Leukemia Research 35 (2011) 1074–1079

Contents lists available at ScienceDirect

Leukemia Research

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

A small molecule significantly inhibits the bcr/abl fusion gene at the mRNA level in human chronic myelogenous leukemia

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ARTICLE INFO

Article history:
Received 19 October 2010
Received in revised form
16 November 2010
Accepted 19 November 2010
Available online 15 December 2010

Keywords:
H89465
2-Aminosteroid
K562
Meg-01
bcr/abl
T315I
CML

ABSTRACT

Bcr/abl fusion gene is the marker gene in chronic myelogenous leukemia (CML) and becomes the target for CML therapy. Although Imatinib opened a new way to treat CML, the resistance to the drug caused by bcr/abl fusion protein mutation stimulated search for new molecules to inhibit bcr/abl expression. In our research, it was found that a novel 2-aminosteroid (H89465) possessed special mechanism in treating CML. H89465 inhibits the proliferation of both non-resistant and resistant CML cells such as K562, Meg-01 and clinical primary CML cells. It prolongs the survival time of NOD/SCID mice inoculated with K562 leukemia cells. The mechanism underlying the effects is concerned with down-regulation of bcr/abl mRNA expression followed by decreasing the BCR/ABL protein expression and tyrosine kinase activity in CML cells. Our results demonstrate that H89465 possesses the therapeutic potential in treating human CML.

1. Introduction

Human chronic myelogenous leukemia (CML) was caused by the reciprocal gene translocation between chromosome 9 (abl) and 22 (bcr) [1] and among above 95% of patients who suffered CML could be found bcr/abl fusion gene [2]. Therefore, bcr/abl was recognized as the pathogenic gene for CML [3]. The tyrosine kinase inhibitor (TKI) Imatinib, a small molecule in its chemical structure, could inhibit the proliferation of CML cells and opened the new era in the treatment of CML in clinic [4]. Nowadays TKIs were widely used in clinic for treating CML and the mechanism referred the binding of TKI molecule with BCR/ABL protein followed by inhibiting tyrosine kinase activity in CML cells [5]. However, some CML cells could change the configuration of the BCR/ABL protein and obstacle the binding of the protein with TKI molecules, thus conferring drug resistance in the following treatment [6–8]. Therefore, the second and third generations of TKIs were developed, in which of them, some could circumvent the resistance of Imatinib [9]. However, no drug, which acted on bcr/abl mRNA level for inhibition, was reported until now except siRNA, which could inhibit bcr/abl oncogene at the mRNA level, but these kinds of molecules faced instant degradation by enzymes in human body and thus prevented their application in clinical treatment [10].

After screening, we discovered a small molecule, designated as H89465 (chemical structure in Fig. 1), that significantly inhibited the mRNA expression of bcr/abl fusion gene at the rooted level, both in non-resistant and in resistant CML cells, followed by inhibiting BCR/ABL protein expression and tyrosine kinase activity in leukemia cells. This small molecule also inhibited the growth of leukemia cells in NOD/SCID mice and eradicated the drug resistance caused by Imatinib in resistant leukemia cells. The data presented here suggest that this small molecule may hold promise for the treatment of human CML.

2. Materials and methods

2.1. Reagents and leukemia cells

The 2-aminosteroid H89465 was synthesized in our laboratory and its chemical structure was elucidated by LC/MS and other spectroscopy methods. The agent was dissolved in alcohol to make the stock solution at the concentration of 10^{-2} mol/L and stored at −20 °C in the dark for preparation. Imatinib was purchased from Novartis Company and dissolved in DMSO as the 10^{-2} mol/L stock solutions for application. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma–Aldrich (USA). The c-Abl mouse-anti-human antibody was purchased from Santa Cruz Biotechnology. K562 [11] and Meg-01 [12] leukemia cell lines were purchased from Shanghai Cell Collections and were maintained in RPMI 1640 medium (GIBCO, USA) with 10% fetal bovine serum (Sijiqing, China) in humidified air with 5% CO_2 at 37 °C. K562 and Meg-01 cells were seeded in 24-well plates (Corning, USA) at the final density of 5×10^4 cells/ml. The clinical primary CML and Imatinib-resistant CML cells were obtained with informed consent from peripheral

