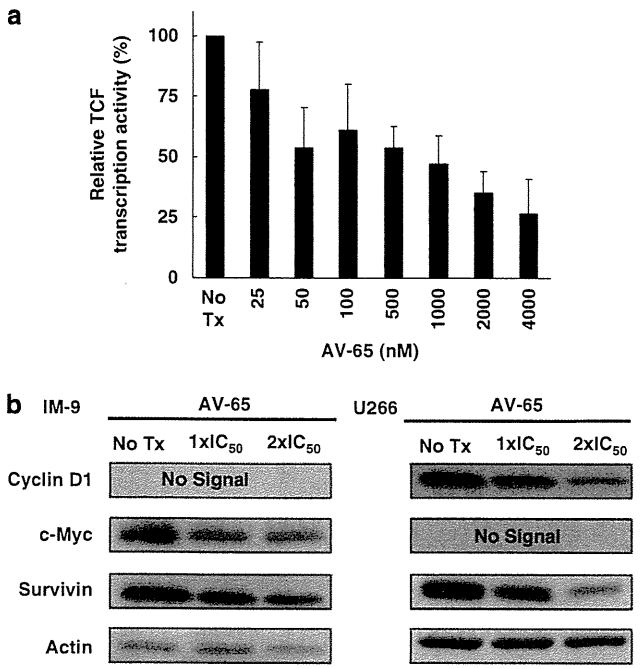


Figure 3 Inhibition of β-catenin-TCF signaling by AV-65 treatment. (**a**) Inhibition of TCF transcriptional activity in HCT-15 colorectal cancer cells. Cells were co-transfected with a TCF/LEF firefly luciferase reporter, and a CMV-driven *Renilla* luciferase reporter. To normalize transfection efficiency, cells were incubated with AV-65 for 14 h, and then luciferase activity was measured. Data represent the mean ± s.d. of three independent experiments. The relative TCF transcription activity represents the difference between the relative luciferase units of treated versus non-treated cells. (**b**) Decreased expression of TCF downstream targets in IM-9 cells. Cells were incubated with serial dilutions of AV-65 and then harvested at 24 h. Harvested cells were lysed in radioimmunoprecipitation assay buffer. Cell extracts (20 μg of protein) was analyzed by western blot using the indicated Abs. Cyclin D1 and c-myc were not expressed in IM-9 and U266 cells, respectively.

