Biochemical Pharmacology 81 (2011) 848–855

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

FNC, a novel nucleoside analogue inhibits cell proliferation and tumor growth in a variety of human cancer cells

Qiang Wang [a], Xiaobing Liu [b], Qingduan Wang [c], Yan Zhang [c], Jinhua Jiang [c], Xiaohe Guo [d], Qingxia Fan [e], Liyun Zheng [c], Xuejun Yu [d], Ning Wang [c], Zhenliang Pan [f], Chuanjun Song [a], Wenqing Qi [b,*], Junbiao Chang [a,**]

[a] Department of Chemistry, Zhengzhou University, Zhengzhou, Henan Province 450001, PR China
[b] Arizona Cancer Center, The University of Arizona, 1515 N Campbell Avenue, Tucson, AZ 85724, USA
[c] Henan Academy of Medical and Pharmaceutical Sciences, Henan Key Laboratory for Pharmacology of Liver, Zhengzhou 450052, PR China
[d] Hennan Key Laboratory of Fine Chemicals, Henan Academy of Science, No. 56 Hongzhuan Road, Zhengzhou 450002, PR China
[e] The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province 450001, PR China
[f] School of Pharmaceutical Science, Zhengzhou University, Zhengzhou, Henan Province 450001, PR China

ARTICLE INFO

Article history:
Received 9 November 2010
Accepted 4 January 2011
Available online 8 January 2011

Keywords:
Nucleoside analogues
Apoptosis
DNA synthesis
FNC
Tumor growth inhibition

ABSTRACT

Inhibition of cellular DNA synthesis is a strategy to block cancer cell division. Nucleoside analogues can incorporate into DNA and terminate DNA strand elongation. So far, several nucleoside analogues have been successfully used as anticancer drugs. FNC, 2′-deoxy-2′-β-fluoro-4′-azidocytidine is a novel cytidine analogue which demonstrated potent activity against hepatitis C virus (HCV). To investigate the therapeutic potential of FNC in human cancers we studied its activity in a number of cancer cells *in vitro* and *in vivo*. FNC potently inhibited cell proliferation with an IC_{50} of 0.95–4.55 μM in a variety of aggressive human cancer cell lines including B-cell non-Hodgkin's lymphomas, lung adenocarcinoma and acute myeloid leukemia. Cells treated with FNC exhibited G1 and S cell cycle arrest at high and low dose, respectively, which confirms the mechanism of action of nucleoside analogues. Treatment of B-NHL cell lines with FNC induced apoptosis in a dose and time dependent manner. Finally, mouse xenograft models of hepatocarcinoma (H22), sarcoma (S180) and gastric carcinoma (SGC7901) demonstrated that FNC had significant tumor growth inhibition activity in a dose-dependent manner with low toxicity. Together, our results suggest that FNC may be a valuable therapy in cancer patients and warrant early phase clinical trial evaluation.

1. Introduction

Human cancer remains a serious disease, and right now, there is still no completely significant chemotherapeutic strategy against it. Therefore, there is an urgent need to develop new and more effective anticancer medication. Cancer cells display uncontrolled cell division and DNA synthesis is essential for this process. Hence, inhibition of nascent DNA formation could prevent cancer growth.

Nucleosides including cytidine, uridine, adenosine, guanosine and thymidine are glycosylamines consisting a nucleobase bound to a ribose or deoxyribose sugar. Nucleosides can be phosphorylated by specific kinases in the cells, producing nucleotides, which are the molecular building-blocks of DNA and RNA [1,2]. The triphosphates of nucleoside analogues compete with the cellular endogenous deoxynucleotides for incorporation into DNA during replication, where they block DNA synthesis through a chain termination mechanism [3–6]. Thus, nucleoside analogues can be used as antiviral or anticancer agents. In medicine several DNA-directed nucleoside analogues, such as ara-C [7,8], fludarabine [9], and gemcitabine [10,11], are antimetabolite effective in the treatment of a variety of malignancies [12–14]. The major mechanism of action of these nucleoside analogue drugs is through incorporation into DNA and inhibiting DNA synthesis [15,16], and thus this class of agents shows specificity for cell growth arrest in S-phase and kills cells by inducing apoptosis [17–23].

FNC (4′-azido-2′-deoxyfluoroarabinocytidine or 2′-deoxy-2′-β-fluoro-4′-azidocytidine) is a novel pyrimidine analogue [24]. Fluoronucleosides have a history of being well phosphorylated by cellular kinases and can be good substrates for RNA and DNA polymerases. It has been demonstrated that FNC is an excellent substrate for deoxycytidine kinase and is phosphorylated with

* Corresponding author. Tel.: +1 520 626 0190; fax: +1 520 626 2225.
** Corresponding author. Tel.: +86 371 67783017; fax: +86 371 67783017.
E-mail addresses: wenqing@email.arizona.edu (W. Qi), changjunbiao@zzu.edu.cn (J. Chang).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.01.001

efficiencies up to 3-fold higher than deoxycytidine. FNC is a highly potent and selective inhibitor of hepatitis C virus (HCV) replication with $IC_{50} = 24 \pm 3$ nM [25]. In this study, we demonstrate that FNC inhibits cell proliferation, induces G1 and S phase arrest and promotes apoptosis in a number of human cancer cell lines. FNC also has potent anti-tumor activity in xenograft mice bearing hepatocarcinoma, sarcoma and gastric carcinoma tumors.

2. Materials and methods

2.1. Cells and reagents

The human non-small cell lung cancer cell line A549, acute myeloid leukemia cell line HL-60 and B-cell non-Hodgkin lymphoma (B-NHL) cell lines RL (DLBC), Granta-519 (MCL), SUDHL-6 (T-FL) (from Dr. Daruka Mahadevan, Arizona Cancer Center, Tucson, AZ) were maintained in RPMI 1640 medium (Mediatech, VA) supplemented with 10% fetal bovine serum, 2 mM sodium pyruvate and 100 units/ml penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. FNC was designed and synthesized in our laboratory (Department of Chemistry, Zhengzhou University, Zhengzhou, PR China) with purity of 95%. The compounds were dissolved at 10 mM in distilled water as a stock solution, and then further diluted to desired concentrations for *in vitro* experiments. Anti-PARP (H-250) and anti-cyclin A (H-432) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-Bcl-xL, anti-phospho-CDK2 (Thr160), anti-cleaved caspase-3 (Asp175) and anti-GAPDH (14C10) antibodies were from Cell Signaling Technology (Danvers, MA).

