

**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.

Key Words: β -catenin, CML, imatinib-resistance, T315I, hypoxia-adaptation

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]. 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% O₂ 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%

1 heat-inactivated fetal calf serum (FSC; Invitrogen, Tokyo, Japan), 2 mM L-glutamine
2 (Gibco), and 1% penicillin-streptomycin (Gibco).

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

9 10 2.2. Growth inhibitory effect of AV65 on CML cells

11 CML cell lines were exposed to AV65 for 72 hours and cell proliferation was
12 assessed using a modified MTT assay as previously described [8]. The combined effect
13 of combination treatment with IM and AV65 was evaluated in K562 cells. Cells were
14 incubated for 72 hours with six concentrations (equivalent to 0.25, 0.5, 0.75, 1.0, 1.5, or
15 2.0 times the IC₅₀) of AV65 alone or in combination with IM. We calculated the
16 combination indexes (CIs) as reported previously [30,31], and the fraction affected (Fa)
17 at each dilution was measured (an Fa of 0.25 equals 75% viable cells). This method
18 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% CO₂. 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).

2.5. Real-time quantitative RT-PCR

Total RNA from K562, BV173, and KBM5 cells was extracted using the QIAamp RNA Blood Mini Kit (QIAGEN, Tokyo, Japan) and subjected to reverse transcription. The mRNA levels of human p21, p27, p57, and cyclin D1 were analyzed using the LightCycler System (Roche Diagnostics, Sandhoferstraße, Mannheim, Germany) with FastStart DNA Master SYBER Green I (Roche Diagnostics). Amplicons were validated by their melting curve and electrophoresis. The expression levels of the

target mRNAs were normalized with that of the housekeeping gene actin. The specific primers for p21 were fwd, 5'-TGGAGACTCTCAGGGTCGAAA-3', and rev, 5'-CGGCGTTTGGAGTGGTAGAA-3'. The specific primers for p27 were fwd, 5'-CCGGCTAACTCTGAGGACAC-3', and rev, 5'-AGAAGAATCGTCGGTTGCAG-3'. The specific primers for p57 were fwd, 5'-GCGGCGATCAAGAAGCTGTC-3', and rev, 5'-CCGGTTGCTGCTACATGAAC-3'. The specific primers for β -catenin were fwd, 5'-GCCGGCTATTGTAGAAGCTG-3', and rev, 5'-GAGTCCCAAGGAGACCTTCC-3'. The specific primers for actin were fwd, 5'-CATGTACGTTGCTATCCAGGC-3', and rev, 5'-CTCCTTAATGTCACGCACGAT-3'.

2.6. Measurement of caspase activity

Caspase-3 activity in the presence of AV65 with or without zVAD was evaluated using a fluorometric protease assay kit (MBL, Aichi, Japan) as previously describe [34].

3. Results

3.1. AV65 inhibited the growth of IM-sensitive human CML cell lines

Examination of β -catenin expression in IM-sensitive CML cell lines showed that the expression levels of β -catenin increased by 20- to 45-fold in the K562, BV173, KT-1, and MYL CML cell lines compared with the total BM cells from healthy volunteers (Fig. 1A). Assessment of the effect of AV65 showed that the inhibitor reduced β -catenin expression in K562 CML cells in a time- and dose-dependent manner in nuclear and cytosolic fractions, as well as in whole cell lysates (Fig. 1B).

The effect of AV65 on the growth inhibition of IM-sensitive CML cells was examined by exposing 7 IM-sensitive human CML cell lines to AV65 for 72 hours and assessing the anti-proliferative effect of this inhibitor using modified MTT assay. AV65 inhibited the growth of all 7 CML cell lines with IC_{50} values ranging from 9.8 to 33.1 nM (Fig. 1C). To investigate the inhibitory effect of AV65 on primary CML cells, the number of colony-forming units (CFUs) observed following AV65 treatment of hematopoietic progenitor cells obtained from 3 healthy individual donors. CML cells obtained from 2 patients with CML in chronic phase was examined by colony assay on day 14. When normal progenitor cells were treated with 1, 3, 10, 30, 50, 70, 100 nM of AV65, the CFUs were 93.9 ± 5.8 , 91.4 ± 7.8 , 62.1 ± 13.4 , 37.6 ± 10.3 , 12.5 ± 9.1 , 1.2 ± 2.0 , and 0 ± 0 % of the control, respectively. When primary CML cells were treated with 1, 3, 10, 30, 50, 70, and 100 nM of AV65, the CFUs were 79.9 ± 2.7 , 45.8 ± 26.1 , $22.8 \pm$

