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Effect of 5-fluoro-2′-deoxycytidine (FdCyd) on p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase 1, 3a, and 3b Genes Expression, Apoptosis Induction, and Cell Growth Inhibition in Pancreatic Cancer AsPC-1 and Hepatocellular Carcinoma LCL-PI 11 Cell Lines

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Abstract

Background: Aberrant DNA methylation of the promoter region is one of the most epigenetic changes in numerous cancers. DNA methyltransferase inhibitors (DNMTIs) can revert DNA hypermethylation in tumor suppressor genes (TSGs). The present study was designed to investigate the effect of 5-fluoro-2′-deoxycytidine (FdCyd) on p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase 1, 3a, and 3b genes expression, apoptosis induction, cell growth inhibition in pancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cell lines.

Materials and Methods: The cells were treated with FdCyd at different periods. Then, the MTT assay, cell apoptosis assay, and qRT-PCR were done to determine cell viability, cell apoptosis, and the relative gene expression level respectively.

Results: The FdCyd decreased DNA methyltransferase 1, 3a, and 3b and increased p16INK4a, p14ARF, and p15INK4b genes expression significantly ($P<0.001$). Besides, LCL-PI 11 cell was more sensitive to FdCyd in comparison to AsPC-1 cell. FdCyd induced significant cell growth inhibition with a dose- and time-dependent manner ($P<0.001$). The IC50 value of FdCyd was obtained with approximately 1µM. Further, FdCyd induced cell apoptosis significantly as a time-dependent manner. The number of apoptotic cells was significantly increased in all groups. The percentage of apoptotic cells after 24 and 48 h were 13.86 and 29.6 % in AsPC-1 and 21.04 and 41.52 % in LCL-PI 11 cell line respectively ($P<0.001$).

Conclusion: The FdCyd can reactivate the p16INK4a, p14ARF, and p15INK4b through inhibition of DNA methyltransferase 1, 3a, and 3b gene expression.

Keywords: 5-fluoro-2′-deoxycytidine, Genes, Neoplasm, Onco-Suppressor

Introduction

The mammalian cell cycle is controlled by several molecular mechanisms and pathways ensuring correct progression and cell division. This division is divided into two stages, mitosis and interphase leads to DNA replication and segregation of replicated chromosomes into two new cells. The interphase comprises G1, S, and G2 phases (1). To date, many studies have reported several genetic and epigenetic alterations of cell-cycle regulators in various cancers. DNA hypermethylation of tumor suppressor genes (TSGs) such as the INK4 family has been indicated in numerous cancers. In humans, the INK4b/ARF/INK4a gene locus located on chromosome 9p21 and encodes potent TSGs including p15ink4b, p14ARF, and p16ink4a. The p15ink4b and p16ink4a bind to CDK4 and 6 and act as cell cycle inhibitors in the G1 phase cell cycle (2). Global cytosine methylation patterns of the promoter region in mammalian cells are established by a complex interplay of at least three independently encoded DNA methyltransferases (DNMTs) consists of DNMT1, DNMT3A, and DNMT3B (3). The levels of these enzymes are often increased in numerous cancer cell lines, which may account for the DNA methylation of TSGs in numerous cancers

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(4). Hypermethylation of CpG islands of TSGs may be involved in carcinogenesis in gastric cancer, breast cancer, pancreatic cancer, head and neck cancer, hepatic cancer, lung cancer, colorectal cancer, bladder cancer, and renal cancer (5). Several compounds act as the DNA methyltransferase inhibitor (DNMTI) comprising 5-Aza-2'-deoxycytidine (5-AZA-CdR, Decitabine), 5-azacytidine (azacitidine), 1-beta-D-arabinofuranosyl-5-azacytosine (fazarabine), dihydro-5-azacytidine (DHAC), 5-fluoro-2′-deoxycytidine (FdCyd) (6) and zebularine (7). Previously, we reported the effect of 5-AZA-CdR on hepatocellular carcinoma (HCC) (8, 9) and colon cancer (10). The present study aimed to investigate the effect of 5-fluoro-2′-deoxycytidine (FdCyd) *on* p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase 1, 3a, and 3b genes expression, apoptosis, cell growth inhibition induction *in* pancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cell lines.

