32]
Forward	CAGGTCTCCAAGACGACATAGA		
Reverse	CGCCTTTTCGATTCATGTACTGC		
p53		153	[33]
Forward	ATGTTTTGCCAACTGGCCAAG		
Reverse	TGAGCAGCGCTCATGGTG		
GAPDH		148	[34]
Forward	TGTTGCCATCAATGACCCCTT		
Reverse	CTCCACGACGTACTCAGCG		

These primer sequences were obtained from previously published articles addressed in this table[26-34]

10, and 25 μM), and sodium butyrate (0, 1, 2.5, 5, 10, 25, and 50 μM) was investigated by MTT assay. As shown in Figure 1, FdCyd and sodium butyrate induced significant cell growth inhibition ($P < 0.0001$). The IC50 value was calculated by Graph pad prism 8, as indicated in Table 1.

Cell apoptosis

To determine cell apoptosis, the Hep3B, SMMC-7721, and HA22T/VGH cells were treated with FdCyd and sodium butyrate, based on IC 50 values, for 24 h and then stained using annexin-V-(FITC) and PI to determine apoptotic cells in early and late apoptosis stage. As indicated in Figures 2-4, both compounds induced cell apoptosis significantly ($P < 0.0001$).

Genes expression in FdCyd treated cell lines

Hep3B, and SMMC-7721 cell lines

The effect of FdCyd on BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, p27, and p53 gene expression was evaluated by quantitative real-time RT-PCR analysis. The result demonstrated that this compound up-regulated the BAX, BAK,

