The pyrimidine analog FNC inhibits cell proliferation and viral protein synthesis in HTLV-1-infected cells

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Abstract. Human T-cell leukemia virus type 1 (HTLV-1), the first retrovirus to be identified, is the etiological agent of an aggressive clonal malignancy of mature $CD4^+$ T lymphocytes known as adult T-cell leukemia (ATL). The prognosis of ATL patients remains poor despite the availability of a number of clinical chemotherapy drugs. In addition, HTLV-1-infected and ATL cells possess an intrinsic resistance to anticancer drugs. 2'-Deoxy-2'-β-fluoro-4'-azidocytidine (FNC) is a novel pyrimidine analog that is efficiently phosphorylated by cellular kinases and is a substrate for RNA and DNA polymerases. In the present study, the antiviral potential of FNC was investigated in HTLV-1-infected cell lines. Following FNC treatment, the HTLV-1-infected cells underwent G_1 or S phase cell cycle arrest. FNC was also observed to reduce cell growth of the HTLV-1-infected cell lines in a dose-dependent manner. Notably, FNC was found to efficiently inhibit the expression of the viral proteins, Tax and p19Gag, in a dose- and time-dependent manner. Treatment with FNC and the protein biosynthesis inhibitor, cycloheximide (CHX), accelerated the inhibition of viral protein synthesis in the HTLV-1-infected cells. Collectively, these results demonstrated the efficient antiretroviral effect of FNC in HTLV-1-infected cells and indicate that FNC may be utilized as a valuable therapy in HTLV-1-infected patients and those with ATL.

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Introduction

Human T-cell leukemia virus type I (HTLV-1) is an oncogenic retrovirus that causes adult T-cell leukemia/lymphoma (ATL), an aggressive clonal malignancy of mature $CD4^+$ T lymphocytes. ATL has been shown to be the first disease caused by human retroviral infection (1). A number of anticancer drugs have been used in the clinic to treat diseases caused by HTLV-1 infection. However, the prognosis of ATL patients remains poor due to the resistance of ATL cells to anticancer drugs (2). The combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and a protease inhibitor (PI) or a non-NRTI (NNRTI) is used to control retroviral HIV infection and this type of antiretroviral therapy has been found to significantly reduce mortality rates (3). Since ATL is a malignant disease caused by a retrovirus, it is possible that HTLV-infected and ATL patients may be treated by NRTIs, PIs or NNRTIs or the combination of these inhibitors.

At present, 6 NRTIs and 1 nucleotide reverse transcriptase inhibitor are utilized clinically for the treatment of HIV, including 2 thymidine, 2 cytidine, 1 adenine and 2 purine nucleotide analogs (3). Nucleoside analogs may incorporate into nascent viral DNA and cause premature chain termination, leading to an antiviral effect. 2'-Deoxy-2'-β-fluoro-4'-azidocytidine (FNC) is a novel pyrimidine analog that is phosphorylated by cellular kinases and acts as a substrate for RNA and DNA polymerases (4). Previous studies have demonstrated that FNC is a potent inhibitor of hepatitis C virus (HCV) (5) and human and duck hepatitis B virus (HBV) replication (6). In addition, FNC inhibits cell proliferation and promotes apoptosis in a number of human cancer cell lines (4). In the present study, the antiretroviral potential of FNC was investigated in HTLV-1-infected cell lines. Following FNC treatment, the HTLV-1-infected cells underwent G_1 and S phase cell cycle arrest and cell proliferation was inhibited. The expression of the viral proteins, Tax and p19Gag, was repressed by FNC and the combination of FNC and the protein biosynthesis inhibitor, cycloheximide (CHX), was observed to accelerate the inhibition of viral protein synthesis in the HTLV-1-infected cells.