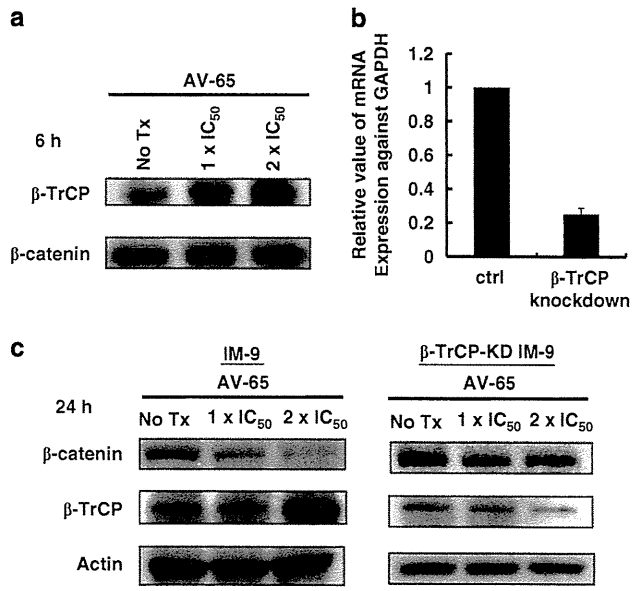


Figure 4 Enhancement of the interaction between β-catenin and β-TrCP by AV-65 treatment. (**a**) Cells were incubated with serial dilutions of AV-65 and then harvested after 6 h. Whole lysate was subjected to immunoprecipitation using an anti-β-catenin Ab, and immune complexes were captured using protein A beads. The protein A slurry (30 μl) was analyzed by immunoblot using anti-β-TrCP Ab to detect binding between β-catenin and β-TrCP. (**b**) IM-9 cells were transduced with lentivirus-based short hairpin RNAs targeting β-TrCP and control vector. β-TrCP levels were measured by real-time polymerase chain reaction. Data represent the means ± s.d. of three independent experiments, and four replicate experiments. (**c**) The alterations of β-catenin expression in IM-9 cells (left panel) and β-TrCP-KD IM-9 cells (right panel) by AV-65 treatment. After 24 h incubation with serial dilutions of AV-65, cells were harvested, and were lysed in radioimmunoprecipitation assay buffer. Cell extracts (20 μg of protein) were analyzed by western blot using an anti-β-catenin Ab and an anti-β-TrCP Ab.

β-Catenin degradation via enhancement of β-TrCP-mediated ubiquitination

We have clarified the mechanism by which AV-65 affects β-catenin degradation. β-Catenin is a target for the ubiquitin–proteasome pathway with β-TrCP, an ubiquitin ligase, triggering the ubiquitination of β-catenin. We examined the interaction between β-TrCP and β-catenin. The immunoprecipitation-western blotting analysis revealed that AV-65 enhanced the interaction of β-TrCP and β-catenin (Figure 4a).

We next established, using a lentivirus vector, a stable IM-9 cells containing a constitutive RNA interference capable of knockdown of β-TrCP (β-TrCP-KD IM-9) (Figure 4b). In contrast to IM-9 cells, the decrease of β-catenin was suppressed in β-TrCP-KD IM-9 cells after exposure to AV-65 (Figure 4c). In β-TrCP-KD IM-9 cells, AV-65-induced ubiquitination of β-catenin was suppressed (Supplementary Figure 3). These observations suggested that AV-65 degrades β-catenin through enhancing β-TrCP-mediated ubiquitination.

Induction of apoptosis by AV-65

Upon cell cycle analysis, we detected an increase in the sub-G1 phase fraction by AV-65 treatment in a dose- and a time-dependent manner (Supplementary Figures 4a and b, respectively). We also examined the induction of apoptosis in AV-65-treated cells by Annexin-V/propidium iodide staining. Early apoptotic cells (Annexin-V-positive/propidium iodide -negative), late apoptotic cells and necrotic cells (Annexin-V-positive/propidium iodide -positive) increased in a dose- and time-dependent manner (Figure 5a and Supplementary Figure 5, respectively). These results indicated that AV-65 inhibits the proliferation of MM cells through the induction of apoptosis.

We next investigated the underlying mechanism of apoptosis induced by AV-65 using a fluorometric protease assay. AV-65 treatment activated caspase-3, caspase-8 and caspase-9, indicating that apoptosis by AV-65 involves the activation of both intrinsic and extrinsic apoptotic pathways (Figure 5b). Western blot analysis showed that the expression of Noxa was increased in IM-9, AMO-1 and OPM-2 cells by AV-65 treatment, and the expression of Bad and Bim_S was increased in U266 cells. In AMO-1 cells, tBid and Noxa levels were increased, and the expression of Noxa was increased in OPM-2 cells. No significant changes in the expression of antiapoptotic proteins such as Bcl-2, BCl-xL and the long isoform of Mcl-1 were observed (Supplementary Figure 6). These findings suggested that AV-65 induces apoptosis accompanied with the activation of caspases through the cell type-dependent activation of proapoptotic BH-3-only proteins.

Inhibitory effects on myeloma in vivo

We assessed the *in vivo* effects of AV-65 on the growth of MM cells *in vivo* in an orthotopic mouse model of MM.

