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Growth inhibition of imatinib-resistant CML cells with the T315I mutation and hypoxia-adaptation by AV65 – a novel Wnt/β-catenin signaling inhibitor

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ABSTRACT

We investigated the effect of a novel Wnt/β-catenin signaling inhibitor, AV65 on imatinib mesylate (IM)-sensitive and -resistant human chronic myeloid leukemia (CML) cells *in vitro*. AV65 inhibited the proliferation of various CML cell lines including T315I mutation-harboring cells. AV65 reduced the expression of β-catenin in CML cells, resulting in the induction of apoptosis. Moreover, AV65 inhibited the proliferation of hypoxia-adapted primitive CML cells that overexpress β-catenin. The combination of AV65 with IM had a synergistic inhibitory effect on the proliferation of CML cells. These findings suggest that AV65 could be a novel therapeutic agent for the treatment of CML.

1. Introduction

Chronic myeloid leukemia (CML) is a disorder of hematopoietic stem cells caused by constitutive activation of the Bcr-Abl tyrosine kinase [1]. Imatinib mesylate (IM) has dramatically improved the management of CML [2,3], but IM resistance is frequently observed, especially in patients with advanced-stage disease [4]. The second-generation Abl tyrosine kinase inhibitors (TKIs) including dasatinib [5], nilotinib [6], bosutinib [7], and bafetinib (INNO-406) [8], have been shown to overcome IM-resistance in CML. These agents, however, are ineffective in CML cells harboring the T315I mutation [9,10]. Another important cause of recurrence of CML is the existence of CML stem cells that are resistant to TKIs [11,12]. Granulocyte–macrophage progenitors from patients in the blast crisis phase of CML or with IM-resistant CML have elevated levels of nuclear β-catenin [13]. Recently, a microarray study of cells from CML patients in blast crisis revealed an activation of the Wnt/β-catenin pathway [14]. A recent gene profile study revealed the upregulation of β-catenin target genes in IM-resistant CML patients in the chronic phase [15]. Moreover, loss of β-catenin impairs the self-renewal of CML stem cells [16]. These observations indicate that Wnt/β-catenin signaling play a role in the maintenance of CML stem cells as well as IM-resistance. Moreover, Bcr-Abl stabilizes β-catenin through tyrosine phosphorylation [17].

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Therefore, the Wnt/β-catenin signaling pathway could be a promising therapeutic target for the treatment of CML.

Activation of Wnt/β-catenin signaling is closely linked to the process of carcinogenesis in solid tumors [18] as well as leukemia [19,20]. Using high-throughput transcriptional screening (HTS) technology, effective inhibitors of Wnt/β-catenin signaling were identified from a library of more than 100,000 chemical compounds [21,22]. From this initial series, a novel Wnt/β-catenin signaling pathway inhibitor named AV65 was selected and optimized. In the present report, the inhibitory effect of AV65 on the proliferation of various IM-sensitive and -resistant CML cell lines is demonstrated.

2. Material and methods

2.1. Reagents and cell lines

The human CML cell lines K562 and, MEG01, and the HL60 acute myeloid leukemia (AML) cell line, were obtained from the American Type Culture Collection (Manassas, VA). The KU812 and BV173 CML cell lines were obtained from the Japanese Collection of Research Biosources (Osaka, Japan) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH (Braunschweig, Germany), respectively. The KCL22 CML cell line was kindly provided by Dr. Tadashi Nagai (Jichi Medical School, Tochigi, Japan). The MYL and MYL-R1 CML cell lines were kindly provided by Dr. Hideo Tanaka (Hiroshima University, Japan). The MYL-R1 is a Lyn-overexpressing subline of MYL [23]. The KT-1 cell line was provided by Dr. Masaki Yasukawa (Ehime University, Japan) [24]. K562-IMR cells with Bcr-Abl upregulation and K562/D1-9 cells with P-glycoprotein (P-gp)-overexpression were kindly provided by Dr. Yoshimasa Urasaki and Dr. Dr. Takahiro Yamauchi, respectively (Fukui University, Japan). The KBM5 cell line and the KBM5/STI-R subclone harboring the T315I mutation were kindly provided by Dr. Miloslav Beran (MD Anderson Cancer Center, Houston, TX) [25,26]. Ba/F3 cell lines expressing Bcr-Abl/wild-type (wt), G250E, Q252H, Y253F, E255K, T315I, T315A, F317L, F317V, M351T, or H396P were established as previously described [8]. The parental Ba/F3 cell line was maintained in 10% WEHI-conditioned medium as a source of IL-3. Two hypoxia-adapted (HA-) CML cell lines were generated, and these hypoxia-adapted sublines from K562 and KCL22 are denoted as K562/HA and KCL22/HA, respectively. Both cell lines proliferate continuously under 1.0% O_2 for more than 1 year without any additional nutrient supplies. These cell lines are resistant to IM [27,28]. Cells were maintained as suspension cultures in RPMI1640 (Gibco, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FSC; Invitrogen, Tokyo, Japan), 2 mM L-glutamine (Gibco), and 1% penicillin–streptomycin (Gibco).