19.4, 26.2 ± 1.5 , 11.0 ± 15.52 , 1.626 ± 2.3 , and 0 ± 0 % of the control, respectively (Fig. 1d). These percentages are the mean $\pm$ standard error of the individuals tested. These observations indicate that AV65 was approximately 5 times more effective at inhibiting colony formation in cells derived from CML patients than in those from healthy volunteers.

The effect of AV65 treatment on the expression of β -catenin and its downstream targets was investigated by Western blot analysis (Fig. 2A). AV65 downregulated the expression of phosphorylated and total β -catenin. Moreover, the expression of c-myc and survivin were also reduced by AV65 treatment. Another work from our group demonstrated that AV65 promotes the degradation of β -catenin via the ubiquitin-proteasome pathway (Yao, in revision). Therefore, we investigated the inhibitory effect of the proteasome inhibitor MG132 on the degradation of β -catenin by AV65. MG132 expectedly inhibited the degradation of β -catenin (Supplementary Fig. S3).

3.2. AV65 caused cell arrest at the G1 phase to S phase transition and induced apoptosis in CML cells

To investigate the cell cycle phases involved in cell death induced by AV65in

1 CML cells, co-staining of cells by PI and TUNEL was performed to detect DNA
2 fragmentation. Double staining revealed that both K562 and BV173 arrested at the G1
3 to S phase transition in response to AV65 treatment at 30 nM for 12 hours (Fig. 3A).
4 Cell cycle analysis also showed that AV65 treatment increased the G1 phase population
5 in these 2 cell lines in a time- and dose-dependent manner, coincident with an increase
6 in the number of cells in subG1 phase (Fig. 3B). To further identify the mechanism of
7 cell cycle-dependent cell death, the transcript levels of cyclin-dependent kinase
8 inhibitors (CKIs) were assessed during the G1 phase in CML cell lines. Real time PCR
9 analysis showed that the transcripts of p21, p27, and p57 were increased by AV65
10 treatment in K562, BV173, and KBM5 cells. However, p53 transcript levels in these
11 cell lines were not altered by AV65 treatment (Fig. 3C). These data indicate that AV65
12 induced cell cycle arrest in a p53-independent manner.

13 Assessment of apoptosis by PI/Annexin V double staining revealed that AV65
14 induced apoptosis in K562 and BV173 cell lines in a time- and dose-dependent manner
15 (Fig. 4A). Treatment with zVAD partially suppressed AV65-induced apoptosis in
16 BV173 cells, which indicates that AV65 induced caspase-dependent apoptosis in BV173
17 cells (Fig. 4B). In K562 cells, however, the effect of AV65 on the induction of apoptosis
18 did not change with zVAD treatment (Fig. 4B), despite the inhibition of caspase-3 by

1 zVAD (data not shown). These results show that AV65 inhibited β -catenin/T cell factor
2 (TCF) transcription signaling, which result in cell cycle arrest in the G1 phase through
3 the upregulation of CKIs and the induction of apoptosis in CML cells.

4 5 *3.3. AV65 enhanced the effect of imatinib*

6 Bcr-Abl is reported to stabilize β -catenin in CML cells through tyrosine
7 phosphorylation [17]. The expression pattern of Bcr-Abl and its downstream effector
8 proteins was therefore examined (Supplementary Fig. S1). Interestingly, the expression
9 of Bcr-Abl and its phosphorylated form was downregulated by AV65, and the levels of
10 phosphorylated Erk1/2, Akt, and Stat5 were also decreased. To investigate the combined
11 effects of AV65 and IM on K562 cells, cell proliferation was assessed using a modified
12 MTT assay with 6 concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the IC₅₀) of each
13 agent or a combination using a constant ratio of one of the drugs. The IC₅₀ value for
14 AV65, which was obtained as described above, was 10 nM, and that of IM was 100 μ M.
15 The CIs and the Fa values at each dilution were calculated using the CalcuSyn software.
16 Dose-effects and CI-Fa plots describing the effects of AV65 and IM combinations are
17 shown in Fig. 5. As shown in Fig. 5A, combination treatment with AV65 and IM caused
18 a greater inhibition of cell growth each agent alone. Data derived from the analysis of

CI-Fa plots are shown in Fig. 5B. The CIs at Fa 0.5 and Fa 0.8 were 1.07 and 0.88, respectively, indicating that AV65 and IM had an additive effect at lower concentrations and a synergistic effect at higher concentrations.