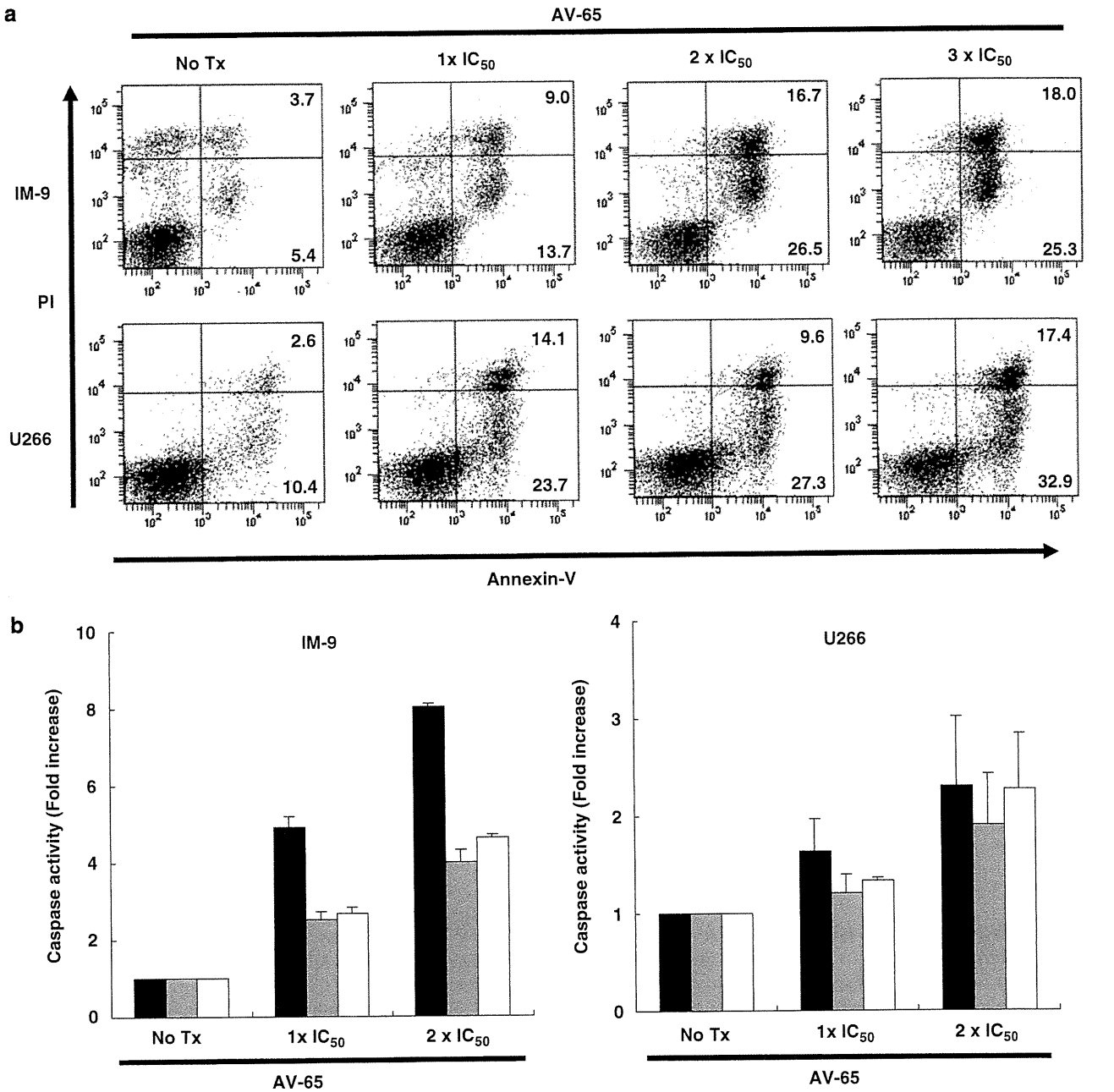
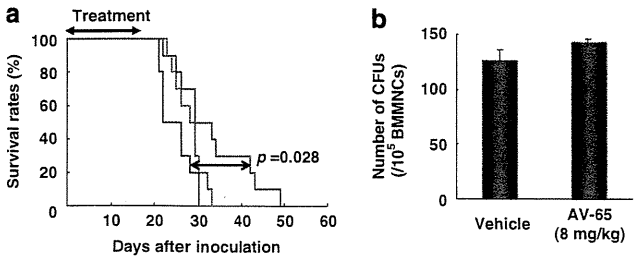