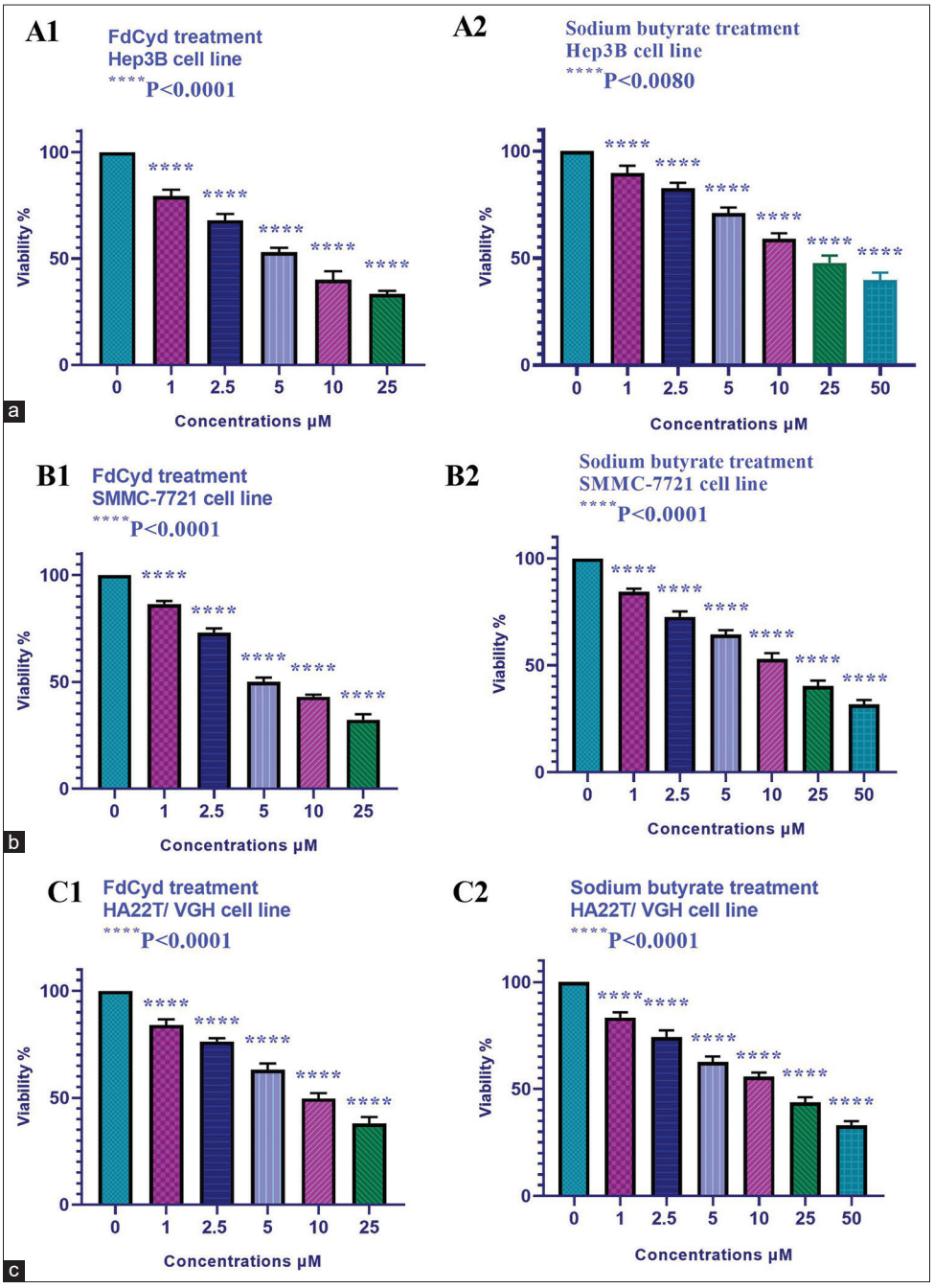

Figure 1: *In vitro* effects of 5'-fluoro-2'-deoxycytidine (0, 1, 2.5, 5, 10, and 25 μM), and sodium butyrate (0, 1, 2.5, 5, 10, 25, and 50 μM) on Hep3B (a), SMMC-7721 (b), and HA22T/VGH (c) cell viability determined by MTT Assay at 24 h. Both compounds inhibited the growth of all three cell lines significantly in a dose-dependent manner