3.4. AV65 inhibited the growth of IM-resistant CML cells

The effect of AV65 was investigated in 4 IM-resistant CML cells, namely, KBM5/STI-R (harboring the T315I mutation), K562/IMR (containing a Bcr-Abl amplification), MYL-R1 (Lyn overexpressing), and K562/D1-9 (P-gp overexpressing). The IC₅₀ values for AV65 in KBM5/STI-R, K562/IMR, MYL-R1, and K562/D1-9 cells were 16.0, 10.0, 47.7, and 60.1 nM, respectively, and the response of these IM-resistant cell lines were similar to those of their parental cell lines with the exception of K562/D1-9 (Fig. 6A). AV65 induced apoptosis in KBM5/STI-R and KBM5 cells (Fig. 6B). Evaluation of effects of AV65 on Ba/F3 cells expressing 10 different Bcr-Abl mutations showed that AV65 inhibited the growth of Ba/F3 cells harboring various mutations, including T315I with IC₅₀ values ranging from 21.6 to 46.5 nM (Supplementary Fig. S2).

3.5. AV65 is also effective in primitive HA-CML cells

1 The effect of AV65 was examined in the HA-CML cell lines, K562/HA and
2 KCL22/HA. These cells have a phenotype that mimics characteristics of primitive
3 leukemia cells [27,28]. In Western blotting analysis, K562/HA and KCL22/HA cells
4 expressed higher levels of β -catenin than their respective parental cells. AV65 inhibited
5 the growth of K562/HA and KCL22/HA cells at a concentration similar to that effective
6 in the inhibition of growth in the parental cell lines (Fig. 6C). These observations
7 indicate that AV65 could be effective in the inhibition of the growth of primitive CML
8 cells that overexpress β -catenin.

10 4. Discussion

11 Recently, activation of the Wnt/ β -catenin signaling pathway has been
12 implicated in the progression of CML. The granulocyte-macrophage progenitors from
13 patients with CML in the blast crisis phase and IM-resistant CML have elevated levels
14 of nuclear β -catenin, resulting in the transcriptional activation of TCF proteins [13].
15 Gene expression profile studies demonstrated that Wnt/ β -catenin signaling is activated
16 in IM-resistant and advanced-stage CML [14,15], and this effect could be caused by
17 glycogen synthase kinase 3 β missplicing [35].

18 β -catenin is a downstream effector of the canonical Wnt signaling pathway.

1 The activation of the Wnt pathway is closely linked to carcinogenesis [18,36]. While the
2 N-terminal phosphorylation of β -catenin triggers its ubiquitination and degradation by
3 the 26S proteasome [37], the stabilized form of β -catenin translocates into the nucleus
4 and activates the transcription of Wnt target genes including c-myc, survivin, and cyclin
5 D1, resulting in the proliferation of cancer cells. β -catenin therefore is considered a
6 therapeutic target for the development of anticancer drugs[32,38-40], In prior studies,
7 AV65 was identified as a novel inhibitor of Wnt/ β -catenin signaling using
8 transcriptional profiling and HTS technology [21,22], Gene expression profiles before
9 and after siNRA-mediated knockdown of β -catenin were compared, and candidate
10 genes that were modulated by the inhibition of the pathway were identified. A
11 consensus set of candidate genes was identified and their transcriptional profiles were
12 validated using a series of small molecule probes capable of inhibiting the
13 Wnt/ β -catenin pathway. An early stage series of compounds was selected for further
14 analysis as inhibitors of the Wnt/ β -catenin pathway. Ultimately, AV-65 was generated
15 (Yao, in revision).