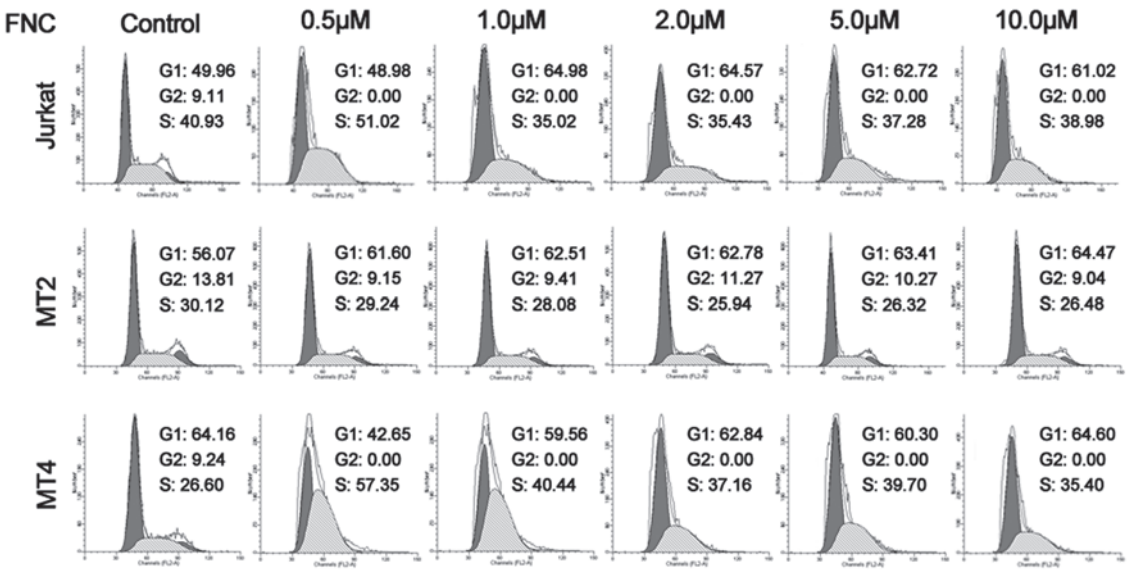

Figure 1. Cell cycle analysis following FNC treatment. Jurkat, MT2 and MT4 cells were treated with the indicated concentrations of FNC for 24 and 48 h. Untreated and treated cells were stained with a mixture of propodium iodide buffer followed by a cell sorting analysis. Acquired data were analyzed by ModFit LT software and the percentages of cells in each cell cycle phase (G_1, G_2, S) were presented. FNC, 2'-deoxy-2'-β-fluoro-4'-azidocytidine.

Materials and methods

Cells and reagents. The human T leukemia cell line, Jurkat, and the HTLV-1-infected cell lines, MT2 and MT4, were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine and antibiotics at 37˚C in 5% CO_2. The FNC was kindly provided by Professor Junbiao Chang (Zhengzhou University, Zhengzhou, China) and the CHX was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). The study was approved by the ethics committee of Xinxiang Medical University.

Antibodies. The anti-Tax mouse monoclonal antibody (mAb) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and the anti-HTLV-1 p19Gag mAb was purchased from Abcam (Cambridge, UK). The anti-β-actin mouse mAb and horseradish peroxidase-linked goat anti-mouse immunoglobulin G were purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China).

Cell cycle analysis. Cells treated with FNC for 24 h were collected and washed twice with PBS, fixed in cold ethanol (100%) to a final concentration of 70% and incubated at 4˚C overnight. The fixed cells were pelleted and incubated with RNase A (10 μg/ml) and propidium iodide (50 μg/ml). The DNA content was determined on a BD FacsCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the cell cycle profile was analyzed by ModFit LT software (Verity Software House, Topsham, ME, USA).

Cell proliferation assay. The effect of FNC on the cell proliferation was assayed by Cell Counting kit-8 (Beyotime, Shanghai, China). In brief, $1x10^4$ cells were incubated in a 96-well plate in the absence or presence of various concentrations of FNC. Following 48 h or the indicated culture times, 10 μl WST-8 solution was added and the cells were incubated for a further 2 h. The number of cells was measured with a microplate reader at a test wavelength of 450 nm. Cell viability was determined as a percentage of the control. All cell proliferation assays were performed in triplicate and repeated in three independent experiments.