2.2. Analysis of cell proliferation inhibition (MTS assay)

Cells were seeded at 8000 per well in 96-well culture plates and allowed to grow for 24 h followed by the desired treatment with increasing concentrations (0.001–20 μM) of FNC for 4 days. Viable cell densities were determined using a CellTiter 96 Cell Proliferation Assay (Promega, Madison, WI). The studies were performed in triplicates and IC_{50} values were estimated by Calcusyn software (Biosoft, UK).

2.3. Apoptosis assay

Using Annexin V staining to detect apoptosis, treated cells were harvested and rinsed with cold PBS once. After centrifugation for 5 min, cells were resuspended in 500 μl of 1× Annexin V binding buffer (BioVision, Annexin V-FITC Reagent Kit, Cat.#1001-1000) and then added 5 μl of Annexin V-FITC and 5 μl of propidium iodide (BioVision, Annexin V-FITC Reagent Kit). After incubation for 5 min at room temperature in the dark, the samples were analyzed by flow cytometry.

2.4. Cell cycle analysis

Cells were treated with different concentrations of FNC for 24 h and 48 h and then the cells were centrifuged at 1500 × *g* for 5 min at 4 °C and resuspended in PBS, fixed by drop wise addition of ice-cold ethanol (100%) to a final concentration of 70%, and incubated for 30 min on ice. Fixed cells were pelleted and treated with 100 μl of RNase A (0.2 mg/ml in PBS) for 5 min at room temperature, then suspended in 1 ml ddH_2O. After staining with 4 μg/ml propidium iodide, the DNA content was determined using a Becton Dickson flow cytometer and the cell cycle profile was analyzed by ModFit software. Cell aggregates were gated out of the analysis, based on the width of the propidium iodide fluorescence signal. Each profile was compiled from 10,000 gated events.

2.5. Immunoblotting

The cells were lysed in NP-40 lysis buffer containing 50 mM Tris–Cl (pH 7.4), 0.15 M NaCl, 0.5% NP-40, 1 mM DTT, 50 mM sodium fluoride, and 2 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations were determined using the BioRad protein assay kit (Hercules, CA) and 50 μg of protein was resolved by electrophoresis on a 10% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane and nonspecific binding was blocked by incubating with 5% nonfat milk in TBST buffer (0.01 M Tris–Cl, 0.15 M NaCl, 0.5% Tween-20, pH 8.0) at room temperature for 1 h. The membrane was subjected to the indicated antibodies and the proteins were detected by a LI-COR Odyssey Infrared Imaging System.

2.6. Mouse xenograft studies

Animal care and treatment were performed at Zhengzhou University's experimental mouse core facility. All mice were maintained under barrier conditions and experiments were conducted using protocols and conditions approved by the institutional animal care. Kunming mice (including male and female, body weight 20 ± 2 g from Shanghai Sikelai Co., Shanghai, China) were injected with 1×10^7 sarcoma (S-180) and hepatoma (H22) cells subcutaneously into the right front flank and divided randomly into several different test groups with 8–10 mice per cohort. One day after implantation of tumor cells, the mice were treated daily by IV or IG with vehicle (saline) or 5-FU (15 mg/kg/day), cisplatin (1.0 mg/kg/day), capecitabine (400 or 600 mg/kg/day) and FNC (0.5, 1.0, 2.0 mg/kg/day) formulated in saline or distilled water (for capecitabine) for 8 days. Then the mice were sacrificed and the tumors were excised and weighed for evaluating the tumor growth inhibition at 24 h after the end of treatment. BALB/c nu/nu mice were provided by Shanghai Sikelai Co. and human gastric cancer cells (SGC7901) were subcutaneously implanted in the right hind back using 200 μl of a 1×10^7 cell/ml suspension in PBS. When tumors reached an average diameter of 5–8 mm, mice were weighed, randomized by tumor size, assigned to the various study groups, and treated with vehicle (saline), capecitabine (600 mg/kg/day), or FNC (0.5, 1.0, 2.0 mg/kg/day) by IG daily for 20 days. After treatment, mice were sacrificed and the tumors were excised and weighed for evaluating the tumor growth inhibition. All results are represented as mean ± SEM of eight or ten animals.

2.7. Statistical analysis

Analysis was done using multiple *t*-test (with Bonferroni correction) with the STATA software package (StataCorp LP, College Station, TX). Data was analyzed by group, $p \leq 0.05$ was considered significant.

3. Results

3.1. FNC induces cell cycle arrest in G1 and S phases

FNC is a nucleoside analogue which should incorporate into DNA synthesis and affect cell cycle progression. To investigate this, we treated three different types of aggressive B-cell non-Hodgkin's lymphomas Granta-519 (mantle cell lymphoma, MCL), RL (diffuse large B-cell lymphoma, DLBCL) and SUDHL-6 (transformed follicular lymphoma, T-FL) with FNC at concentrations of 0.5 μM, 1.0 μM, 2.0 μM and 5.0 μM for 24 h and 48 h. As expected, S phase arrest was induced by FNC at low doses in Granta-519 and SUDHL-6 cells even at the early time course of 24 h. However, G1 growth arrest was observed when cells treated with FNC at high doses of ≥2 μM (Fig. 1A). G1 arrest was also