16 In the present study, the effect of AV65 on the suppression of the proliferation
17 of IM-sensitive and -resistant CML cells was demonstrated. AV65 decreased β -catenin
18 protein levels in CML cell lines in a time- and dose-dependent manner (Fig. 1B). Work

1 from our group revealed that AV65 promotes the degradation of β -catenin via the
2 ubiquitin-proteasome pathway (Yao, in revision). β -catenin translocation to the nucleus
3 decreased in response to AV65 treatment (Fig. 1B). As we have demonstrated that AV65
4 decreased TCF transcriptional activity (Yao, in revision), the expression of its
5 downstream proteins including c-myc and survivin were also reduced (Fig. 2A),
6 resulting in cell growth inhibition. As expected, the proteasome inhibitor suppressed the
7 degradation of β -catenin by AV65 (Supplementary Fig. S3). Flow cytometric analysis
8 showed that AV65 induced caspase-dependent apoptosis in BV173 cells, but apoptosis
9 was caspase-independent in K562 cells. These results suggest that the effect of AV65 on
10 induction of apoptosis in CML cells may or may not be accompanied by the activation
11 of caspases and that apoptosis with caspase activation varies depending on the CML cell
12 type, as previously observed [29]. The present results, together with other data from our
13 group (Yao, in revision), showed that AV65 inhibited TCF transcriptional activity by
14 promoting the degradation of β -catenin, which resulted in the induction of cell death.
15 The inhibition of TCF transcriptional activity by AV65 caused the downregulation of the
16 expression of survivin and c-myc, which are downstream effectors of β -catenin.
17 Interestingly, AV65 also decreased the expression of the phosphorylated forms of
18 Bcr-Abl, Erk1/2, Akt, and Stat5 (Supplementary Fig. S1). Although this mechanism

1 remains still unclear, these observations suggest that Wnt/ β -catenin signaling might play
2 a role in the stabilization of Bcr-Abl. Further investigations are necessary to clarify this
3 issue.

4 Frizzled2 and Lymphoid enhancer-binding factor-1 as well as β -catenin are
5 upregulated in CD34-positive CML cells during the disease progression from chronic
6 phase to blastic crisis, and Wnt3a increases β -catenin expression in CD34-positive CML
7 cells [41]. These observations suggest that Wnt/ β -catenin signaling plays an important
8 role in the CML progression. Therefore, the strategy targeting Wnt/ β -catenin signaling
9 might be also effective for the treatment of advanced-stage CML.

10 In the present study, the effects of AV65 were shown to be independent from
11 Bcr-Abl expression levels, Lyn overexpression, or the Abl T315I mutation in human
12 CML cell lines. AV65 is also effective against Ba/F3 cells expressing different mutant
13 forms of Bcr-Abl, including T315I. These observations indicate that AV65 is effective
14 against IM-resistant CML cells. Importantly, AV65 inhibited the growth of CML cells
15 harboring the T315I mutation. To overcome IM-resistance in CML, a second generation
16 of TKIs has been developed. However, the T315I mutation confers resistance to all
17 known TKIs [9,42]. Moreover, studies have demonstrated that patients with the T315I
18 mutation have a poor prognosis [43,44]. The development of novel agents directed

1 against the T315I clone is therefore important, and several multi-targeted kinase
2 inhibitors have recently been shown to be effective against the T315I mutation [45-47].
3 The present data demonstrate the potential of the AV65 compound as a novel agent
4 against CML with the T315I mutation. However, the IC₅₀ value of AV65 in K562/D1-9
5 cells (P-gp overexpressing) was approximately 60.1 nM, which was higher than the IC₅₀
6 in the parental cell line and in other IM-resistant cells. This observation suggests that
7 AV65 is a substrate of P-gp. As IM is currently the drug of choice for the treatment of
8 CML, the effects of combination treatment with AV65 and IM were investigated and the
9 results showed that AV65 enhanced the inhibitory effects of IM (Fig. 5).