Materials and Methods

Materials

Human pancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cell lines were purchased from the National Cell Bank of Iran-Pasteur Institute. FdCyd and Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma (St. Louis, MO, USA). The compound was dissolved in dimethyl sulfoxide (DMSO) to make a work stock solution. Further concentrations of the agent were obtained by diluting the provided solution. Other compounds including materials and various kits were purchased as provided for previous works (11, 12). The 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 was approved in the Ethics Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC. 1399.009.

Cell culture and cell viability

Human pancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cell lines were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in 5% CO2 overnight and then seeded into 96-well plates (3×10^5 cells per well). After 24 h, the medium was replaced with an experimental medium containing various concentrations of 5-fluoro-2′-deoxycytidine (0, 0.5, 1, 2.5, 5, and 10 μM). The control groups received DMSO only, at a concentration of 0.05 %. After a period of 24 and 48h, the cells were investigated by MTT assay according to Standard protocols to determine cell viability. Therefore, MTT solution was added to each well for 4 h at 37°C and then the MTT solution was changed by DMSO and shaken for 10 min to dissolve all of the crystals. Finally, the optical density was detected by a microplate reader at a wavelength of 570 nM. Each experiment was repeated three times (triplicates).

Cell apoptosis assay

To determine AsPC-1 and LCL-PI 11 apoptotic cells, the cells were cultured (3×10^5 cells/well) and incubated overnight and then treated with FdCyd (1 μM) for different time periods (24 and 48 h). Subsequently, the treated and untreated cells were harvested by trypsinization, washed with cold PBS, and resuspended in Binding buffer (1x). Finally, Annexin-V-(FITC) and PI were used to determine the apoptotic cells by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

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

To determine the relative expression level of p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase *1, 3a, and 3b genes* qRT-PCR was done. The AsPC-1 and LCL-PI 11 cells were treated with FdCyd (1 μM) for 24 and 48 h and then qRT-PCR was done as our previous works (13). The primer sequences are indicated in Table I.

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Statistical analysis

The database was set up with the SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA) for analysis. The data were acquired from three separate tests and are indicated as means ± standard deviations. The comparisons between groups were achieved with ANOVA (one-way ANOVA) and Turkey test. A significant difference was considered as $P < 0.05$.

Results

Result of cell viability by the MTT assay

The cell viability of the AsPC-1 and LCL-PI 11 cells treated with FdCyd (0, 0.5, 1, 2.5, 5, and 10 μM) was investigated by MTT assay. This technique showed that the activity of cellular enzymes reduced the tetrazolium salt MTT and produced a dark-blue formazan crystal. The crystal was dissolvable in DMSO by which the number of viable cells can be investigated. As indicated in Figure. 1, FdCyd induced significant cell growth inhibition with a dose-dependent manner ($P< 0.001$). The IC50 value of FdCyd was obtained with approximately 1μM.

Result of cell apoptosis assay

To determine apoptosis, the pancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cells were treated with FdCyd (1 μM) for 24 and 48 h and then stained using annexin-V-(FITC) and PI to determine apoptotic cell in early and late apoptosis stage. As indicated in Figures 2 and 3, FdCyd induced cell apoptosis significantly as a time-dependent manner in all experimental groups ($P<0.001$).

Result of determination of genes expression

The effect of FdCyd (1 μM) on p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase *1, 3a, and 3b genes expression* was investigate by quantitative real-time RT-PCR analysis. The result indicated that treatment with FdCyd (1 μM) up-regulated p16INK4a, p14ARF, p15INK4b, and down-regulated DNA methyltransferase *1, 3a, and 3b genes expression* significantly, Figures 4 and 5 and Table II. FdCyd had a significant time-dependent manner ($P<0.001$).