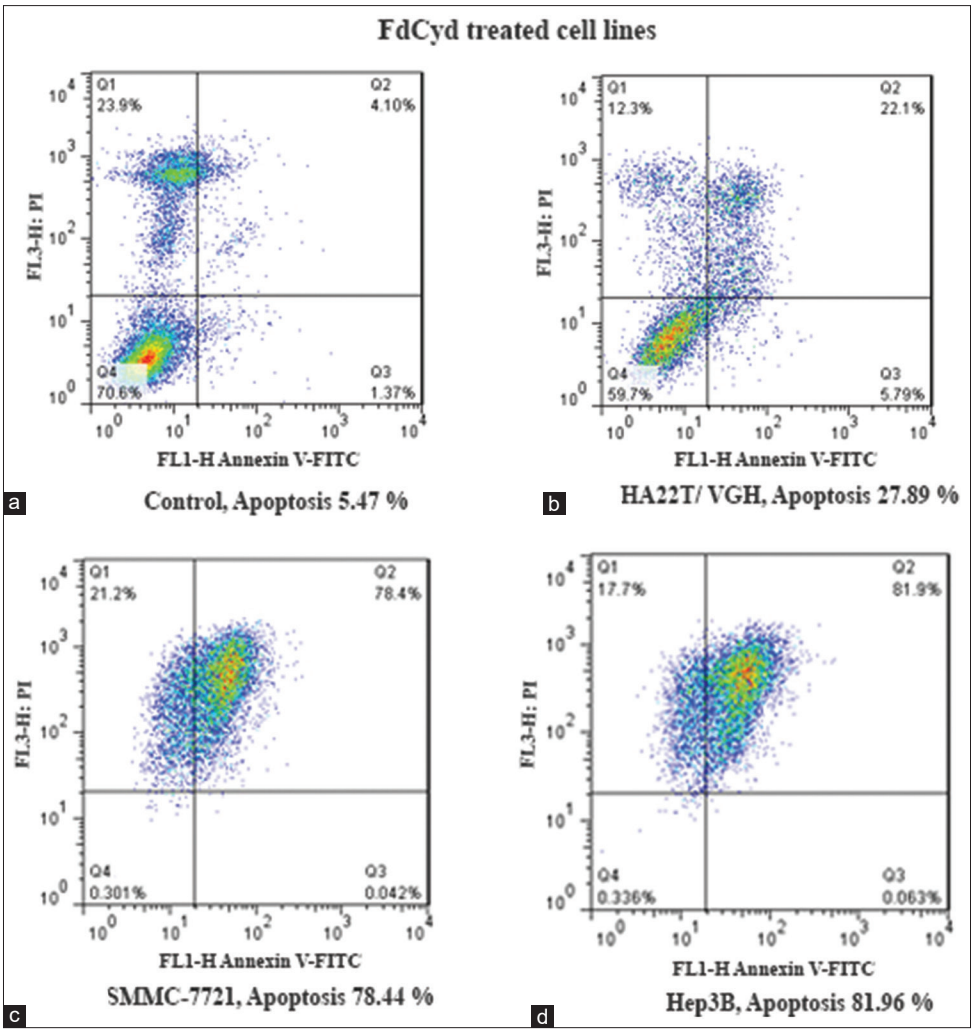

Figure 2: The apoptotic effect of 5' fluoro 2' deoxycytidine on treated cell lines versus control groups (a) at 24 h. Treated cell lines include HA22T/VGH (b), SMMC 7721 (c), and Hep3B (d). The 5'-fluoro-2'-deoxycytidine induced significant apoptosis. The results were obtained from three independent experiments. Maximal apoptosis was seen in the Hep3B cell line after 24 h

APAF1, p21, p27, 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, as indicated in Figure 5. $P < 0.05$.

HA22T/VGH cell line

The effect of FdCyd on BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, p27, and p53 gene expression was evaluated by quantitative real-time RT-PCR analysis. The result demonstrated that this compound up-regulated the BAX, BAK, and APAF1, p21, and p27 and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in HA22T/VGH cell line as indicated in Figure 5. It has no significant effect on p53 gene expression $P < 0.05$.

Genes expression in sodium-butyrate treated cell lines Hep3B, SMMC-7721, and HA22T/VGH

The effect of sodium butyrate on BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, p27, 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, p27, and p53 and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in all three cell lines, Hep3B, SMMC-7721, and HA22T/VGH, as indicated in Figure 6 $P < 0.05$.

In the current study, we did not investigate the protein level of the mentioned genes because of technical limitations. Therefore, protein level evaluation is recommended.

Discussion

Recent *in vitro* studies have shown that HDACIs and DNMTIs induce different phenotypes in various tumor cells, comprising growth arrest, activation of the extrinsic/cell death receptor and/or intrinsic/mitochondrial apoptotic pathways autophagic cell death, mitotic cell death, and reactive oxygen species-induced cell death.[35,36] In addition, these compounds can induce apoptosis through the reactivation of cyclin-dependent kinase inhibitors,[37,38] such as p21, p27, and