Figure 5 Induction of apoptosis in myeloma cells by AV-65. (**a**) Cells were incubated with serial dilutions of AV-65 and harvested after 24 h. Data are representative of three independent experiments. The numbers inside each quadrant indicate the percentage of the cell population with the quadrant characteristic. (**b**) Cells were incubated with serial dilutions of AV-65 and harvested after 24 h. Harvested cells were lysed in lysis buffer, and then caspase-3, -8 and -9 activity was evaluated using a fluorometric protease assay. Data represent the means ± s.d. of three independent experiments. Solid, gray and open bars indicate caspase-3, -8 and -9, respectively.

Following engraftment of IM-9 cells into NOD/SCID mice, all animals died of MM by approximately 32 days after transplantation (Figure 6a and Supplementary Figure 7). When we administered AV-65 to MM-bearing mice intravenously, the survival of mice treated with the higher doses of AV-65 was significantly prolonged as compared to vehicle-treated mice ($P=0.028$; Figure 6a). To investigate the adverse effects of AV-65 in treated mice, we assayed colony-forming activity in BM and other biochemical parameters in AV-65 and vehicle-treated mice. There were no statistical differences in body weight (data not shown), the complete blood cell counts between the groups (Figure 6b) or the numbers of colony-forming units in BM (Figure 6c). These observations indicated that AV-65 is a potent therapeutic agent for MM and does not induce severe adverse effects.

Discussion

Over the past 20 years, drug discovery has been dominated by phenotypic screening based on cancer cell biology. Using a traditional, pharmacology-driven approach, compounds that



c

	Vehicle	AV-65 (8 mg/kg)
WBC (/μl)	800 ± 75	725 ± 88
Hb (g/dl)	12.4 ± 0.5	13.3 ± 0.3
PLT (x10^4/μl)	84.6 ± 6.0	90.5 ± 5.4

Figure 6 The *in vivo* inhibitory effects of AV-65 on MM cells in an orthotopic mouse model. Following irradiation (2 Gy), specific pathogen-free 7- to 8-week old female NOD/SCID mice were inoculated intravenously with 5×10^6 IM-9 cells in 100 μl phosphate-buffered saline through the tail vein. The day after inoculation, AV-65 was administered intravenously for four cycles of 4 days on/1 day off. (**a**) Survival of IM-9-bearing mice treated with higher doses of AV-65 was significantly prolonged compared with vehicle-treated mice ($P = 0.028$). Red, green and blue lines represent the survival rates of high dose of AV-65 (8 mg/kg), low dose of AV-65 (4 mg/kg) and vehicle-treated groups, respectively (10 mice per group). (**b**) The complete blood counts of vehicle- of AV-65-treated mice. There was no significant difference between mice treated with the higher doses of AV-65 and vehicle. Data represent the means ± s.d. of four mice in each group. (**c**) Influence on BM hematopoiesis in treated mice. After four courses of AV-65 treatment, NOD/SCID mice were killed, and BM cells were obtained. Mononuclear cells were obtained by density centrifugation and analyzed by colony-forming assay. Colony-forming assay was performed in duplicate for each mouse (4 mice per group). There was no significant difference between mice treated with the higher doses of AV-65 and vehicle. Data represent the means ± s.e. of four mice in each group.

directly affected the pathophysiology of cancer cells were identified. Advances in molecular biology and genetic engineering has brought out the era of chemical genetics,[29] leading to well-characterized selective cancer-specific drugs such as imatinib and trastuzumab, which target specific molecular pathways. In recent years, the Human Genome Project and related efforts have led to the establishment of databases based on genomics and transcriptome analyses, and transcriptional profiling and high-throughput technology using microarrays have been applied to systemic drug screening.[30] Profiling the expression of numerous gene sets in normal versus pathological states can provide critical clues to intracellular signaling pathway function, as well as to identities of genes that play important roles in carcinogenesis.