Table I. The primer sequences of p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase 1, 3a, and 3b genes.

Primer	Primer sequences (5' to 3')	Reference
DNMT1		13
Forward	GAGGAAGCTGCTAAGGACTAGTTC	
Reverse	ACTCCACAATTTGATCACTAAATC	
DNMT 3a		13
Forward	GGAGGCTGAGAAGAAAGCCAAGGT	
Reverse	TTTGCCGTCTCCGAACCACATGAC	
DNMT 3b		13
Forward	TACACAGACGTGTCCAACATGGGC	
Reverse	GGATGCCTTCAG GAATCACACCTC	
$p14^{ARF}$		14
Forward	GTGGGTTTTAGTTTGTAGTT	
Reverse	AAACCTTTCCTACCTAATCT	
p15INK4b		15
Forward	AAGCTGAGCCCAGGT CTCCTA	
Reverse	CCACCGTTGGCCGTAAACT	
p16INK4a		16
Forward	CCCGCTTTCGTAGTTTTCAT	
Reverse	TTATTTGAGCTTTGGTTCTG	
GAPDH		12
Forward	AAC GTG TCA GTO GTG GAC CTG	
Reverse	GGG TGT CGC TGT FGA AGT	

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Table II. The relative expression level of p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase 1, 3a, and 3b genes.

Cell line	Gene	Drug	Dose (µM)	Duration (h)	Expression	P-value
AsPC-1	p16INK4a	FdCyd	1	24	2.3	P<0.001
AsPC-1	p16INK4a	FdCyd	1	48	2.7	P<0.001
AsPC-1	p14ARF	FdCyd	1	24	1.8	P<0.001
AsPC-1	p14ARF	FdCyd	1	48	2.3	P<0.001
AsPC-1	p15INK4b	FdCyd	1	24	2.1	P<0.001
AsPC-1	p15INK4b	FdCyd	1	48	2.5	P<0.001
AsPC-1	DNMT1	FdCyd	1	24	0.54	P<0.001
AsPC-1	DNMT1	FdCyd	1	48	0.47	P<0.001
AsPC-1	DNMT3a	FdCyd	1	24	0.55	P<0.001
AsPC-1	DNMT3a	FdCyd	1	48	0.46	P<0.001
AsPC-1	DNMT3b	FdCyd	1	24	0.48	P<0.001
AsPC-1	DNMT3b	FdCyd	1	48	0.42	P<0.001
LCL-PI 11	p16INK4a	FdCyd	1	24	2.6	P<0.001
LCL-PI 11	p16INK4a	FdCyd	1	48	2.7	P<0.001
LCL-PI 11	p14ARF	FdCyd	1	24	2.1	P<0.001
LCL-PI 11	p14ARF	FdCyd	1	48	2.3	P<0.001
LCL-PI 11	p15INK4b	FdCyd	1	24	2.5	P<0.001
LCL-PI 11	p15INK4b	FdCyd	1	48	2.8	P<0.001
LCL-PI 11	DNMT1	FdCyd	1	24	0.46	P<0.001
LCL-PI 11	DNMT1	FdCyd	1	48	0.34	P<0.001
LCL-PI 11	DNMT3a	FdCyd	1	24	0.42	P<0.001
LCL-PI 11	DNMT3a	FdCyd	1	48	0.39	P<0.001
LCL-PI 11	DNMT3b	FdCyd	1	24	0.39	P<0.001
LCL-PI 11	DNMT3b	FdCyd	1	48	0.32	P<0.001