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doi:10.1016/j.leukres.2010.11.012

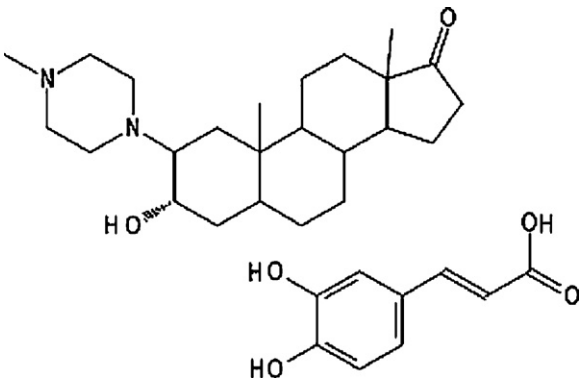

Fig. 1. Chemical structure of H89465.

blood of leukemia patients in Hunan Malignancy Hospital according to the regulations concerning human samples and were cultured in DMEM medium (GIBCO, USA) with 10% fetal bovine serum after MNCs were isolated by density centrifugation. In the experiments, the different CML cells were treated with H89465 or Imatinib for five days at 10^{-5} mol/L doses to obtain different data. All data represented were from at least three separate experiments.

2.2. Real-time quantitative RT-PCR

Total RNA from human K562, Meg-01 and CML leukemia cells was prepared using Trizol (Invitrogen, USA) and analyzed using an Agilent 2100 bioanalyzer (Agilent Technologies, USA). cDNA was synthesized from isolated RNA using random hexanucleotides and Superscript II reverse transcriptase (Invitrogen) on Gene Amp PCR System 9700 (Applied Biosystems, USA). Real-time PCR was performed on Rotor-Gene 3000 Real-time PCR analyzer (Corbett Research, USA) using the reverse-transcribed cDNAs and was monitored quantitatively by β-actin as the internal control. The gene-specific primers were designed by Primer Premier 5.0 software and were synthesized by KangChen Biotech (Shanghai, China). The relative amounts of amplified gene expressions were calculated by the fluorescence ratio between the treated and the untreated cell samples normalized by the β-actin housekeeping gene signal. The bcr/abl fusion gene and other gene primers are as follows in Table 1.

2.3. Western blot

Harvested cells (at least 1×10^{-6} cells for one isolation) were washed and the total cellular proteins were isolated by using the KC-415 ProtSep Kit according to the included instructions (KangChen, Shanghai). The protein was quantified by BCA KC-430 Kit and BSA was taken as the standard. The sample protein was separated by SDS-PAGE (Bio-Rad, USA) and transferred onto PVDF membrane (Bio-Rad). The membrane was incubated with anti-Abl antibody from Santa Cruz Biotechnology (Santa Cruz Biotechnology, USA) and stained with the second antibody conjugated with HRP for quantification. The gray value was scanned and analyzed by image software (ImageJ, USA).

2.4. Co-immunoprecipitation

Harvested cells were lysed in the lysis buffer (1% Triton X-100, 150 mM NaCl, 0.5% deoxycholate, 2.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml Pepstin-A, 2 μg/ml Aprotinin, 25 mM NaF, 0.5 mM sodium orthovanadate, 1.0 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride, 20 mM P-nitrophenyl phosphate) for 30 min, and the nuclear and cellular debris cleared by centrifugation. Anti-Abl antibody (Santa Cruz Biotechnology, USA) was added into the lytic solution and protein A-agarose beads were incubated with anti-Abl monoclonal antibody at 4 °C for overnight. After washing the mix of the protein A-beads and the antibody with the lysis buffer, the immunoprecipitates were eluted with the SDS sample-loading buffer. Proteins were separated by SDS-PAGE as described above and immunoprecipitates were examined by Western blot analysis after transfer of proteins onto the nitrocellulose membranes. Western blot analyses were performed with anti-phosphotyrosine antibody (anti-pTyr, PharMingen, USA) and with anti-Actin antibody as the control. Tyrosine kinase activity was quantified by DAB staining scan after the second antibody conjugated with HRP was added. The amounts of phosphorylated tyrosine residues were taken as the activities of tyrosine kinase in leukemia cells.