β-Catenin is a downstream effector of the canonical Wnt signaling pathway. The activation of Wnt signaling is closely linked to carcinogenesis,[13,31,32] and β-catenin is considered a valid drug target known to regulate the Wnt pathway.[27,33] In support of this, downregulation of β-catenin by small interfering RNA inhibits the proliferation of cancer cells.[16,34,35] Recently, a study describing the high-throughput transcriptional screening analysis (Supplementary Figure 1) of HT29 colon adenocarcinoma cells transfected with an small interfering RNA against β-catenin was reported.[18] In HT-29 cells, β-catenin overexpression correlates with carcinogenesis due to mutation of adenomatous polyposis coli,[11] making these cells a useful model system. Transcriptional changes before and after knockdown of β-catenin were profiled using a gene expression microarray, and nine genes (*CTNNB1*, NM_001904; *CEACAM6*, NM_002483; *EMP1*, NM_001423; *IHH*, NM_002181; *MYEF2*, NM_016132; *NTN4*, NM_021229; *DHRS9*, NM_005771; HMGB1, NM_002128; and *GAPDH*, NM_002046; *GAPDH* was used as control) were identified as candidate genes that were modulated by inhibition of the Wnt/β-catenin pathway. The gene set was then validated using a series of small-molecule probes that are known inhibitors of the Wnt/β-catenin signaling (that is, indomethacin and retinoic acid).[36–38] A random forest algorithm developed during the signature validation phase was used to identify hit compounds from the screening library. Hit compounds and closely related analogs were re-tested in escalating doses to confirm activity and measure potency, and several early-stage compound series was selected for further analysis as inhibitors of the Wnt/β-catenin pathway. Ultimately, the LC-363 compound series (Supplementary Figure 2) were selected for further analysis as an inhibitor of the Wnt/β-catenin pathway. AV-65 was generated based on initial LC-363 series. Our preliminary data show that this AV-65 compound was more potent in inhibiting the Wnt/β-catenin signaling than the early-stage compound series (data not shown). Lepourcelet *et al.*[39] screened several antagonistic compounds for their ability to inhibit the β-catenin pathway using a high-throughput method for immunoenzymatic detection based on protein–protein interactions. The screened compounds were from natural sources, and several of them shared polyhydroxylated planar features. One of these compounds, PKF115-584, inhibited the proliferation of MM cells.[15] SDX-308, an etodolac analog, also induced cytotoxicity in MM cells through inhibition of the β-catenin pathway.[40] By comparison, this novel compound of this study is fundamentally different from these earlier compounds in both structure and function.

We first examined the *in vitro* effects of AV-65 on MM cells. AV-65 inhibited the proliferation of MM cells in a dose- and a time-dependent manner (Figures 1a and b, respectively). Moreover, β-catenin was degraded in AV-65-treated cells through an increase in ubiquitination (Figure 2b). The results of a dual luciferase reporter assay indicated that AV-65 inhibits TCF transcriptional activity, and that c-myc, cyclin D1 and survivin expression is decreased by AV-65 (Figures 3a and b). Moreover, β-catenin was degraded in AV-65-treated cells through an increase in ubiquitination (Figure 2b). Moreover, AV-65 enhanced the interaction between β-catenin and β-TrCP, resulting in the increased ubiquitination and degradation of β-catenin. The involvement of β-TrCP was verified by experiments showing knockdown of β-TrCP (Figure 4). AV-65 inhibited TCF/LEF transcriptional activity, resulting in a decrease in the level of TCF downstream targets and the induction of apoptosis through the activation of caspases (Figures 5a and b). Thus, AV-65 inhibits the Wnt/β-catenin signaling through the decrease of β-catenin/TCF transcriptional activity, resulting in the suppression of MM cell proliferation.