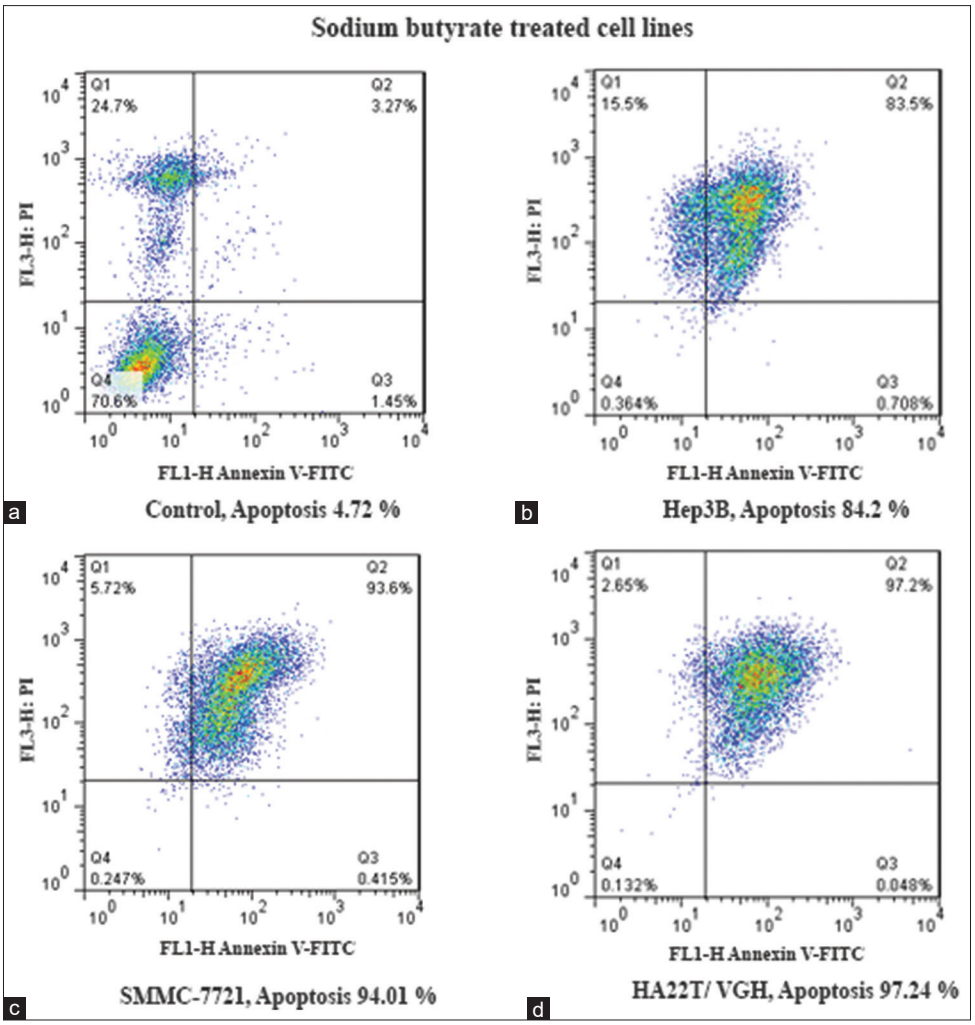

Figure 3: The apoptotic effect of sodium butyrate on treated cell lines versus control groups (a) at 24 h. Treated cell lines include Hep3B (b), SMMC 7721 (c), and HA22T/VGH (d). The sodium butyrate induced significant apoptosis. The results were obtained from three independent experiments. Maximal apoptosis was seen in HA22T/VGH cell line after 24 h

p57.[39] Our findings indicated that FdCyd and sodium butyrate can induce cell growth inhibition and apoptosis induction in Hep3B, SMMC-7721, and HA22T/VGH through intrinsic apoptotic pathway, cyclin-dependent kinase inhibitors (p21 and p27) reactivation, and p53-dependent and independent pathways. This compound up-regulated the BAX, BAK, APAF1, p21, p27, 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. Similar to our report, several studies have indicated that the mitochondrial apoptotic pathway is activated by DNMTIs such as zebularine and decitabine in leukemic T cells.[40] Similarly, it has also been demonstrated that DNMTI decitabine induces apoptosis in human leukemia cell lines U937 and HL60 which is correlated with the downregulation of anti-apoptotic Bcl-2, cIAP-1, XIAP, and cIAP-2 protein levels, the activation of caspases, and the collapse of mitochondrial membrane potential (MMP).[41] Further, DNMTIs such as EGCG reactivates cyclin-dependent kinase inhibitors such as $p16^{INK4a}$, and $p15^{INK4b}$ in colon cancer.[42] Several *in vitro* studies have shown that 5-Aza-CdR treatment up-regulates the Bax gene in the human pancreatic cancer cell line (PANC-1). This gene is the first pro-apoptotic member of the BCL^{-2} family, which acts as the heart of the intrinsic apoptosis pathway. It is inserted tightly within the outer mitochondrial membrane and is involved in promoting death during apoptosis.[43] Other researchers have reported that this agent significantly reduces MCL-1 levels in acute myeloid leukemia (AML).[44]

As we reported in this article, numerous works have indicated that sodium butyrate can induce apoptosis through mitochondrial pathway and also cyclin-dependent kinase inhibitors reactivation.