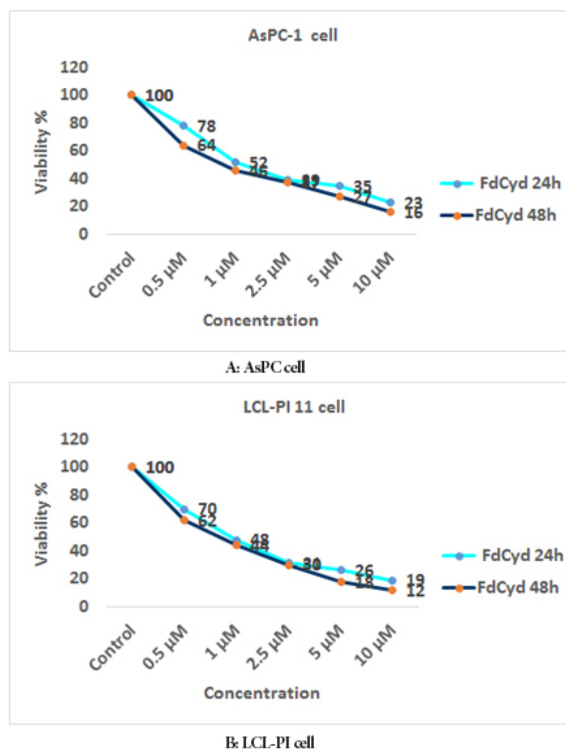

Figure1. In vitro effects of FdCyd (0, 0.5, 1, 2.5, 5, and 10 µM) on AsPC-1 (A) and LCL-PI 11 (B) cell viability determined by MTT Assay at 24 and 48 h. The FdCyd inhibited the growth of both cell lines significantly as a dose-dependent manner.

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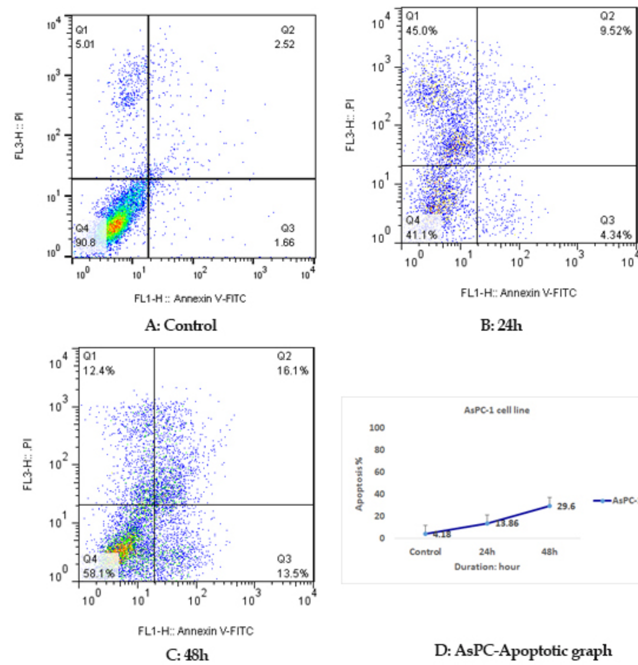

Figure 2. The apoptotic effect of FdCyd (1 µM) on AsPC-1 cell versus control groups at different periods (24 and 48h). The FdCyd induced significant apoptosis as a time-dependent manner. The results were obtained from three separate experiments and were presented as mean ± standard error of the mean.