We also investigated the *in vivo* effects of a water-soluble formulation of AV-65. Survival of MM-bearing mice was significantly prolonged by administration of AV-65 (Figure 6a). AV-65 had no adverse effects on the mice, in terms of body weight (data not shown), the number of the peripheral blood cells or the colony-forming units in the BM (Figures 6b and c). We are planning to carry out a phase I study with this compound series in solid and hematopoietic malignancies.

In conclusion, a novel Wnt/β-catenin signaling inhibitor, AV-65, inhibited the proliferation of MM cells *in vivo* and *in vitro*. Thus, β-catenin represents a promising molecular target for therapy against MM, and AV-65 a promising agent against MM.

Conflict of interest

H Yao, E Ashihara, Y Nakagawa, J Kuroda, R Nagao, R Tanaka, A Yokota, M Takeuchi, K Sakai, C Shimazaki, M Taniwaki, H Hirai, S Kimura and Taira Maekawa disclose no financial conflict of interest. JW Strovel, Kathyrn Strand and Janak Padia are employees of PGx Health, a Division of Clinical Data Inc.

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Supplementary Information accompanies the paper on Blood Cancer Journal website (http://www.nature.com/bcj)

caused 53% neutrophil activation compared to non-preabsorbed plasma. On the other hand, preabsorption by KY-mock cells caused 83% neutrophil activation compared to no preabsorption (Fig 1B). We observed similar results for release of heparin-binding protein using the same experimental procedures described above (data not shown) (Yasui *et al*, 2008). We reported previously that neutrophils are activated by anti-HLA Class I alloantibodies via their surface Fc receptors CD16 and CD32 (Yasui *et al*, 2008), and accordingly, we examined whether anti–Siglec-14 alloantibodies activated neutrophils via Fc receptors. Pretreatment with blocking monoclonal antibodies for Fc receptors (Tamm & Schmidt, 1996, Aicher *et al*, 2000) caused 95% neutrophil activation compared to no Fc blocking (data not shown). These results indicate that anti–Siglec-14 alloantibodies are responsible for activation of neutrophils, not only by an Fc receptor-dependent mechanism, but more predominantly by an Fc receptor-independent mechanism. Yamanaka *et al* reported that stimulation by LPS, a ligand of Siglec-14, resulted in Siglec-14 initiating TNF-α secretion in transduced monocytic cell line. This effect was dependent on the interaction of Siglec-14 with activating adaptor protein DAP-12 (Yamanaka *et al*, 2009). These results suggest that DAP-12 may mediate the activation of neutrophils by anti–Siglec-14 alloantibodies.

We conclude that in addition to anti-HLA and anti-HNA alloantibodies, anti–Siglec-14 alloantibodies might also be involved in the development of some NHTRs, especially TRALI.

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A phase I/IIa clinical trial of immunotherapy for elderly patients with acute myeloid leukaemia using dendritic cells co-pulsed with WT1 peptide and zoledronate

Immunotherapy, which is less toxic and is tumoricidal through mechanisms different from those of chemotherapy, has the potential capacity to confer clinical benefits on elderly patients with acute myeloid leukaemia (AML), in whom