10 AV65 also inhibited the growth of hypoxia-adapted CML cell lines at
11 concentrations comparable with those shown to be effective in the respective parental
12 cell lines. Although a definite CML stem cell niche has not been identified, leukemic
13 stem cells are located in an osteoblastic niche [48,49], which is a hypoxic region of the
14 BM [27], The self-renewal of normal hematopoietic stem cells favors hypoxia [50] and
15 resistance to hypoxia is one of the defining features of leukemic stem cells [51]. HA
16 cells survive long-term under hypoxic conditions (1% O₂) and include a large number of
17 cells in a dormant state and resistant to Abl TKIs. Furthermore, these cells exhibit a
18 higher engraftment activity than their parental cells and possess stem cell-like

characteristics [27]. Interestingly, these HA cells showed a higher level of β -catenin expression (Fig. 6C). This observation is consistent with the results reported by Dr. Jamieson [13]. Assuming that HA-CML cells exhibit characteristics similar to those of CML stem cells, the effects of AV65 on CML-HA cells were examined. AV65 inhibited the growth of HA-CML cells at similar concentrations to those inhibiting the growth of parental cells. In conclusion, AV65 inhibited the growth of CML cells harboring the T315I mutation and primitive CML cells. The present findings indicate that β -catenin could be a therapeutic target in CML, and suggest that AV65 is a potential novel therapeutic agent for the treatment of CML.

Conflicts of interest

R. Nagao, E. Ashihara, S. Kimura, H. Yao, M. Takeuchi, R. Tanaka, Y. Hayashi, H. Hirai, and Taira Maekawa disclose no financial conflict of interest. J.W. Strovel, Janak Padia, and Kathryn Strand are employees of PGx Health, A Division of Clinical Data, Inc.

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Figure legends.

Fig. 1. Growth inhibitory effect of AV65 in human CML cells. (A) Expression of β -catenin in the K562, BV173, KT-1, and MYL CML cell lines and total BM cells from a healthy volunteer (ctrl) as a control. (B) K562 cells were treated with AV65 and the expression of β -catenin was detected by Western blotting. Results represent the means of 3 independent experiments. (C) Seven imatinib-sensitive human CML cell lines were exposed to AV65 for 72 hours and anti-proliferative effects were examined using a modified MTT assay. (D) The colony-forming assay was performed in duplicate in primary CML cells obtained from patients with CML in the chronic phase and bone marrow mononuclear cells obtained from healthy volunteers was performed in duplicate. After 14 days of culture, colonies were evaluated under an inverted microscope. Data represents the mean $\pm$ SD of 3 independent experiments.

Fig. 2. Effect of AV65 on the expression of β -catenin and its downstream targets. K562 CML cells were treated with AV65 at the indicated concentrations for 72 hours. Changes in the expression of β -catenin and its downstream effector proteins were evaluated.

Fig 3. Cell cycle analysis and transcript levels of CDK inhibitors in AV65-treated CML cells. (A) K562 and BV173 were exposed to AV65 at a concentration of 10 nM for 12 hours. Apoptosis induced by AV65 was analyzed by PI and TUNEL double staining. Each dot line in the scattergram indicates G1 phase. (B) CML cells treated with AV65 were fixed and stained with PI and analyzed for DNA content by FACS Canto II. The numbers inside each histogram indicate the percentage of the subG1 fraction. The results shown in Figure 2a and b are representative of 3 independent experiments. (C) Transcript levels of CKIs in CML cells detected by real time PCR analysis. The results represent the means + SD of 3 independent experiments.

Fig. 4. Induction of apoptosis by AV65 treatment in CML cells. (A) K562 and BV173 cells were exposed to AV65 for 24, 48, and 72 hours at concentrations of 10 nM and 30 nM. Cells were stained with PI and Annexin V-FITC and subjected to flow cytometric analysis for the determination of apoptosis. The numbers inside each histogram indicate the percentage of early apoptotic cells (Annexin-V+/PI-) and late apoptotic/necrotic cells (Annexin-V+/PI+). (B) K562 and BV173 were treated with AV65 in the presence of zVAD. Cells were stained with PI and Annexin V-FITC. The numbers inside each

1 histogram indicate the percentage of early apoptotic cells (Region A) and late
2 apoptotic/necrotic cells (Region B). Results are representative of 3 independent
3 experiments.

4
5 **Fig. 5.** The inhibitory effects of AV65 in combination with IM on K562 cells. (A) K562
6 cells were incubated for 72 hours with 6 concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0
7 times the IC_{50}) of AV65 and IM or a combination of the 2 agents using the constant ratio
8 design of a modified MTT assay. The IC_{50} values of AV65 and IM were 10 nM and 100
9 μ M, respectively. The killing curves of the concurrent administration of AV65 and IM
10 are shown. (B) Plots of combination index (CI) against fraction affected (Fa). CIs were
11 determined with the nonlinear regression program CalcuSyn.