Western blot analysis. Whole cell lysates were extracted from cells suspended in radioimmunoprecipitation assay buffer supplemented with 1 mM PMSF (both Beyotime). The lysates were resolved by electrophoresis on polyacrylamide gels containing 0.1% SDS, prior to being transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in Tris Buffer Saline Tween 20 (TBST) buffer. The blots were incubated with the appropriate primary antibody diluted in TBST and then exposed to the appropriate second antibody conjugated with horseradish peroxidase following a TBST wash. The bands on the membrane were visualized and captured using the ECL reagent (Beyotime) and X-ray films.

Results

FNC induces G_1 or S phase cell cycle arrest. As FNC is a nucleoside analog that induces DNA synthesis blockage, the present study performed a cell cycle analysis to investigate the effect of FNC on cell cycle progression. The HTLV-1-uninfected cell line, Jurkat, and two HTLV-1-infected cell lines, MT2 and MT4, were treated with FNC at various concentrations for 24 h (Fig. 1). As predicted, S phase arrest was induced by FNC at low doses of 0.5 μM in the Jurkat and MT4 cells. However, no S phase arrest was observed in the MT2 cells even when they were treated with FNC at high doses. The cell numbers in the G_1 phase were decreased by treatment with FNC at a low dose of 0.5 μM, followed by a weak increase in the G_1 phase arrest in the MT2 cells when higher concentrations of FNC were delivered. Notably, as the concentration of FNC increased, the

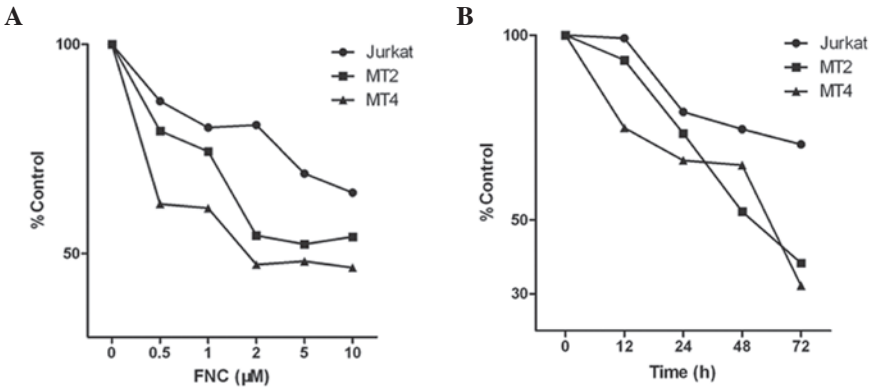

Figure 2. FNC reduces cell proliferation. The Jurkat, MT2 and MT4 cell lines were (A) cultured in the presence or absence of FNC for 48 h or (B) treated with 5 μM FNC for 12, 24, 48 and 72 h, with cell growth assessed using CCK-8. Results are expressed as the percentage of the control and represent the mean of 3 independent experiments. FNC, 2'-deoxy-2'-β-fluoro-4'-azidocytidine; CCK-8, Cell Counting kit-8.

FNC-induced S phase arrest decreased and the G_1 phase arrest increased. These results indicate that FNC induces G_1 or S phase cell cycle arrest in leukemia and HTLV-1-infected cells.

FNC inhibits the proliferation of HTLV-1-infected cell lines. To evaluate the role of FNC in the cell growth of HTLV-1-infected cell lines, the cells were treated with various concentrations of FNC for 48 h. A WST-8 assay revealed that FNC reduced the cell growth of the 2 HTLV-1-infected cell lines, MT2 and MT4, in a dose-dependent manner. However, FNC only weakly affected the cell growth of the uninfected T-cell line, Jurkat, even at high concentrations (Fig. 2A). Since the inhibition of cellular DNA synthesis by FNC is associated with the arrest of cell division, the inhibitory effect of FNC on cell growth was investigated at various times. Following 72 h of the FNC (5 μM) treatment, significant inhibition of cell proliferation was identified in the HTLV-1-infected cell lines compared with the uninfected cells (Fig. 2B). These results indicate that FNC effectively inhibits the cell growth of HTLV-1-infected cells and that these cells are more sensitive to FNC than uninfected cells.