AV65, a novel Wnt/β-catenin inhibitor, was dissolved in dimethyl sulfoxide to a stock of 1 mM and stored in aliquots at −20 °C until use. The caspase inhibitor zVAD, which was purchased from the Peptide Institute (Osaka, Japan), was dissolved in dimethyl sulfoxide and stored at −20 °C until required for use. zVAD was used at 50 μM for K562 and BV173, as previously described [29]. MG132, a proteasome inhibitor, was purchased from Sigma–Aldrich (Tokyo, Japan).

2.2. Growth inhibitory effect of AV65 on CML cells

CML cell lines were exposed to AV65 for 72 h and cell proliferation was assessed using a modified MTT assay as previously described [8]. The combined effect of combination treatment with IM and AV65 was evaluated in K562 cells. Cells were incubated for 72 h with six concentrations (equivalent to 0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the IC_{50}) of AV65 alone or in combination with IM. We calculated the combination indexes (CIs) as reported previously [30,31], and the fraction affected (Fa) at each dilution was measured (an Fa of 0.25 equals 75% viable cells). This method provides a quantification of the synergism (CI < 1) and antagonism (CI > 1) between two drugs at different doses. Calculations of the CI were made under the assumption that the mechanisms of action of the evaluated drugs were not mutually exclusive. The inhibitory effects of AV65 on primary CML cells were also investigated using a colony assay. Bone marrow (BM) mononuclear cells obtained from CML patients and healthy volunteers (ALLCells, Emeryville, CA) were plated in duplicate in MethoCult H4434 Classic (StemCell Technologies Inc, Vancouver, Canada) and cultured at 37 °C in 5% CO_2. After 14 days of culture, colonies were evaluated under an inverted microscope.

2.3. Western blot analysis

Following treatment with AV-65 compounds, more than 1×10^6 cells were collected by centrifugation. Western blotting analysis was performed as previously described [27,32]. Antibodies (Abs) against β-catenin, cyclinD1, phosphorylated Erk1/2 (pT202/pY204) (BD, Tokyo, Japan), Oct-1, c-Myc, Stat5 (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated β-catenin (Ser33/37/Thr41), survivin, Erk1/2, Akt, c-Abl, phosphorylated Akt (Ser473), phosphorylated c-Abl (Tyr245), phosphorylated Stat5 (Tyr694) (Cell Signaling Technology, Danvers, MA), Actin (Sigma–Aldrich, Tokyo, Japan), and GAPDH (CHEMICON, MA, USA) were used as primary Abs. Horseradish peroxidase-coupled IgG (Amersham Biosciences, Tokyo, Japan) was used as a secondary Ab, and immunoreactive proteins were detected by enhanced chemiluminescence (ECL) or ECL-plus kits (Amersham Biosciences).

2.4. Flow cytometric analysis

Cells were fixed and stained with propidium iodide (PI). Apoptosis induced by AV65 was determined using the Annexin V-FITC Apoptosis Detection Kit I (BD Bioscience), according to the manufacturer's instructions. Apoptosis was also evaluated using PI and TdT-mediated dUTP-biotin nick-end labeling (TUNEL) to detect fragmented DNA as previously described [33]. Cells were analyzed by FACS Canto II using the Diva software (BD Bioscience).