In the U937 human leukemic cell line, sodium butyrate treatment induces apoptosis by upregulation of pro-apoptotic BAX and down-regulation of anti-apoptotic Bcl-2 and Bcl-xl.[45] It decreases both Bcl-XL and Bcl-Xs expression in human hepatoma HuH-6 and HepG2 cells.[46] Furthermore, the Bcl-2 expression is decreased, and caspase-3 expression increased after sodium butyrate treatment in bladder cancer

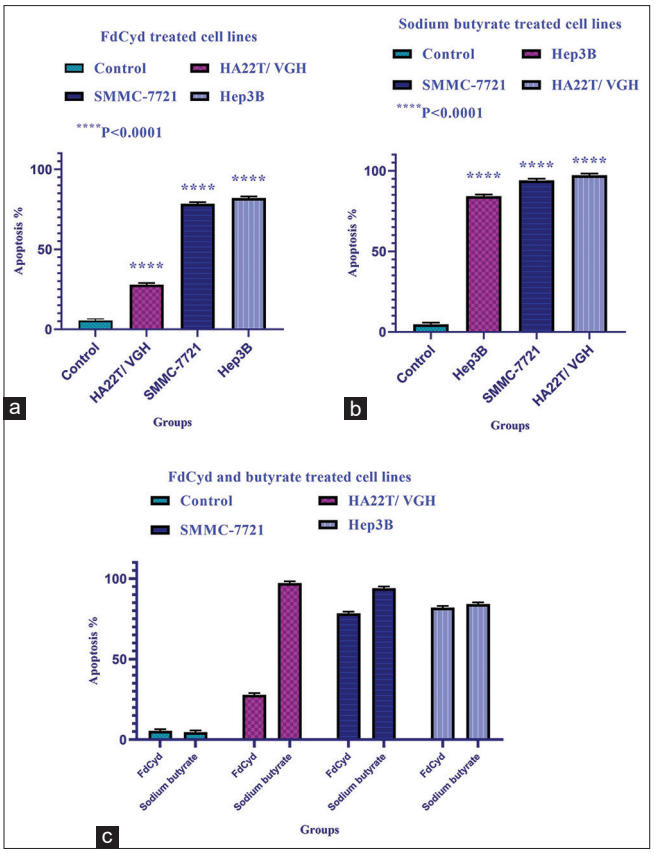

Figure 4: The effect of 5' fluoro 2' deoxycytidine (a), sodium butyrate (b) and the comparative effects of 5' fluoro 2' deoxycytidine in comparison to sodium butyrate (c) on Hep3B, SMMC 7721, and HA22T/VGH cell lines. Asterisks (*) indicate significant differences between the treated and untreated control groups. As demonstrated above, TSA had a more significant apoptotic effect in comparison to 5'-fluoro-2'-deoxycytidine

cells.[47] In addition to the mitochondrial pathway, this compound can induce apoptosis through reactivation of the cyclin-dependent kinase inhibitor such as p21WAF1 reported in bladder cancer cell lines T24, 253J, and UMUC3 cell lines.[48] In human malignant lymphoma CA46 cells, HDACI TSA plays its apoptotic role via ink4 family (e.g., p16INK4a) reactivation.[49] In the current study, we observed that FdCyd had no significant effect on p53 gene expression in HA22T/ VGH cell line, whereas both compounds, FdCyd, and sodium butyrate, induce significant apoptosis in all three cell lines, Hep3B, SMMC-7721, and HA22T/VGH. Therefore, these compounds can induce apoptosis in a p53-dependent and–independent manner. Further, minimal apoptosis was observed in HA22T/VGH. It may be concluded that the p53-dependent manner is the stronger apoptotic pathway in comparison to the p53-independent pathway. It should be noted that the mentioned molecular mechanisms are not thy only mechanisms of FdCyd and sodium butyrate. Other researchers have shown that DNMTIs and HDACIs can induce apoptosis through the extrinsic apoptotic pathway.[18,50]

We did not evaluate this pathway. Therefore, this assessment is recommended. Besides, in the current study, we did not