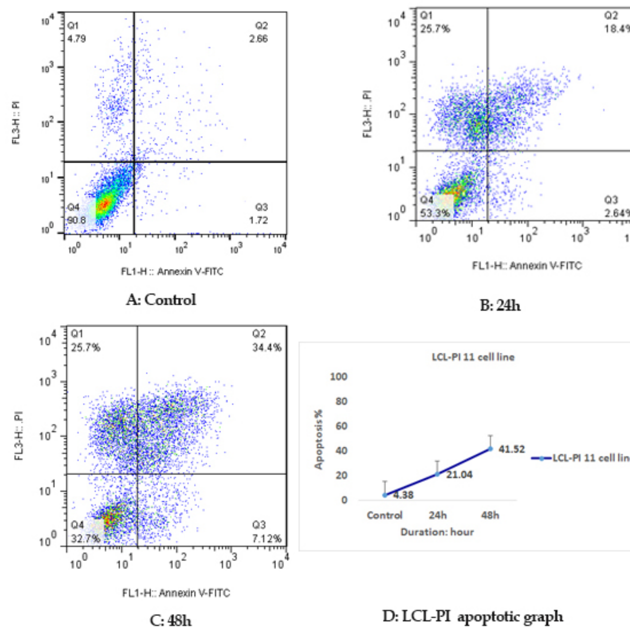

Figure 3. The apoptotic effect of FdCyd (1 µM) on LCL-PI 11 cell versus control groups at different periods (24 and 48h). The FdCyd induced significant apoptosis as a time-dependent manner. The results were obtained from three separate experiments and were presented as mean ± standard error of the mean.

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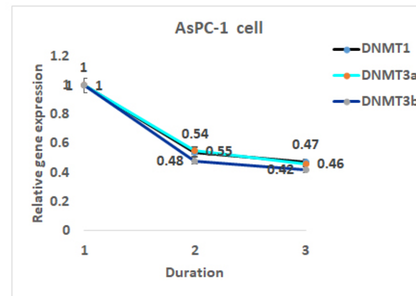
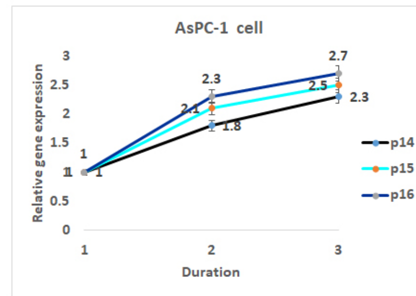
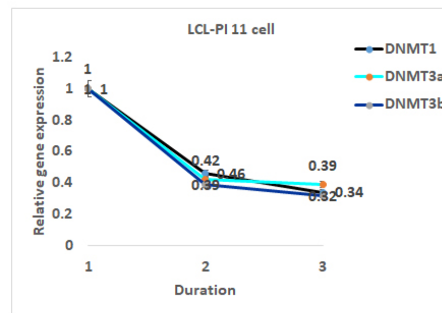


A: Significant down-regulation

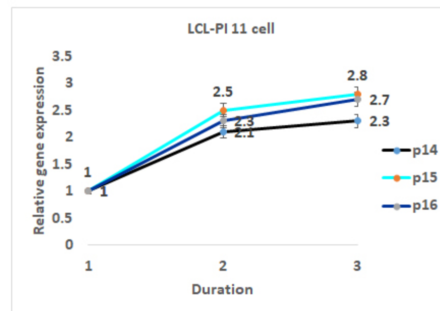


B: Significant up-regulation

Figure 4. The relative expression level of p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase *1, 3a, and 3b* in the pancreatic cancer AsPC-1 cell line treated with FdCyd (1 µM) versus untreated control groups at different periods (24 and 48h). 0: Control, 1: 24 h, 2: 48 h.



A: Significant down-regulation



B: Significant up-regulation

Figure 5. The relative expression level of p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase *1, 3a, and 3b* in the hepatocellular carcinoma LCL-PI 11 cell line treated with FdCyd (1 µM) versus untreated control groups at different periods (24 and 48h). 0: Control, 1: 24 h, 2: 48 h.