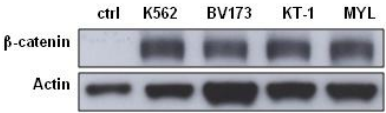
12
13 **Fig. 6.** Inhibitory effect of AV65 on IM-resistant CML cells. (a) Four IM-resistant CML
14 cell lines; K562/IMR (Bcr-Abl amplification), MYL-R1 (Lyn overexpressing),
15 KBM5/STI-R (harboring the T315I mutation), and K562/D1-9 (P-gp overexpressing),
16 were exposed to AV65 for 72 hours and its anti-proliferative effects were examined by a
17 modified MTT assay. The results represent the means $\pm$ SD of 3 independent
18 experiments. (B) KBM5 and KBM5/ STI-R cells were exposed to AV65 for 24, 48, and

72 hours at concentration of 1 x IC₅₀ (20 nM, 15 nM, respectively) and 3 x IC₅₀ (60 nM, 45 nM, respectively). Cell were stained with PI and Annexin V-FITC and subjected to flow cytometric analysis of apoptosis. The numbers inside each histogram indicate the percentage of early apoptotic cells (Annexin-V+/PI-) and late apoptotic/necrotic cells (Annexin-V+/PI+). Results are representative of 3 independent experiments. (C) Total protein lysates were extracted from the hypoxia-adapted CML cell lines, K562/HA and KCL22/HA. Western blotting was performed using antibodies against β -catenin and Actin (upper panel). Anti-proliferative effects were examined by the modified MTT assay. Results represent the means $\pm$ SD of 3 independent experiments (lower panel).

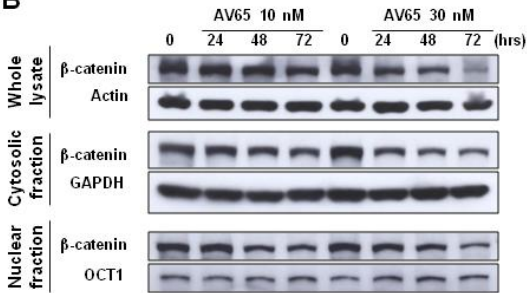
1 **Figures**

Fig. 1.

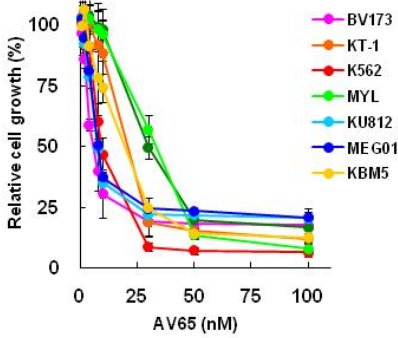
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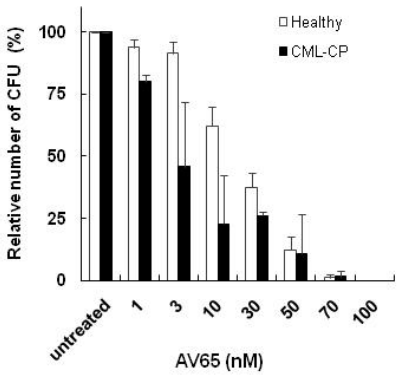
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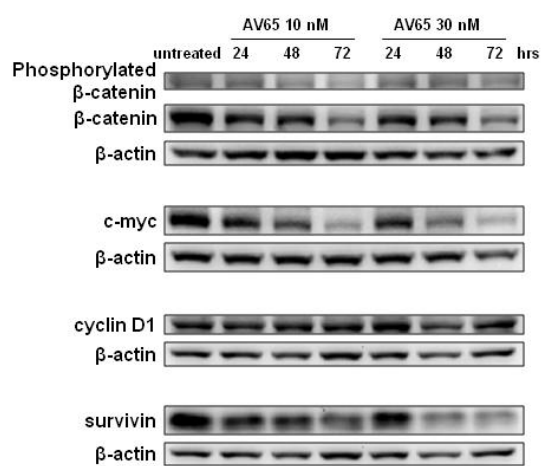
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2

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Fig. 2.



