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

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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

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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