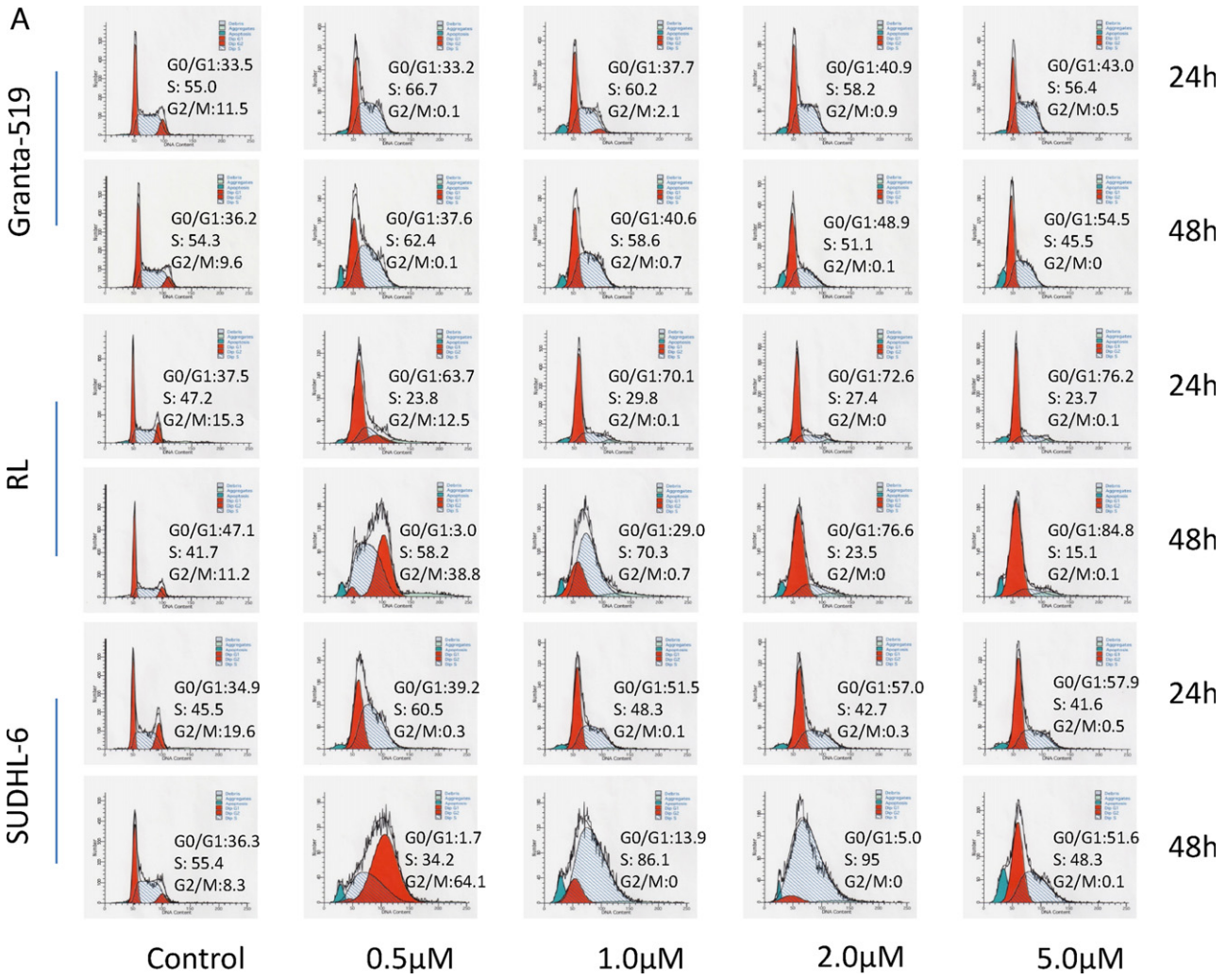

Fig. 1. Cell cycle analysis of lymphoma cell lines after treatment with FNC. (A) Granta-519, RL and SUDHL-6 cells were treated with vehicle (distilled water), FNC at concentrations of 0.5 μM, 1.0 μM, 2.0 μM and 5.0 μM for 24 h and 48 h. Cells were harvested, fixed, treated with RNase, and then labeled with propidium iodide, and analyzed by flow cytometry. (B) Granta-519, RL and SUDHL-6 cells were treated with vehicle (control), nocodazole at 0.2 μg/ml, FNC at 2.0 μM and nocodazole (0.2 μg/ml) plus FNC (2.0 μM) simultaneously for 24 h and DNA content was analyzed by flow cytometry. (C) PANC-1, BxPC-3 and SW480 cells were treated with or without FNC at 15 μM for 72 h and DNA content was analyzed by flow cytometry. *X*-axis: DNA content, *Y*-axis: cell numbers. The number represents the percentage of cell population in G0/G1, S and G2/M phases. (D) Granta-519, RL and SUDHL-6 cells were treated with vehicle and FNC at 2 μM for 8 h, 16 h, 24 h, 32 h and 48 h. Cyclin A and phosphor-CDK2 (Thr160) was evaluated by Western blotting analysis. GAPDH was used as a loading control.

induced by FNC in RL even at the low doses at 24 h, but S phase arrest appeared at 48 h and G1 population was still high when cells treated at high doses of ≥2 μM at this time point (Fig. 1A). To further confirm the G1 or S phase arrest by FNC, Granta-519, RL and SUDHL-6 cells were treated with nocodazole or FNC alone or nocodazole plus FNC simultaneously for 24 h. As shown in Fig. 1B, nocodazole alone synchronized cells in G2/M phase compared to control. FNC still blocked cells in G1/S phase even added the nocodazole, indicating FNC regulates G1/S cell cycle progression. In addition, S phase arrest was examined in different solid tumor cells including pancreatic cancer cell lines PANC-1 and BxPC-3 and colon cancer cell line SW480 72 h after treatment (Fig. 1C). Similar results were also observed in Hela cells no matter how high concentration of FNC used (8–20 μM, data not shown). Moreover, a synchronized G2/M phase wave also appeared in RL and SUDHL-6 cells treated with 0.5 μM of FNC at late time course of 48 h (Fig. 1A) as well as in solid tumors (Fig. 1C). Active CDK2/cyclin A complexes play key roles during S-phase. Consistent with the cell cycle analysis, both cyclin A protein level and CDK2 activity (phosphorylation of Thr160) were decreased in a time-dependent manner after FNC treatment (Fig. 1D). Taken together, the nucleoside analogue FNC has effect on cell cycle progression of G1 or S phase in both aggressive B-cell non-Hodgkin's lymphomas (NHL) and solid malignancies.

3.2. FNC inhibits cell proliferation

FNC regulates cell cycle progression. Next we ask whether it inhibits cell proliferation *in vitro*. Granta-519, RL, SUDHL-6, A549 and HL-60 cell lines were treated with FNC at a varying concentration for 96 h, MTS assays were performed to evaluate cell proliferation. Fig. 2 shows that FNC effectively inhibited the growth of cells with IC_{50} values ranging from 0.95 to 4.55 μM (Table 1). Similar results were also shown in numerous cancer cell lines such as HT-29, MCF-7, Hela, K562, etc. (data not shown), suggesting FNC has extensive anti-tumor activity *in vitro*.