1

2

A K562

untreat AV65 30 nM BV173 untreat AV65 30 nM

TUNEL ↑

PI →

B

AV65 10 nM AV65 30 nM

0 24 48 7 24 48 72 (hrs)

K562

H 3.3 3.5 3.8 7.1 12.8 16.4 20.7

BV173

H 2.3 9.0 10.2 15.9 58.7 69.2 79.7

PI →

C

p21 p27 p57 p53

K562 (p53 deletion)

Relative fluorescent units

untreat 24 hrs 48 hrs 72 hrs

10 nM 30 nM

BV173 (p53 mutation)

untreat 24 hrs 48 hrs 72 hrs

10 nM 30 nM

KBM5

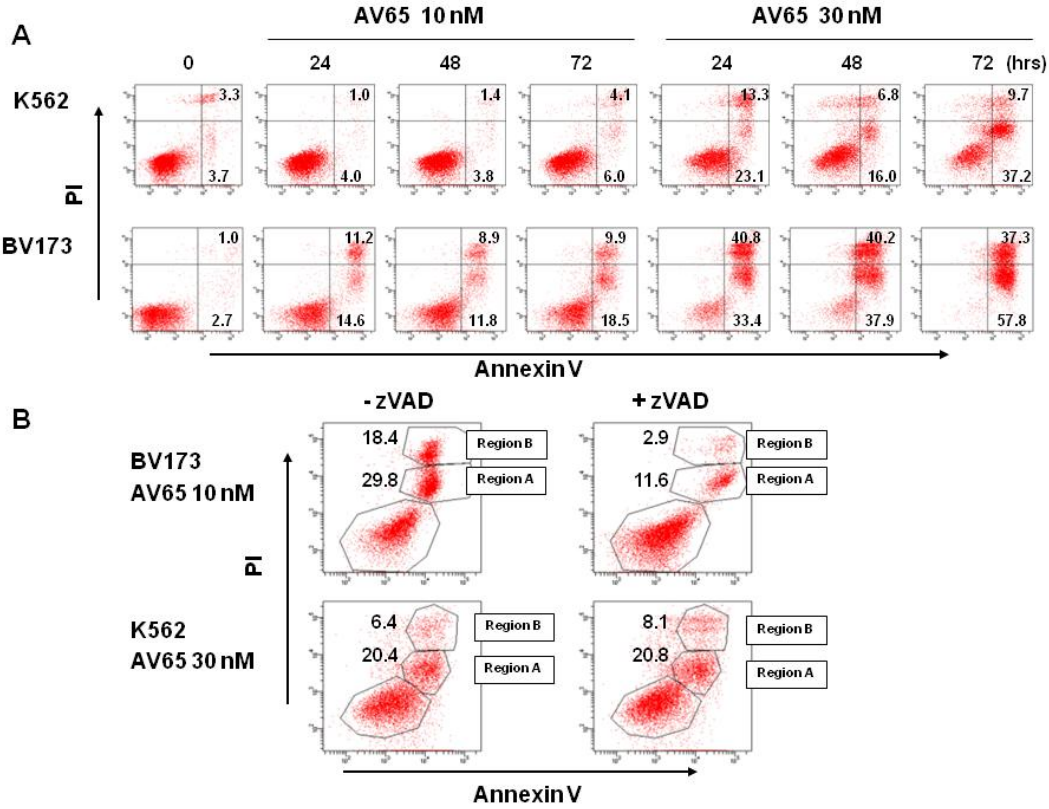
untreat 24 hrs 48 hrs 72 hrs

20 nM 60 nM

AV65 (nM)

