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A novel synthetic novobiocin analog, FM-Nov17, induces DNA damage in CML cells through generation of reactive oxygen species

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ABSTRACT

Objectives: To investigate the cytotoxicity of FM-Nov17 against chronic myeloid leukemia (CML) cells, we explored its underlying mechanisms mediating the induction of DNA damage and apoptotic cell death by reactive oxygen species (ROS).

Methods: MTT assays were used to measure the proliferation-inhibition ratio of K562 and K562/G01 cells. Flow cytometry (FCM) was used to test the level of extracellular ROS, DNA damage, cell cycle progression and apoptosis. Western blotting was used to verify the amount of protein.

Results: FM-Nov17 significantly inhibited the proliferation of K562 cells, with an IC_{50} of 58.28 ± 0.304 μM, and K562/G01 cells, with an IC_{50} of 62.36 ± 0.136 μM. FM-Nov17 significantly stimulated the generation of intracellular ROS, followed by the induction of DNA damage and the activation of the ATM–p53–r-H2AX pathway and checkpoint-related signals Chk1/Chk2, which led to increased numbers of cells in the S and G_2/M phases of the cell cycle. Furthermore, FM-Nov17 induced apoptotic cell death by decreasing mitochondrial membrane potential and activating caspase-3 and PARP. The above effects were all prevented by the ROS scavenger N-acetylcysteine.

Conclusions: FM-Nov17-induces DNA damage and mitochondria-dependent cellular apoptosis in CML cells. The process is mediated by the generation of ROS.

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder that is associated with BCR-ABL, a constitutively activated tyrosine kinase (PTK), which is the product of the Philadelphia (Ph) chromosome [1]. Imatinib (STI571) has proven highly effective in reducing leukemic cells in CML. However, clinically, increasing drug resistance has been attributed to BCR-ABL overexpression, as well as T315I gene mutations [2,3] and leukemia stem cell mutations [4,5]. Therefore, it is imperative to develop novel therapeutic interventions to overcome imatinib resistance.

Excessive production of ROS has been implicated in cell death by causing DNA damage-induced cell cycle arrest in several cancer cells [6–10]. ROS not only decrease the mitochondrial transmembrane potential ($\Delta\Psi_m$) but also stimulate the release of cytochrome c, leading to mitochondrial-dependent apoptosis in a variety of human cancer cells [11,12].

A previous study demonstrated that novobiocin (Nov) was a potent inhibitor of cell growth in Bcr-Abl positive human leukemia cells [13]. Due to its low anti-tumor activation (high IC_{50} value (500 μM)), Nov primarily acts as an adjuvant, enhancing the effects of other cytotoxic chemotherapies and overcoming drug resistance [14–17]. Therefore, it is desirable to develop new and more potent novobiocin derivatives and to further elucidate the underlying anti-tumor mechanisms of the drug. Our group synthesized a series of Nov derivatives, including FM-Nov17 (Fig. 1a), which is one of the most active Nov

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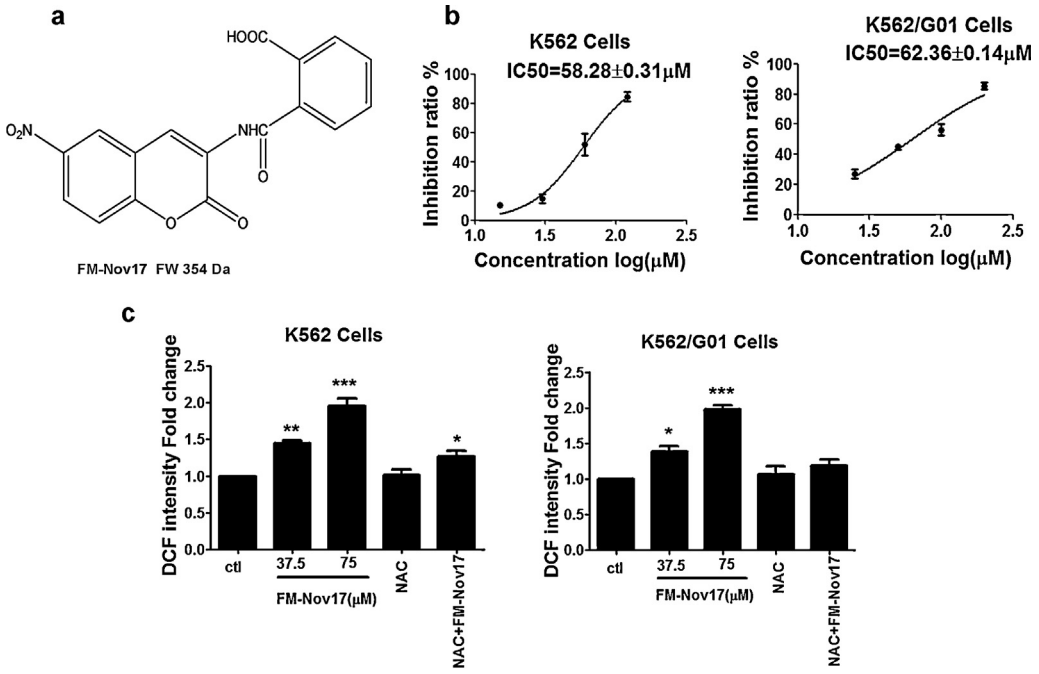