FNC inhibits the synthesis of viral proteins in HTLV-1-infected cell lines. FNC is an excellent substrate for the RNA polymerases and is phosphorylated more efficiently than deoxycytidine (5). As a moderate inhibitor of RNA polymerase, FNC efficiently inhibits the synthesis of RNA, including retroviral RNA and the mRNA of viral proteins. The inhibition of RNA synthesis leads to the blockage of protein expression. Tax, encoded by HTLV-1, has been implicated as the major transforming protein of HTLV-1 and is required for the induction of cellular transformation (7). An additional viral structural protein, $p19^{Gag}$, is also a vital component of HTLV-1. Therefore, the effect of FNC on the expression of these important viral proteins was measured. Following treatment with FNC, the expression of Tax, Tax-env (a known fusion between the envelope and the Tax-coding sequence) (8,9) and $p19^{Gag}$ was examined in the HTLV-1-infected cell lines by western blot analysis. FNC reduced the expression of the viral proteins in a dose-dependent manner in the HTLV-1-infected cell lines, MT2 and MT4 (Fig. 3A and B). The expression levels of Tax-env and p19Gag were decreased by more than half in the MT2 cells when they were treated with high doses of ≥1 μM for 24 h (Fig. 3A). In the MT4 cells, a lower level of Tax expression was detected in the presence of FNC (Fig. 3B). In addition, FNC further inhibited the expression of the viral proteins when the cells were treated for a longer period of time (Fig. 3C and D). Following treatment with 2 and 5 μM FNC, the HTLV-1-infected cells expressed low levels of Tax-env, Tax and $p19^{Gag}$. FNC also inhibited the expression of the viral proteins in a time-dependent manner.

Since FNC inhibits protein expression largely through the blockage of mRNA synthesis, we next examined whether FNC inhibited viral protein expression when combined with other protein biosynthesis inhibitors. The HTLV-1-infected cells were treated with CHX in the absence or presence of FNC and then the expression of the viral proteins was detected by western blot analysis (Fig. 3E and F). Consistent with our predictions, FNC accelerated the inhibition of Tax-env, p19Gag and Tax expression by CHX in the MT2 and MT4 cells. These results demonstrate that FNC has an efficient inhibitory effect on the synthesis of viral proteins in HTLV-1-infected cells.

Discussion

HTLV-1 was the first human retrovirus associated with cancer to be isolated. Currently, it is estimated that 10-20 million individuals worldwide are infected with HTLV-1 (10) and ~3-5% of the infected population develop the T-cell malignancy, ATL (11). Treatments for ATL are extremely limited and the prognosis for this disease has not improved despite the availability of intensive chemotherapy. The poor prognosis is associated with the resistance of ATL cells to chemotherapy drugs. Therefore, it is important to find more effective drugs for the treatment of HTLV-1-infected patients and those with ATL. FNC, a novel pyrimidine analog, exhibits highly potent and selective inhibition of HCV replication. A recent study revealed that FNC inhibits the replication of human HBV *in vitro* and inhibits duck HBV replication *in vivo* (6). Thus, the aim of the present study was to examine the antiretroviral potential of FNC in the HTLV-1-infected cell lines. The present study demonstrated that treatment with FNC induces the G_1 and S phases of the cell cycle, growth arrest and the inhibition of viral protein synthesis *in vitro,* indicative of an efficient antiretroviral effect of FNC in HTLV-1-infected cell lines.