2

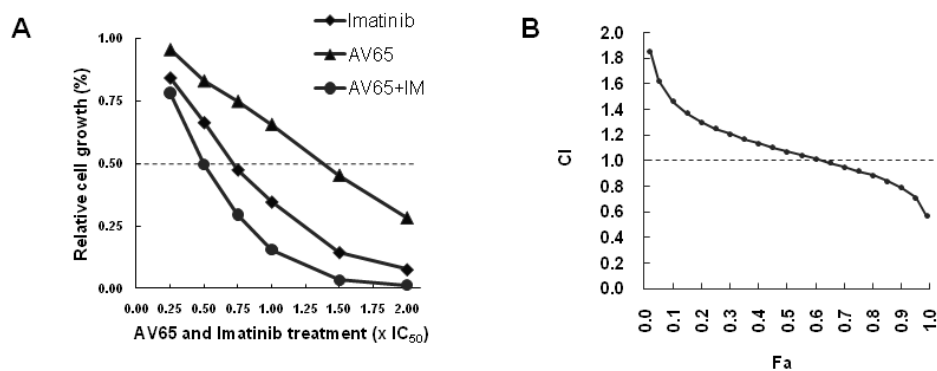
Fig. 4.



1

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Fig. 5.

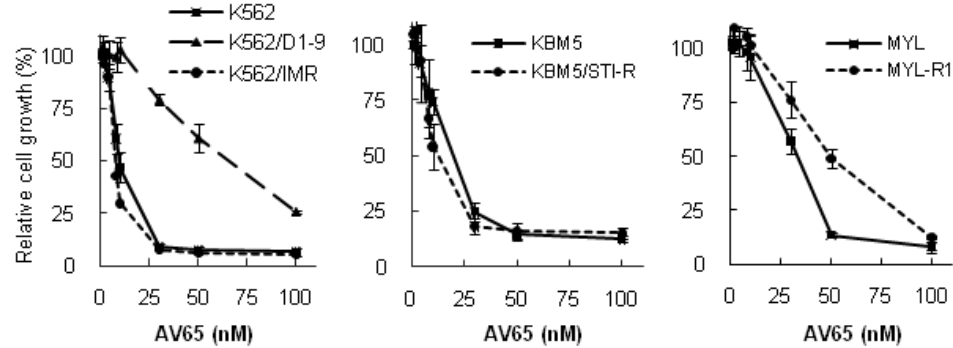


1

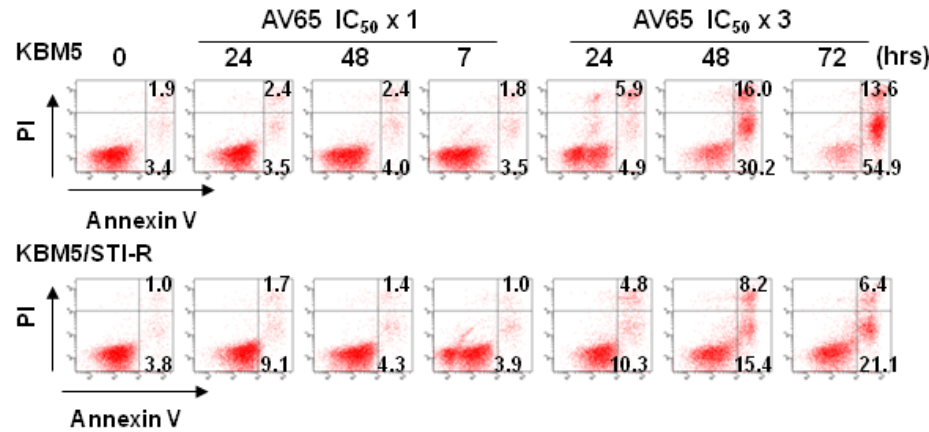
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Fig. 6.

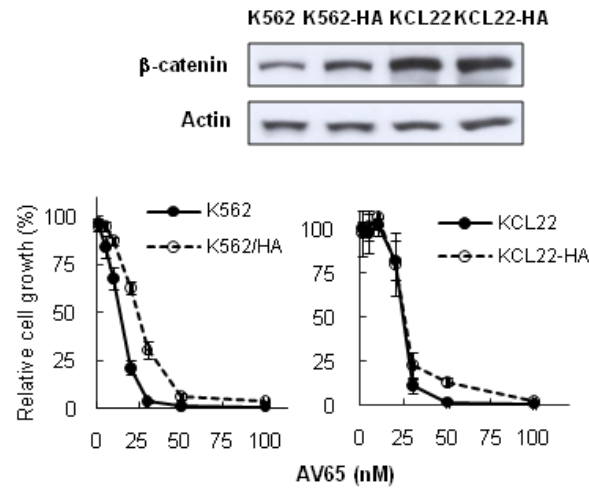
A



B



C



1
2