3.3. FNC induces apoptosis

To examine whether apoptosis is involved in inhibition of cell proliferation by FNC, we further detect apoptosis after FNC treatment. Granta-519, RL and SUDHL-6 cells were treated with

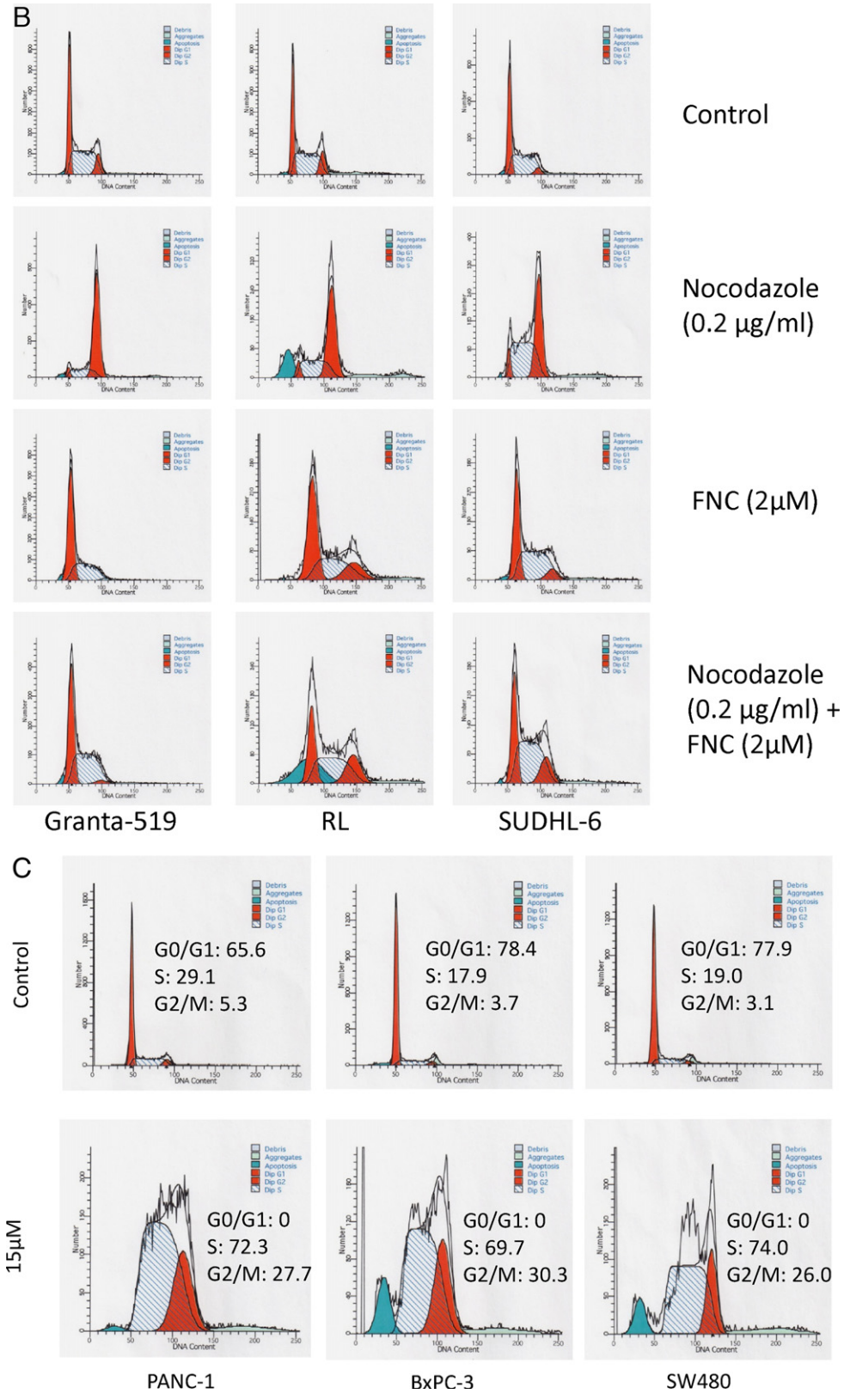

Fig. 1. (*Continued*).

FNC at concentrations of 0.5 μM, 1.0 μM, 2.0 μM and 5.0 μM for 72 h, apoptosis was evaluated by flow cytometry assays following Annexin V and PI staining. As expected, FNC clearly induced apoptosis in all three cell lines in a dose-dependent manner (Fig. 3). Especially, more than 90% of cells go to apoptosis in Granta-519 even at 0.5 μM of FNC, indicating Granta-519 cells are more sensitive to FNC than RL and SUDHL-6 which consists with the MTS assay (Table 1). FNC also induces apoptosis in Hela cells (data not shown). These results were confirmed by demonstrating an increased level of cleaved nuclear enzyme poly (ADP-ribose)

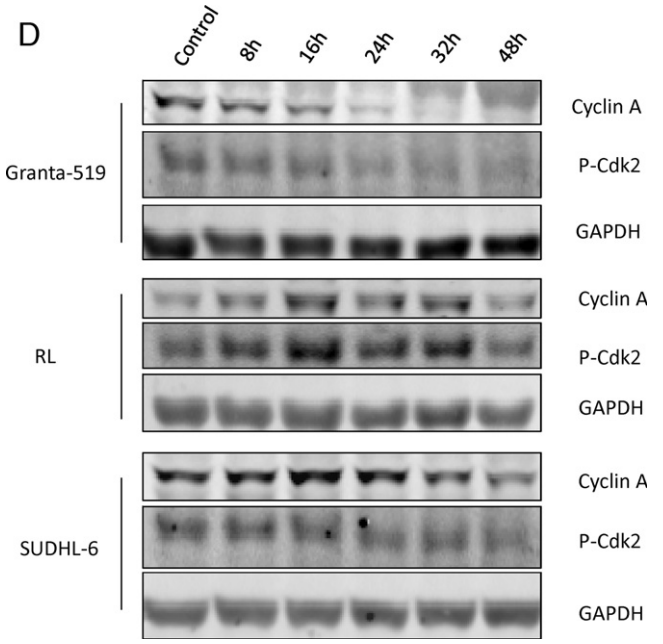

Fig. 1. (*Continued*).

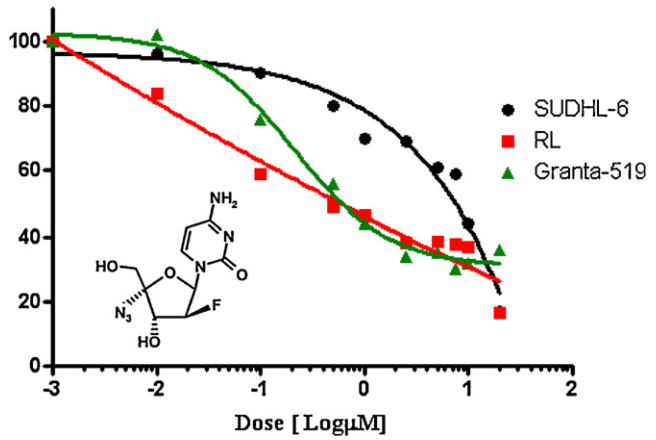

Fig. 2. Effect of FNC on cell proliferation. Granta-519, RL and SUDHL-6 cells were exposed to varying concentrations of FNC for 4 days. Cell viability was assessed by MTS analysis. Points are the means of triplicate determinations ± SD. Inset: the chemical structure of FNC.

polymerase (PARP) in treated Granta-519 and RL cells (Fig. 4A). Caspase-3 is one of the key executioners of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as PARP. As expected, activation of caspase-3 (cleaved caspase-3) was observed in Granta-519 and RL cells after FNC treatment (Fig. 4A). In addition, apoptosis occurred as early as 16 h in Granta-519, 32 h in RL and 48 h in SUDHL-6 cells (Fig. 4B) indicating apoptosis induction is in a time-dependent manner in different B-NHL subtypes. Moreover, Bcl-xL, a Bcl-2 family member which prevents apoptosis was decreased by FNC in all three lymphoma cell lines (Fig. 4B). Together, the data demonstrate that FNC leads to promotion of apoptosis in dose and time-dependent manner in aggressive B-NHL cells.