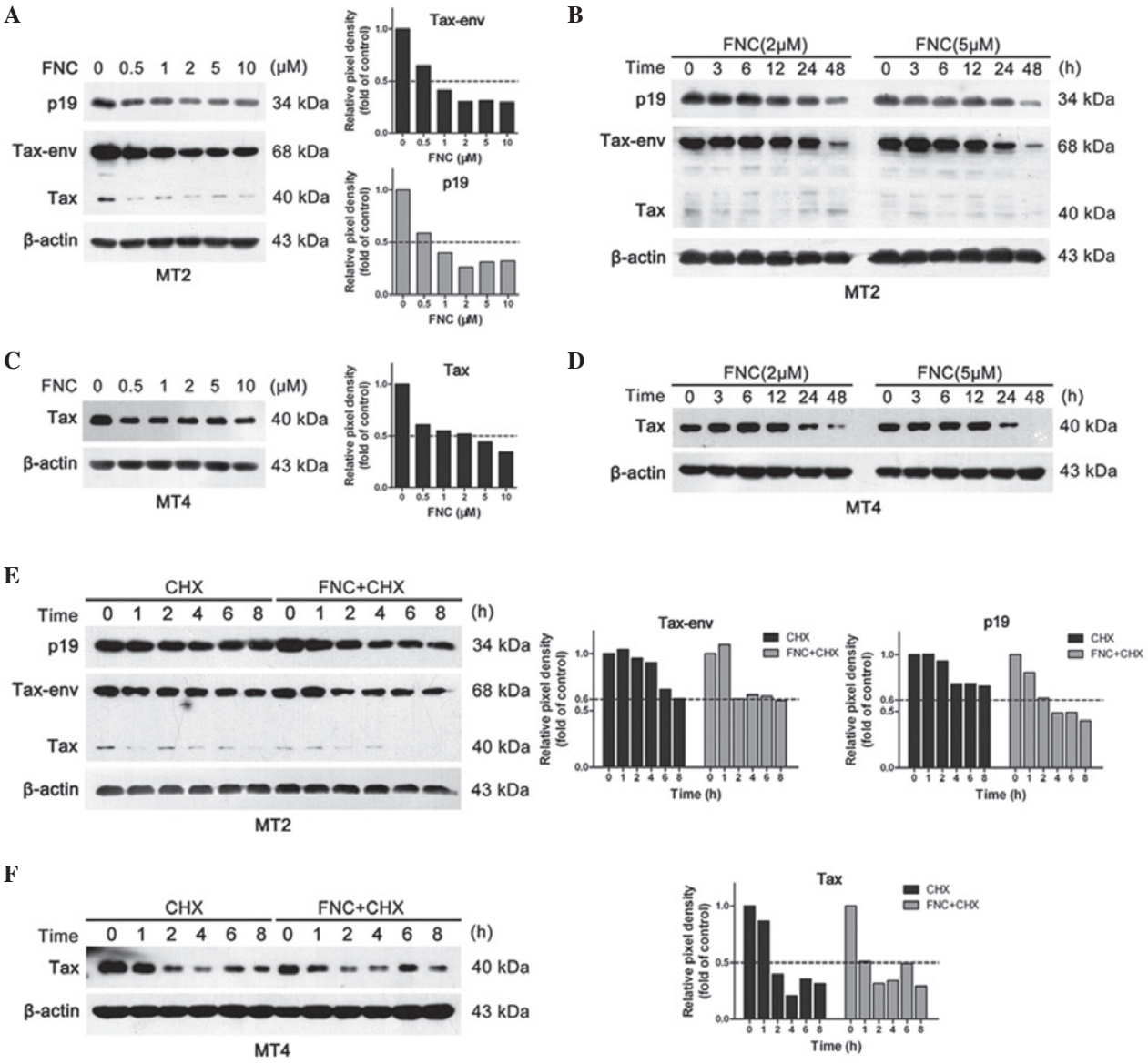

Figure 3. FNC inhibits the expression of viral proteins. (A) MT2 and (B) MT4 cells were treated with FNC at the indicated concentrations for 24 h. Whole cell lysates were extracted and assessed by western blot analysis for Tax and HTLV-1 $p19^{Gag}$ protein expression. (C) MT2 and (D) MT4 cells were treated with 2 and 5 μM FNC for the indicated times. Whole cell lysates were extracted and Tax and HTLV-1 p19Gag protein expression was assessed by western blot analysis. (E) MT2 and (F) MT4 cells were treated with CHX (50 μg/ml) alone or in combination with FNC (5 μM) for the indicated times. Whole cell lysates were extracted and assessed by western blot analysis for Tax and HTLV-1 $p19^{Gag}$ expression. Pixel densities of all the blots were quantified using Image J 1.44 software. The graphs show the pixel density for various proteins normalized to β-actin. FNC, 2'-deoxy-2'-β-fluoro-4'-aziocytidine; CHX, cycloheximide; HTLV-1, human T cell leukemia virus type 1.

As a nucleoside analog, FNC may incorporate into DNA and block the extension of the DNA strand. Therefore, a cell cycle analysis was performed in the present study in order to confirm the inhibitory effect of FNC on cell mitosis. The expected G_1 and S growth arrest was observed when the uninfected and HTLV-1-infected cells were treated with FNC. Fludarabine, an adenine nucleoside analog, induces cell cycle arrest at G_0/G_1 (12) and FNC has previously been reported to induce cell cycle arrest in a number of human B-cell lines (4). Numerous nucleoside analogs are used in the treatment of cancer and virus-associated diseases, and abnormal mitosis may always be induced by these analogs (13). FNC may be phosphorylated by cellular kinases and this FNC triphosphate may be then be utilized by DNA replication as a decoy substrate, which once incorporated into a DNA strand causes premature chain termination due to the absence of a 3'-hydroxyl group on the nucleoside sugar.

Cell cycle arrest constantly results in a failure of cell mitosis, which induces the inhibition of cell proliferation. In the present study, FNC was found to suppress the cell growth of three human T-cell lines by the induction of cell cycle arrest. FNC-induced cell growth arrest was observed when the cells were treated with low dose FNC (0.5 μM) and the inhibition occurred within 12 h in the HTLV-1-infected cell lines (Fig. 2B). In the Jurkat cells, only a weak inhibition was observed, whereas intense suppression was detected in the HTLV-1-infected cell lines, indicating that infected cells are more sensitive to FNC. A significant difference in