Fig. 1. FM-Nov17 inhibits the proliferation and intracellular ROS levels of K562 and K562/G01 cells. K562 and K562/G01 cells were incubated with 37.5 μM and 75 μM FM-Nov17 for 48 h. (a) The chemical structure of FM-Nov17. The molecular weight of FM-Nov17 is 354 Da. (b) Cell viability was evaluated by the MTT method. Data were plotted as a function or percentage of cell viability based on controls *vs.* drug concentrations. All studies were performed in triplicate. The concentration of drug required for 50% growth inhibition (IC50) was estimated using GraphPad Prism5 software. (c) K562 and K562/G01 cells were incubated with 37.5 μM and 75 μM FM-Nov17 for 24 h. Quantification of DCF intensity fold increase after FM-Nov17 treatment. All data were expressed as the mean ± SE ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, NS $p > 0.05$, $n = 3$).

derivatives. In this study, we investigated the anti-tumor mechanisms of FM-Nov17. We demonstrated that FM-Nov17 induces DNA damage, increases the number of cells in the G_2/M phases, and increases mitochondrial-dependent apoptotic cell death by generating ROS in both imatinib-sensitive and -resistant CML cells.

Materials and methods

Cell lines and reagents

Human leukemic K562 cells were cultured and passaged in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine (medium A) in a 5% humidified CO_2 atmosphere at 37 °C. Imatinib-resistant K562/G01 cells were purchased from the Institute of Hematology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China. The K562/G01 cells were maintained in medium A containing or lacking 4 μM imatinib. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) was purchased from Sigma–Aldrich, Inc. (MO, USA). FM-Nov17 was synthesized by our lab with 95% purity, and dissolved in 0.1% dimethyl sulfoxide (DMSO) solution, with DMSO serving as a vehicle control. Monoclonal antibodies against poly(ADP-ribose) polymerase (PARP), cleaved-caspase3, cytochrome c, r-H2AX (pS139), p-ATM (ser 1981), CDC25A, CDC25C, and β-actin were purchased from Cell Signaling Technology, Inc. (MA, USA). A polyclonal secondary antibody to mouse/rabbit IgG-H&L was purchased from Nanjing Keygen Biotech Co. Ltd (Nanjing, China). An Annexin-V-Fluos staining kit was purchased from Roche Diagnostics (IN, USA). A DNA damage detection kit was purchased from BD Corporation, and an ROS detection kit was purchased from Beyotime Company (Jiangsu, China).

Cell proliferation and viability assays

Cells were plated in 96-well plates (5×10^3 cells/well) in growth media and treated with varying concentrations of FM-Nov17 for 48 h. After treatment, the number of remaining viable cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric dye-reduction method. All studies were performed in triplicate. The concentration of the drug at which cell growth was inhibited by 50% (IC_{50}) was estimated using GraphPad Prism 5 software.

Cell apoptosis examined by Annexin V-FITC and PI staining

After 48 h treatment with FM-Nov17, cells were harvested and suspended with 5 mL of phosphate-buffered saline (PBS), followed by the addition of 5 μL of Annexin V-FITC and 5 μL of PI. The mixture was incubated at 37 °C in the dark for 15 min and transferred to the FCM tubes to measure cell fluorescence. Cells undergoing early stage apoptosis were initially Annexin-V positive. As apoptosis increased, the cells were positive for both Annexin-V and propidium iodide (PI).

Flow cytometry

K562 and K562/G01 cells were seeded onto six-well plates and treated with FM-Nov17 for different time periods. The cells were collected and fixed overnight in 70% ethanol at −20 °C. Then, after two washes with 1× PBS, the cells were incubated at room temperature in the dark for 20 min with 1 μL of PI solution (50 μg/mL). FACScan cytometry (Becton–Dickinson) based on red fluorescence was used to analyze the relative DNA content of these cells. Quantification of the fraction of cells in each cell cycle stage was performed with ModFit LT for Mac 3.0 software (Becton–Dickinson).