3.4. FNC inhibits tumor growth in xenograft mice

Based on our *in vitro* data that FNC was effective in regulating cell cycle, inducing apoptosis and inhibiting cell proliferation, we evaluated this compound in mouse xenograft models of sarcoma

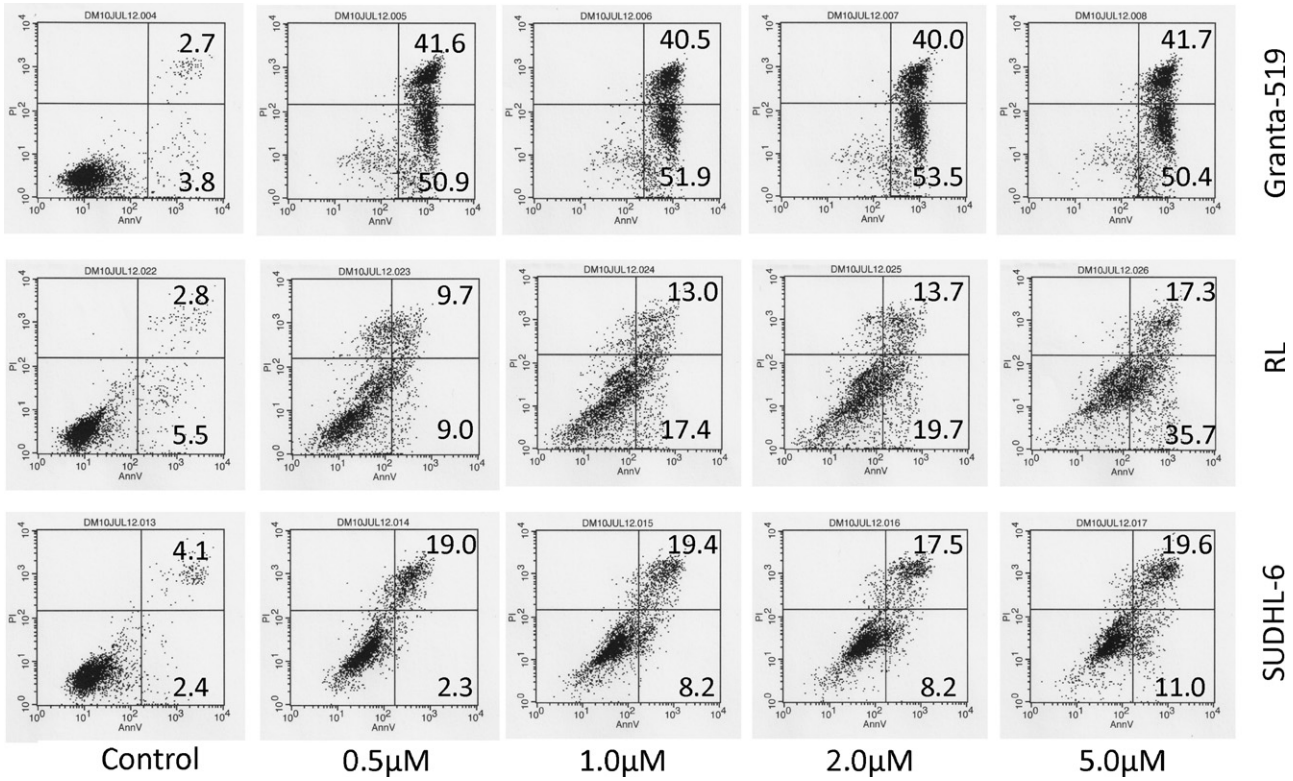

Fig. 3. FNC induces apoptosis. Granta-519, RL and SUDHL-6 cells were treated with vehicle (control) or FNC at various concentrations as indicated for 72 h. Apoptosis was detected by flow cytometric analysis based on propidium iodide (*Y*-axis) and Annexin V staining (*X*-axis). Percentages of apoptotic cells (the lower and upper right quadrants) are indicated.

Table 1
Antiproliferative activity of FNC in a panel of human cancer cell lines.

Cell lines	IC50 (μM)
SUDHL-6	4.55
RL	1.74
Granta-519	0.95
A549	1.22
HL60	3.30

(S-180), hepatoma (H22) and gastric cancer (SGC7901). The mice bearing tumors were treated with vehicle control (saline), positive controls 5-FU (15 mg/kg), cisplatin (1.0 mg/kg) and capecitabine (600 mg/kg) and FNC at different doses of 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg daily for 8 days. Dose-dependent tumor growth inhibition (TGI) of FNC was demonstrated compared with saline vehicle-treated controls regardless of that drug was administered by IV (Table 2A) or IG (Table 2B). The efficiency of TGI by FNC at high dose of 2.0 mg/kg was up to 60% compared to control. However, there was no significant difference of TGI between FNC at 2.0 mg/kg and positive controls (Tables 2A and 2B). Nude mice bearing SGC7901 were treated with vehicle (saline), capecitabine (400 mg/kg) and FNC (0.5, 1.0 and 2.0 mg/kg) daily for 20 days by IG. Similar to S-180 and H22 xenograft models, FNC inhibited tumor growth in a dose-dependent manner (Table 2C). The efficiency of TGI was 68% when mice were treated with FNC at high dose which is same as capecitabine treatment. Importantly, the body weights of all mice in both models did not change significantly (within 10%) during the study and mice appeared to tolerate treatment(s) well.