FNC-induced inhibition was identified in the uninfected and HTLV-1-infected cells when the treatment lasted 72 h.

It has also been reported that the HTLV-1 virus contains a number of structural and enzymatic proteins that are essential for retrovirus formation, infection and transmission (1). Among the proteins encoded by HTLV-1, Tax is critical for viral transcription and cell transformation (14,15). It has also been reported that FNC is an excellent substrate for deoxycytidine kinase and RNA polymerase. The present results reveal that FNC inhibits Tax synthesis in two HTLV-1-infected cell lines, as well as the viral matrix protein, $p19^{Gag}$, in MT2 cells. Unexpectedly, $p19^{Gag}$ (34kDa) was not detected in the cell lysate of the MT4 cells. This may be due to a reduction in $p19^{Gag}$ production in the MT4 cells (16). However, the $p19^{Gag}$ expression was markedly inhibited by FNC in the MT2 cells, indicating that FNC efficiently inhibits the replication of HTLV-1. Notably, MT2 cells possess a marked ability to induce transformation, indicating that $p19^{Gag}$ expression may be essential for viral transmission. In addition, the FNC-induced inhibition was sustained over a significant period of time (Fig. 3C and D), indicating that FNC is a drug with prolonged effects. The inhibition of viral protein synthesis and replication is due to the premature termination of mRNA synthesis caused by FNC. The decreased levels of viral proteins observed in the present study were caused by the inhibition of new protein synthesis and protein degradation. In addition, FNC was identified to accelerate the CHX inhibition of protein synthesis.

In conclusion, FNC is a novel and potent inhibitor of viral protein synthesis in HTLV-1-infected cells. As a potential antiviral drug, FNC efficiently inhibits HTLV-1 replication and the synthesis of the key viral protein, Tax, *in vitro*. Further investigation must be performed to develop FNC as an antiviral agent for the treatment of HTLV-1-infected patients and those with ATL.

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