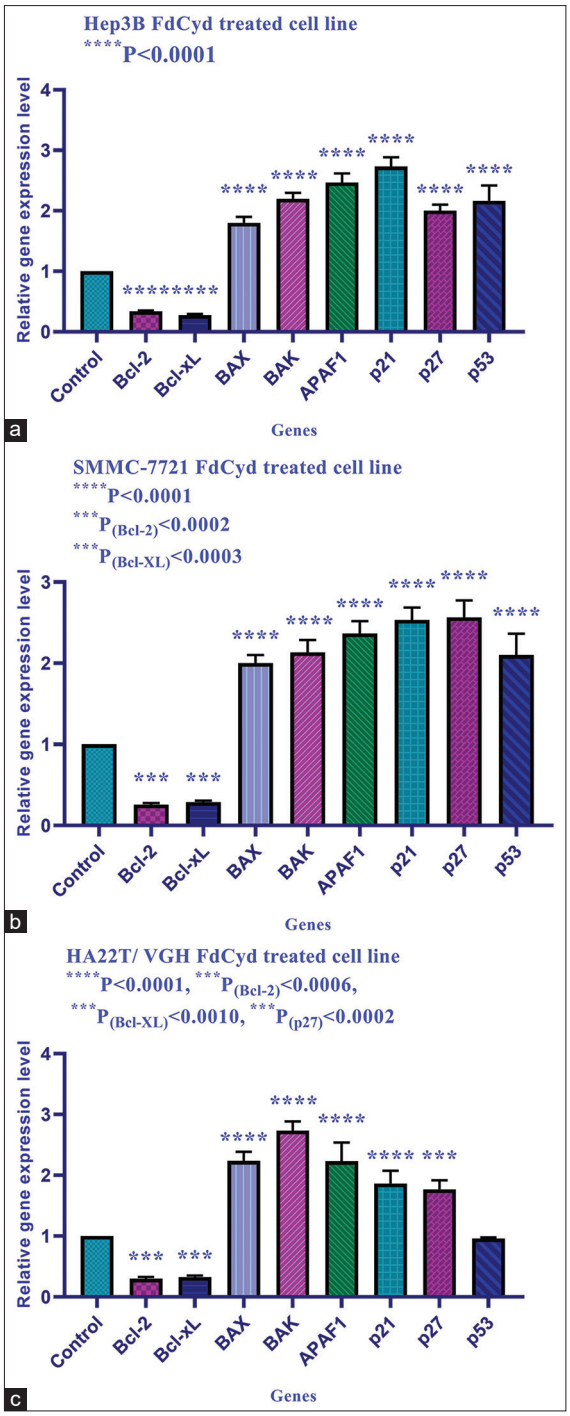

Figure 5: The relative expression level of BAX, BAK, APAF1, Bcl 2, Bcl xL, p21, p27, and p53 in the Hep3B, SMMC 7721, and HA22T/VGH cell line treated with 5' fluoro 2' deoxycytidine versus untreated control groups at 24 h. As indicated in this Figure, this compound up regulated the BAX, BAK, APAF1, p21, p27, and p53 and down regulated Bcl 2, and Bcl xL gene expression significantly after 24 h of treatment in Hep3B (a), and SMMC 7721 (b) cell lines. Additionally, this compound up regulated the BAX, BAK, and APAF1, p21, and p27 and down regulated Bcl 2, and Bcl xL significantly after 24 h of treatment in the HA22T/VGH (c) cell line, it has no significant effect on p53 gene expression. *P* values includes Part A: ****$P < 0.0001$; Part B: ****$P < 0.0001$, ***P (Bcl-2) <0.0002, *P* (Bcl-XL) <0.0003; Part C: ****$P < 0.0001$, ***P (Bcl-2) <0.0006, ***P (Bcl-XL) <0.0010, ***P (p27) <0.0002