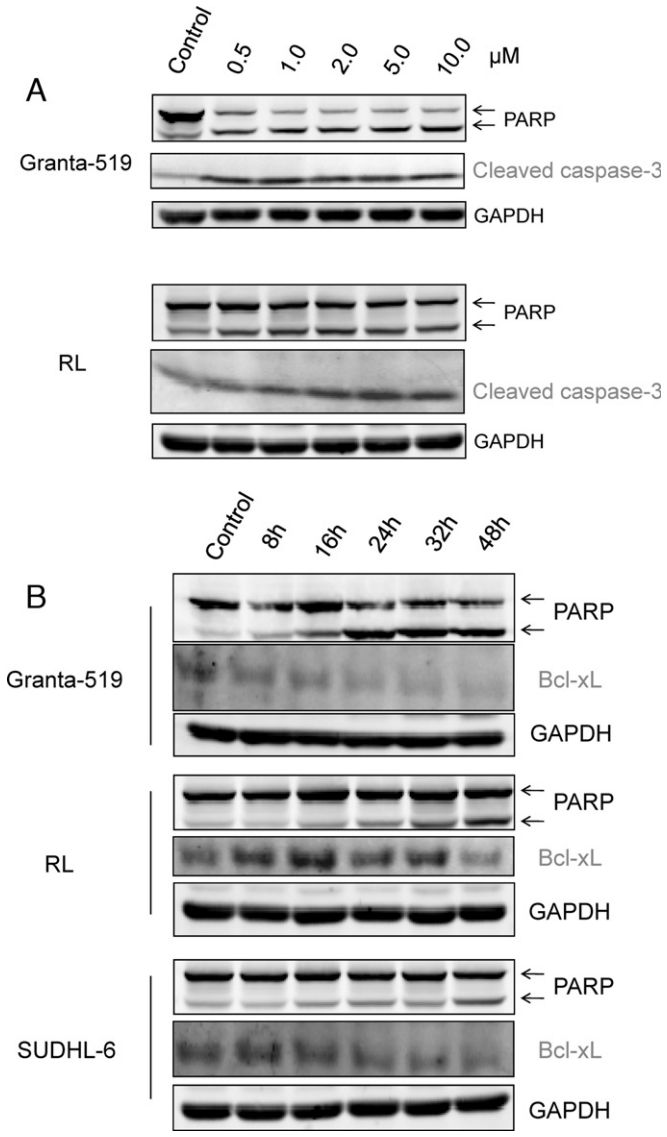

Fig. 4. FNC enhances PARP cleavage. (A) Granta-519 and RL cells were treated with FNC at 0.5 μM, 1.0 μM, 2.0 μM, 5.0 μM and 10.0 μM for 72 h. Apoptosis was evaluated with PARP and caspase-3 cleavage utilizing Western blotting analysis. (B) Granta-519, RL and SUDHL-6 cells were treated with vehicle and FNC at 2 μM for 8 h, 16 h, 24 h, 32 h and 48 h. PARP cleavage and Bcl-xL were evaluated by Western blotting analysis. GAPDH was used as a loading control.

4. Discussion

Every year millions of people are diagnosed to have cancer in the world. The development of novel anticancer agents is paramount to achieve improved response rates to therapy among cancer persons. Nucleoside analogues have provided key medicines to treat various malignancies, including non-small cell lung cancer [26], pancreatic cancer, acute myeloid leukemia, bladder cancer, breast cancer and lymphomas [9]. The mechanism of action of nucleoside analogues is to replace molecular building-blocks of DNA, terminate DNA chain elongation and consequently inhibit cell division. FNC is a novel 2′-deoxy-2′-fluoro-4′-azido cytidine analogue [24]. It has been reported that FNC is an excellent substrate for deoxycytidine kinase and is phosphorylated with higher efficiency than deoxycytidine [25]. FNC is found to be a highly potent inhibitor of HCV replication [25]. In this study, we investigate the effect of FNC on human cancers and demonstrate that it regulates cell cycle, induces apoptosis and inhibits cancer cell growth *in vitro* and *in vivo*. Thus, FNC could be a new regimen for cancer patients.

Fludarabine, an adenine nucleoside analogue, induces cell cycle arrest at G0/G1 at 15 μM in human B cells [21]. However, gemcitabine, 2′,2′-difluoro-2′-deoxycytidine, results in an increase in the S-phase in a human acute myelogenous leukemia cell line, ML-1 and a pancreatic cancer cell line, MiaPaCa [11,18]. This S-phase arrest is the dose response in exponentially growing populations. Maximum S-phase arrest for a 24 h gemcitabine treatment occurred with 6–15 nM gemcitabine. Lower concentrations failed to arrest cells, whereas higher concentrations caused accumulation at the G1-S boundary in ML-1 cells [18]. In addition, Ara-C, or cytosine arabinoside, an another deoxycytidine analogue, is also found to regulate cell cycles at G1-S phase or S phase depending on dosage of Ara-C and cell cycle regulatory protein expression in acute myeloid leukemia (AML) cells [20] and the status of *Ras* oncogene in NSCL and colon carcinoma cell lines [8]. Interestingly, fludarabine also demonstrate to delay cell cycle progression, and subsequent parasynchronization of tumor cells into G2-M phase in the C3Hf/Kam mice bearing SA-NH sarcoma cells [22]. Consistent with above previous studies, our data demonstrated that FNC induced G1 and S phase arrest at high and low dose, respectively, in B-cell NHL cell lines. G2/M synchronization was also found in RL and SUDHL-6 cells treated with low dose (0.5 μM) at late time course (48 h). Accumulation of G2/M population is probably due to S phase arrest by FNC. Similarly, FNC induced S phase arrest and subsequent G2/M synchronization in solid tumors including pancreatic cancer cell lines, PANC-1 and BxPC-3 and colon cancer cell line SW480 even at high concentration. S phase arrest is important for killing tumor cells by nucleoside analogues because previous studies show gemcitabine and fludarabine induce apoptosis of cells in S phase [10,22].

Induction of apoptosis is a common cytotoxic property of gemcitabine, fludarabine and ara-C. Gemcitabine has been found to induce apoptosis *in vitro* and *in vivo* [10,17]. Incorporation of gemcitabine into DNA and S-phase arrest is critical in gemcitabine-

Table 2A
FNC (IV) inhibits tumor growth in H_{22} and S_{180} xenograft mice.

	Drug	Dosage ($mg\,kg^{-1}\,d^{-1} \times 8$)	Tumor weight ($\bar{x} \pm S$, g)	Tumor growth inhibition (%)
H_{22}	Control	Saline	1.21 ± 0.65	–
	5-Fu	15	0.46 ± 0.18	61.9*
	Cisplatin	1.0	0.58 ± 0.35	52.1*
	FNC	2.0	0.44 ± 0.50	63.6*
	FNC	1.0	0.72 ± 0.47	40.5*
	FNC	0.5	0.87 ± 0.59	28.1*
S_{180}	Control	Saline	1.64 ± 0.52	–
	5-Fu	15	0.62 ± 1.20	62.2*
	Cisplatin	1.0	0.63 ± 0.15	61.6*
	FNC	2.0	0.58 ± 0.17	64.6*
	FNC	1.0	1.00 ± 0.27	39.0*
	FNC	0.5	1.21 ± 0.35	26.2*

* Treatment versus control $p < 0.01$.