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Discussion

Aberrant DNA methylation of the promoter region is one of the most epigenetic changes in numerous cancers. The activity of DNMTs leads to DNA hypermethylation and cancer induction (17). Meanwhile, the methylation of CpG islands affects the transcription and expression of specific genes such as p16INK4a, p14ARF, p15INK4b leads to cancer induction. However, DNMTIs can revert DNA hypermethylation in tumor suppressor genes (TSGs) and suppress cancer-specific cellular phenotypes (18). In the current study, we indicated that FdCyd down-regulated DNA methyltransferase *1, 3a, and 3b genes expression, up-regulated* p16INK4a, p14ARF, and p15INK4b, inhibit cell growth and induced apoptosis in pancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cell lines. Similarly, our previous studies indicated that DNA demethylating agent 5-aza-CdR (3 μM) can induce apoptosis by p16INK4a, p14ARF, p15INK4b genes reactivation in HCC, PLC/PRF5, and pancreatic cancer, MIA Paca-2, cell lines (9). In line with our report, it has been demonsterated that DNA demethylating agent 5-Aza-CdR can induce p14ARF mRNA expression in esophagus cancer cell lines (TE-1 and Eca-9706) (19), p16INK4a in pancreatic cancer cell lines SW1990 and AsPC-1 (20), and P15/INK4b and P16/INK4a in acute myeloid leukemia (AML) KBM3/Bu250^{6}, OCI-AML3, and HL60 cell lines (21). We did further investigation and found that FdCyd played its role through the down-regulation of DNA methyltransferase *1, 3a, and 3b gene expression. Inconsistent with our results, in vitro study has shown that zebularine [1-(beta-D-ribofuranosyl)-1, 2-dihydropyrimidin-2-one] reactivates a silenced* p16INK4a *gene and demethylates its promoter region in T24 bladder cancer cell line (22).* It has been shown that DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (decitabine) caused the DNA demethylation of the all CpG sites in the p15INK4b CpG islands in hematopoietic cells (23). Furthermore, other researchers have reported that FdCyd induces p21 gene reactivation which correlates with the arrest of AML cells in the G_1 cell cycle phase (24). It has also been shown that the p21$^{\mathbf{WAF1/CIP1}}$ expression in metastatic prostate cancer PC3, LNCaP, and DU145 cell lines is enhanced as a result of treatment with 5-Aza-CdR (25). Besides, 5-Aza-dC can increase the up-regulation of p21^{WAF1}, p27^{KIP1}, and p57^{KIP2} genes expression in pancreatic cell lines AsPC-1, PANC-1, BxPC-3, HPAC, Capan-2, and HPAF-II (26). Our previous work indicated that 5-AZA-CdR can down-regulate DNMT1, DNMT3a, DNMT3b in HCC LCL-PI 11 cell line [27]. Furthermore, we indicated that 5-Aza-CdR can induce apoptosis through the re-activation of p15INK4, p16INK4, p18INK4, and p19INK4 genes expression in HCC PLC/PRF/5 cell line [28]. It has been reported that 5-Aza-CdR significantly increases the expression of P16 leads to apoptotic induction in pancreatic cancer [29]. In T24 human bladder carcinoma, it has been shown that 5-Aza-CR and zebularine induce apoptosis by up-regulation of p16 [30]. Finally, DNA demethylating agents can reactivate cyclin-dependent kinase inhibitors by inhibition of DNA methyltransferase inhibitors activity. It should be noted that our report is the first report of the evaluation of the effect of FdCyd *on* p16INK4a, p14ARF, p15INK4b, and DNA methyltransferase *1, 3a, and 3b genes expression, apoptosis, and cell growth inhibition induction in p*ancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cell lines. Therefore, it is the novelty of the current work. We did not assess the effect of the compound at 72 h because of budget limitation. This evaluation is recommended.

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Conclusion

In summary, our findings indicated that FdCyd can reactivate the p16INK4a, p14ARF, and p15INK4b through inhibition of DNA methyltransferase 1, 3a, and 3b genes expression in pancreatic cancer AsPC-1 and hepatocellular carcinoma LCL-PI 11 cell lines resulting in cell growth inhibition and apoptosis induction. Further, the LCL-PI 11 cells were more sensitive to FdCyd in comparison to the pancreatic cancer AsPC-1 cells.

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Conflict of interest

The authors declare no conflict of interest.

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