Table 1
Primers used in human gene amplifications for real-time RT-PCR.

Gene	Primer	Amplified base (bp)
Bcr/abl		
Sense	5′-GGAGCTGCAGATGCTGACCAAC-3′	200 (K562, CML)
Antisense	5′-TCAGACCCTGAGGCTCAAAGTC-3′	125 (Meg-01)
β-Actin		
Sense	5′-CCTGTACGCCAACACAGTGC-3′	211
Antisense	5′-ATACTCCTGCTTGCTGATCC-3′	

2.5. MTT assay

Leukemia cells were plated in triplicate at 5×10^4 per well on 24-well plate and exposed to increasing concentrations of H89465. The growth inhibition was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay after treatment for 5 days. The absorbance of MTT was read at 546 nm on a microreader. The doses that inhibited 50% proliferation (IC_{50}) were analyzed by the median-effect method (CalcuSyn Software, Biosoft, USA).

2.6. NOD/SCID mice inoculation

The NOD/SCID mice were purchased from Chinese Academy of Medical Sciences (Beijing, China) and were raised under the condition of giving commercial food and water ad libitum at 23 ± 5 °C and 55 ± 5% RH through the research. NOD/SCID mice 6–8 weeks of age were individually sublethally irradiated (2 Gy) and administered 1×10^6 human K562 leukemia cells via tail vein on day 0. Administration of H89465 and Imatinib were initiated on day 8 and the mice were divided into three groups: (1) the untreated control mice, (2) the mice intravenously via the tail vein administered 6.0 mg/kg of H89465 for every other day per week through 8 consecutive weeks and (3) the mice intravenously via the tail vein administered 60 mg/kg of Imatinib for every other day per week through 8 consecutive weeks. Each group contained ten mice. After 8 weeks of treatment, peripheral blood (PB) of mice was collected via the orbital plexus under anesthesia. The percentages of human K562 leukemia cells in PB were determined by flow cytometry. For survival analysis, mice death was determined either by spontaneous death or elective killing due to pain or suffering according to the established criteria. Survival data was analyzed by the Kaplan–Meier log-rank test.

2.7. Flow cytometry

Peripheral blood (PB) from NOD/SCID mice were suspended in PBS and the cells were mounted onto flow cytometry for event fluorescence determination after double staining with fluorescein isothiocyanate (FITC)-conjugated anti-human CD13 antibody or FITC-conjugated anti-human CD33 antibody and phycoerythrin (PE)-conjugated anti-human CD71 antibody (all from Becton–Dickinson) on ice for 30 min. The FITC- or PE-labeled mouse IgG was used as the negative controls.

2.8. Imatinib-resistant leukemia cells

Imatinib-resistant K562 and Meg-01 cells were established in our laboratory according to the reported methods [13,14] and kept in 1×10^{-6} mol/L Imatinib medium before H89465 treatment. Briefly, wild type K562 and Meg-01 cells were exposed to step-wise increasing concentrations of Imatinib. Subpopulations of cells that were able to grow in the presence of 1×10^{-6} mol/L Imatinib were then selected using limit dilution method. The clinical primary resistant CML cells were from the patients who procured Imatinib resistance after the disease proceeding and whose blood cells were found at a site mutation at the typical T315I position.

2.9. Statistical analysis

Significant differences between values obtained in a population of leukemia cells treated with different experimental conditions were determined using the Student *t*-test. *P* values of less than 0.05 were assigned significance.

3. Results

3.1. H89465 significantly inhibited bcr/abl mRNA expression in CML cells followed by inhibiting BCR/ABL protein expression and tyrosine kinase activity in CML cells

In the experiments, the different CML cells were treated with H89465 for five days at 10^{-5} mol/L doses to obtain different data. It was surprised to discover that H89465 significantly inhibited the bcr/abl mRNA expression in K562, Meg-01 and clinical primary CML cells and the mRNA inhibition was verified by real-time RT-PCR methods (Fig. 2A and D). By using the Western blot method, the BCR/ABL protein expressions in K562, Meg-01 and CML cells were also apparently inhibited by H89465 (Fig. 2B and E). In a typical TK activity assay, it was found that TK activities in K562, Meg-01 and