Table 2B
FNC (IG) inhibits tumor growth in H_{22} and S_{180} xenograft mice.

	Drug	Dosage ($mg\,kg^{-1}\,d^{-1} \times 8$)	Tumor weight ($\bar{x} \pm S$, g)	Tumor growth inhibition (%)
H_{22}	Control	Saline	1.66 ± 0.79	–
	Capecitabine	600	0.63 ± 0.17	62.0*
	FNC	2.0	0.56 ± 0.31	66.3*
	FNC	1.0	1.12 ± 0.70	32.5*
	FNC	0.5	1.44 ± 0.47	13.3
S_{180}	Control	Saline	1.64 ± 0.52	–
	Capecitabine	600	0.59 ± 0.15	64.0*
	FNC	2.0	0.56 ± 0.22	67.7*
	FNC	1.0	0.73 ± 0.16	55.5*
	FNC	0.5	1.09 ± 0.17	33.5*

* Treatment versus control $p < 0.01$.

Table 2C
FNC (IG) inhibits tumor growth in SGC7901 xenograft nude mice.

Drug	Dosage ($mg\,kg^{-1}\,d^{-1} \times 20$)	Tumor weight ($\bar{x} \pm S$, g)	Tumor growth inhibition (%)
Control	Saline	1.63 ± 0.13	–
Capecitabine	400	0.61 ± 0.09	63.2*
FNC	2.0	0.51 ± 0.12	68.7*
FNC	1.0	0.97 ± 0.20	40.5*
FNC	0.5	1.37 ± 0.24	16.0*

* Treatment versus control $p < 0.01$.

induced apoptosis. Moreover, bladder carcinoma cells with wild type *TP53* are more sensitive to apoptosis than *TP53*-mutant cells under gemcitabine treatment [23]. Similarly, the effectiveness of fludarabine and ara-C therapies relies on their abilities to induce apoptosis in tumor cells. Inhibition of DNA synthesis, arrest of cell cycle and activation of *TP53* are key events in the mechanism of induction of apoptosis [7,14,22]. Moreover, ara-C also can induce apoptosis in resting G_0-B-CLL cells using a mechanism independent of cell proliferation and DNA replication but associated with inhibition of RNA synthesis and downregulation of Mcl-1 [19]. As estimated, apoptosis was induced by FNC in both lymphoma and solid tumor cells.

The exisiting nucleoside analogues have critical problems such as emergence of drug-resistant, requirement of frequent and large doses of drugs and side effects of drugs. FNC, 2′-deoxy-2′-fluoro-4′-azido cytidine is designed to solve above problems based on the fundamentals of both organic chemistryand biochemistry, and past findings of relationship between biological activity and structure of nucleoside derivatives. The hypothetical fundamentals comprise the following two ways [27]. (**a**) Prevent emergence of drug-resistant virus: All clinical NRTIs belong to the family of 2′,3′-dideoxy-nucleoside (ddN). The ddN structure has been assumed essential for nucleoside derivative to be anti-viral active, i.e. to be the chain terminator of proviral DNA biosynthesis. However, resistance to all these clinical NRTIs is emerged. Resistance to these ddNs means that virus can acquire the ability to discriminate between ddN and physiologic 2′-deoxynucleoside (dN) and does not accept ddN into the active center of its reverse transcriptase (RT) and/or selectively cut off the incorporated ddN from its proviral DNA terminus. Therefore, the nucleoside (**N**) that could prevent the emergence of drug-resistant must satisfy the following two conditions: **N** must have 3′-OH and **N** must be the chain terminator of proviral DNA biosynthesis. FNC (2′-deoxy-2′-fluoro-4'-azido cytidine) will be difficult for virus to discriminate because it has all the functional groups of dN. And the neopentyl-type secondary 3′-OH of FNC would be too unreactive to be used for elongation of proviral DNA biosynthesis, FNC could be the chain-terminator of proviral DNA biosynthesis. So, FNC was designed as the nucleoside that can prevent emergence of drug-resistant virus. (**b**) Provide nucleosides with stability to both enzymatic and acidic glycolysis: The lone pair of the ring oxygen plays an important role in both enzymatic and acidic glycolysis of nucleosides by participating to form an oxocarbonium ion. The steric hindrance between the 4′-azido and 3′-OH of FNC changes the ring conformation into 3′-endo (N-type). It will be difficult for the lone pair of the ring oxygen of FNC with 3′-endo conformation to form oxocarbonium ion because the three bonds, C4-O-C1-C2, can not be co-planar easily. Thus, the introduction of azido at the 4′-position of nucleosides provide them with stability to both enzymatic and acidic glycolysis.

Given that FNC induces cell cycle arrest and apoptosis, it could inhibit tumor cell growth very well. Indeed, cell proliferation was inhibited by FNC at low μM in numerous cancer cell lines. Importantly, FNC significantly inhibited tumor growth in different mouse models without toxicity, suggesting FNC may be a useful agent for cancer therapy. In conclusion, this is the first time to show FNC, a novel nucleoside analogue, has anticancer activity *in vitro* and *in vivo*. Further studies should be conducted to ensure a clinical trial is warranted.

Acknowledgments

We are grateful to the National Natural Science Foundation of China (Outstanding Young Scholarship to J. Chang, #30825043) and the Outstanding Scholar Foundation of Henan Province (#094100510019) for financial support.