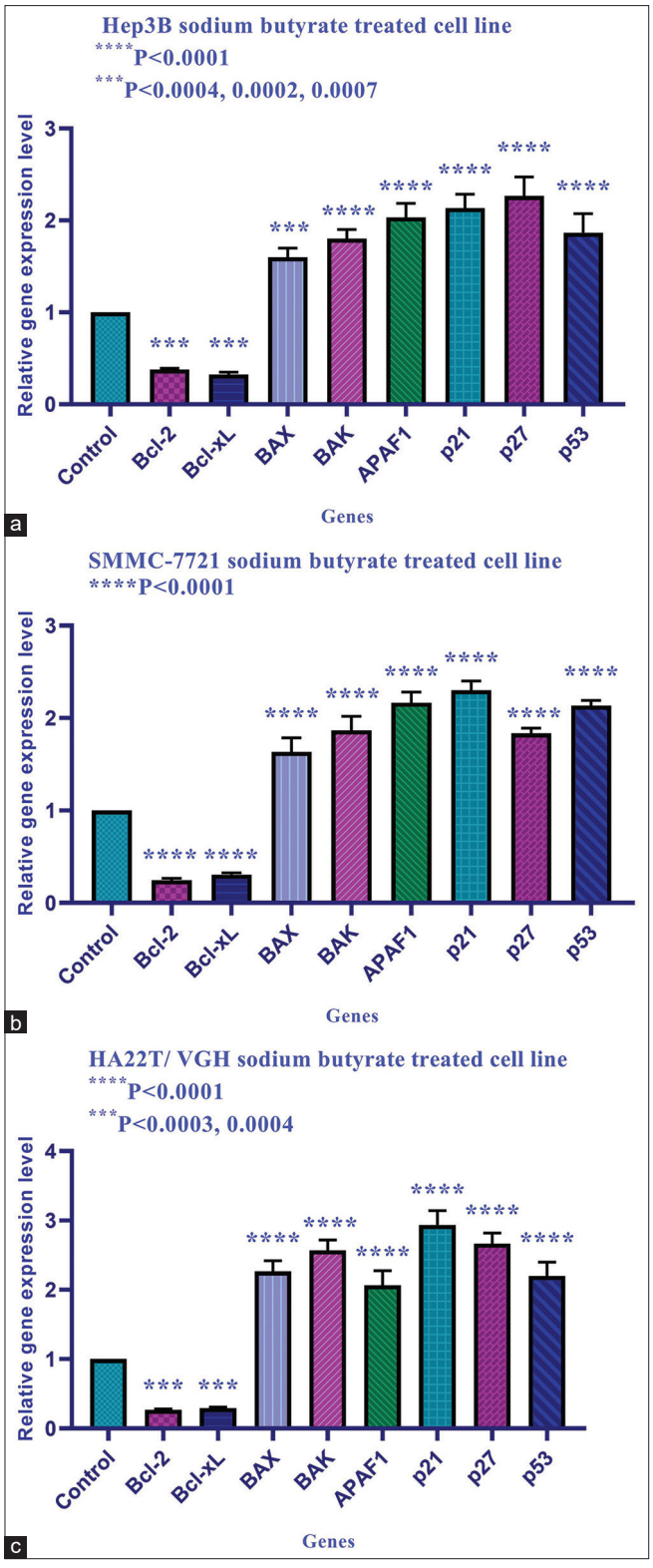

Figure 6: The relative expression level of BAX, BAK, APAF1, Bcl 2, Bcl xL, p21, p27, and p53 in the Hep3B (a), SMMC 7721(b), and HA22T/VGH (c) cell lines treated with sodium butyrate versus untreated control groups at 24 h. As indicated in this Figure, this compound up-regulated the BAX, BAK, APAF1, p21, p27, and p53 and down-regulated Bcl-2, and Bcl-xL significantly after 24 h of treatment in all three cell lines, Hep3B, SMMC-7721, and HA22T/VGH

investigate the protein level of the mentioned genes because of technical limitations. Therefore, protein level evaluation is recommended. Additionally, the investigation of the effect of these compounds on neuroblastoma and glioblastoma with high concentrations and more durations is recommended strongly.

Conclusion

In conclusion, our findings indicated that FdCyd and sodium butyrate can induce their apoptotic effects through extrinsic apoptotic pathways in HCC Hep3B, SMMC-7721, and HA22T/VGH cell lines in a p53-dependent and–independent manner.

Acknowledgments

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Conflicts of interest

There are no conflicts of interest.

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