References

[1] Hoggard PG, Sales SD, Kewn S, Sunderland D, Khoo SH, Hart CA, et al. Correlation between intracellular pharmacological activation of nucleoside analogues and HIV suppression in vitro. Antivir Chem Chemother 2000;11:353–8.
[2] Wurtzer S, Compain S, Benech H, Hance AJ, Clavel F. Effect of cell cycle arrest on the activity of nucleoside analogues against human immunodeficiency virus type 1. J Virol 2005;79:14815–21.
[3] Major PP, Egan EM, Herrick DJ, Kufe DW. Effect of ARA-C incorporation on deoxyribonucleic acid synthesis in cells. Biochem Pharmacol 1982;31:2937–40.
[4] Ross DD, Cuddy DP. Consequences of 2′,2′-difluorodeoxycytidine (gemcitabine) on replicative DNA synthesis in intact HL-60 cells. Semin Oncol 1995;22:26–34.
[5] Bianchi V, Borella S, Rampazzo C, Ferraro P, Calderazzo F, Bianchi LC, et al. Cell cycle-dependent metabolism of pyrimidine deoxynucleoside triphosphates in CEM cells. J Biol Chem 1997;272:16118–24.
[6] Kufe DW, Major PP, Egan EM, Beardsley GP. Correlation of cytotoxicity with incorporation of ara-C into DNA. J Biol Chem 1980;255:8997–9900.
[7] Kobayashi T, Ruan S, Jabbur JR, Consoli U, Clodi K, Shiku H, et al. Differential p53 phosphorylation and activation of apoptosis-promoting genes Bax and Fas/APO-1 by irradiation and ara-C treatment. Cell Death Differ 1998;5:584–91.
[8] Koo HM, McWilliams MJ, Alvord WG, Vande Woude GF. Ras oncogene-induced sensitization to 1-beta-D-arabinofuranosylcytosine. Cancer Res 1999;59:6057–62.
[9] Lin TS, Blum KA, Fischer DB, Mitchell SM, Ruppert AS, Porcu P, et al. Flavopiridol, fludarabine, and rituximab in mantle cell lymphoma and indolent B-cell lymphoproliferative disorders. J Clin Oncol 28:418–23.
[10] Milas L, Fujii T, Hunter N, Elshaikh M, Mason K, Plunkett W, et al. Enhancement of tumor radioresponse in vivo by gemcitabine. Cancer Res 1999;59:107–14.
[11] Sun D, Urrabaz R, Kelly S, Nguyen M, Weitman S. Enhancement of DNA ligase I level by gemcitabine in human cancer cells. Clin Cancer Res 2002;8:1189–95.
[12] Kaye SB. New antimetabolites in cancer chemotherapy and their clinical impact. Br J Cancer 1998;78(Suppl. 3):1–7.
[13] Noble S, Goa KL. Gemcitabine. A review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. Drugs 1997;54:447–72.
[14] Eissner G, Multhoff G, Gerbitz A, Kirchner S, Bauer S, Haffner S, et al. Fludarabine induces apoptosis, activation, and allogenicity in human endothelial and epithelial cells: protective effect of defibrotide. Blood 2002;100:334–40.
[15] Huang P, Chubb S, Plunkett W. Termination of DNA synthesis by 9-beta-D-arabinofuranosyl-2-fluoroadenine. A mechanism for cytotoxicity. J Biol Chem 1990;265:16617–25.
[16] Huang P, Plunkett W. Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. Cancer Chemother Pharmacol 1995;36:181–8.
[17] Huang P, Plunkett W. Induction of apoptosis by gemcitabine. Semin Oncol 1995;22:19–25.
[18] Shi Z, Azuma A, Sampath D, Li YX, Huang P, Plunkett W. S-Phase arrest by nucleoside analogues and abrogation of survival without cell cycle progression by 7-hydroxystaurosporine. Cancer Res 2001;61:1065–72.
[19] de Vries JF, Falkenburg JH, Willemze R, Barge RM. The mechanisms of Ara-C-induced apoptosis of resting B-chronic lymphocytic leukemia cells. Haematologica 2006;91:912–9.
[20] Radosevic N, Delmer A, Tang R, Marie JP, Ajchenbaum-Cymbalista F. Cell cycle regulatory protein expression in fresh acute myeloid leukemia cells and after drug exposure. Leukemia 2001;15:559–66.
[21] Baran-Marszak F, Feuillard J, Najjar I, Le Clorennec C, Bechet JM, Dusanter-Fourt I, et al. Differential roles of STAT1alpha and STAT1beta in fludarabine-induced cell cycle arrest and apoptosis in human B cells. Blood 2004;104:2475–83.
[22] Gregoire V, Van NT, Stephens LC, Brock WA, Milas L, Plunkett W, et al. The role of fludarabine-induced apoptosis and cell cycle synchronization in enhanced murine tumor radiation response in vivo. Cancer Res 1994;54:6201–9.
[23] da Silva GN, de Castro Marcondes JP, de Camargo EA, da Silva Passos Junior GA, Sakamoto-Hojo ET, Salvadori DM. Cell cycle arrest and apoptosis in TP53 subtypes of bladder carcinoma cell lines treated with cisplatin and gemcitabine. Exp Biol Med (Maywood) 235:814–24.
[24] (a). Chang J, Bao X, Wang Q, Guo X, Wang W, Qi X. Preparation of 2′-fluoro-4′-substituted nucleoside analogs as antiviral agents. Chinese Patent Application No: CN 2007-10137548, 20070807; Chinese Patent No: CN 101177442A, 20080514;
(b). Chang J. 2′-Fluoro-4′-substituted nucleosides, the preparation and use. International Application No: PCT/CN2008/001239, 20080627; International Patent No: WO2009009951, 20090122
(c). Chang J. 2′-Fluorine-4′-substituted nucleoside analogues, preparation methods and uses thereof. US Patent No: US 2010/0234584A1, 20100916
(d). Chang J, Wang Q, Wang Q. 2′-fluoro-4′-substituted nucleoside analogs and application. Chinese Patent Application No: CN 200910227556.3, Chinese Patent No: CN 201010506595X;
(e) Smith DB, Kalayanov G, Sund C, Winqvist A, Maltseva T, Leveque VJ, et al. The design, synthesis, and antiviral activity of monofluoro and difluoro analogues of 4′-azidocytidine against hepatitis C virus replication: the discovery of 4′-azido-2′-deoxy-2′-fluorocytidine and 4′-azido-2′-dideoxy-2′,2′-difluorocytidine. J Med Chem 2009;52:2971–8.
[25] Klumpp K, Kalayanov G, Ma H, Le Pogam S, Leveque V, Jiang WR, et al. 2′-Deoxy-4′-azido nucleoside analogs are highly potent inhibitors of hepatitis C virus replication despite the lack of 2′-alpha-hydroxyl groups. J Biol Chem 2008;283:2167–75.
[26] Giovannetti E, Mey V, Danesi R, Basolo F, Barachini S, Deri M, et al. Interaction between gemcitabine and topotecan in human non-small-cell lung cancer cells: effects on cell survival, cell cycle and pharmacogenetic profile. Br J Cancer 2005;92:681–9.
[27] Ohrui H, Kohgo S, Hayakawa H, Kodama E, Matsuoka M, Nakata T, et al. 2′-Deoxy-4′-C-ethnyl-2-fluoroadenosine: A nucleoside reverse transcriptase inhibitor with highly potent activity against all HIV-1 strains, favorable toxic profiles and stability in plasma. Nucleic Acids Symposium Series, Oxford Univeristy Press, 2006;50:1–2.