therapy-related mortality and resistance to chemotherapy are considered to be major reasons for dismal prognosis. We conducted a phase I/IIa clinical trial of immunotherapy for elderly patients with AML, using dendritic cells (DCs) pulsed with HLA-A*2402-restricted modified $WT1_{235-243}$ peptide (CYTWNQMNL) and zoledronate. Wilms' tumour 1 (WT1) is a transcription factor highly expressed in AML (Oka *et al*, 2008). HLA-A*2402-restricted modified $WT1_{235-243}$ peptide, in which an anchor residue of the natural $WT1_{235-243}$ peptide (CMTWNQMNL) was substituted to enhance the affinity to the HLA-A*2402 molecule, has a more potent capacity to activate WT1-specific cytotoxic T lymphocytes (CTLs) *in vitro* than the natural peptide (Tsuboi *et al*, 2002). The peptide vaccine using the modified peptide has shown antitumor activity in patients, indicating that modified peptide-induced CTLs can kill tumour cells that present the natural peptide (Oka *et al*, 2004). Zoledronate is an aminobisphosphonate that induces the accumulation of isopentenyl pyrophosphate, a ligand of Vγ9Vδ2 T-cell receptor, in a variety of cells (Gober *et al*, 2003). We and others have shown that addition of zoledronate to tumour antigen-loaded DCs trigger activation of Vγ9Vδ2 T cells, which produce interferon (IFN)-γ and stimulate the interacting DCs, leading to enhanced activation of tumour antigen-specific $CD8^+$ T cells (Fiore *et al*, 2007; Takahara *et al*, 2008). As Vγ9Vδ2 T cells are located in secondary lymphoid organs (Dieli *et al*, 2003), DCs loaded with tumour antigen and zoledronate may strongly activate antigen-specific $CD8^+$ T cells in draining lymph nodes, owing to the helper effect of Vγ9Vδ2 T cells.

The study protocol was approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University. Written informed consent was obtained from each patient before enrollment in accordance with the Declaration of Helsinki. Three HLA-A*2402-positive patients with ages of 69–77 were enrolled (Table I). WT1 mRNA expressions in the patients' leukemic cells were confirmed by real-time quantitative polymerase chain reaction. Following recovery of normal hematopoeisis after chemotherapies, monocytes were collected by apheresis and elutriation, and cultured with 800 IU/ml granulocyte-macrophage colony-stimulating factor and 500 IU/ml interleukin-4 to generate immature DCs. DCs were matured with 10 ng/ml tumour necrosis factor-α plus 1 μg/ml prostaglandin E_2. The majority of DCs were co-pulsed with 10 μmol/l modified WT1 peptide and 0·1 μmol/l.zoledronate (WT1/ZOL-DC), while the rest of the DCs were pulsed with 2 μg/ml keyhole limpet hemocyanin (KLH) (KLH-DCs). DCs were frozen, and thawed at the time of DC administration. We administered WT1/ZOL-DCs intradermally (1×10^7 cells) and intravenously (1×10^7 cells) in an attempt to stimulate WT1-specific T cells in draining lymph nodes as well as in bone marrow, which is the main tumour site in leukaemia and has been reported to preferentially contain leukaemia-specific T cells (Melenhorst *et al*, 2009). In addition, 5×10^6 KLH-DCs were intradermally administered to monitor immunogenicity of DCs. The administration of DCs was repeated every 2 weeks for a total of five administrations.

The vaccinations were well tolerated and safe. Whereas Patient #1 dropped out of the study after the third vaccination owing to rapid growth of leukemic cells with no inductions of immune responses to either KLH or the modified WT1 peptide, Patients #2 and #3, who completed the five vaccinations, exhibited inductions of immune

Table I. Patient characteristics and results of DC vaccination.

Patient	Age (years)/ Sex	Diagnosis	Number of DC Vacs	Adverse effects*	Immune response		Clinical response†	Died at (after Vacs)
					KLH	WT1		
1	77/F	AML-MRC	3	Injection site reaction (1)	No	No	Progressive disease (2·2% → 18%) Died of leukaemia	8 months
2	69/F	AML M2	5	Injection site reaction (1)	Yes	Yes	Progressive disease (2·0% → 11·5%) Transient decrease in leukaemic cells after the 4th vac (6·9% → 4·4%)‡ Died of pneumonia with leukaemia	4·5 months
3	76/M	AML-MRC	5	Fatigue (1) Injection site reaction (1)	Yes	Yes	Stable disease (0·6% → 1·2%) Alive with disease	–

Vac, vaccination; AML-MRC, acute myeloid leukaaemia with myelodysplasia-related changes.

*Numbers in parenthesis indicate grade of toxicity.

†Percentages in parenthesis indicate those of leukaemic cells in bone marrow before and after the vaccinations.

‡Percentages of leukaemic cells in bone marrow before and 2 weeks after the 4th vaccination.

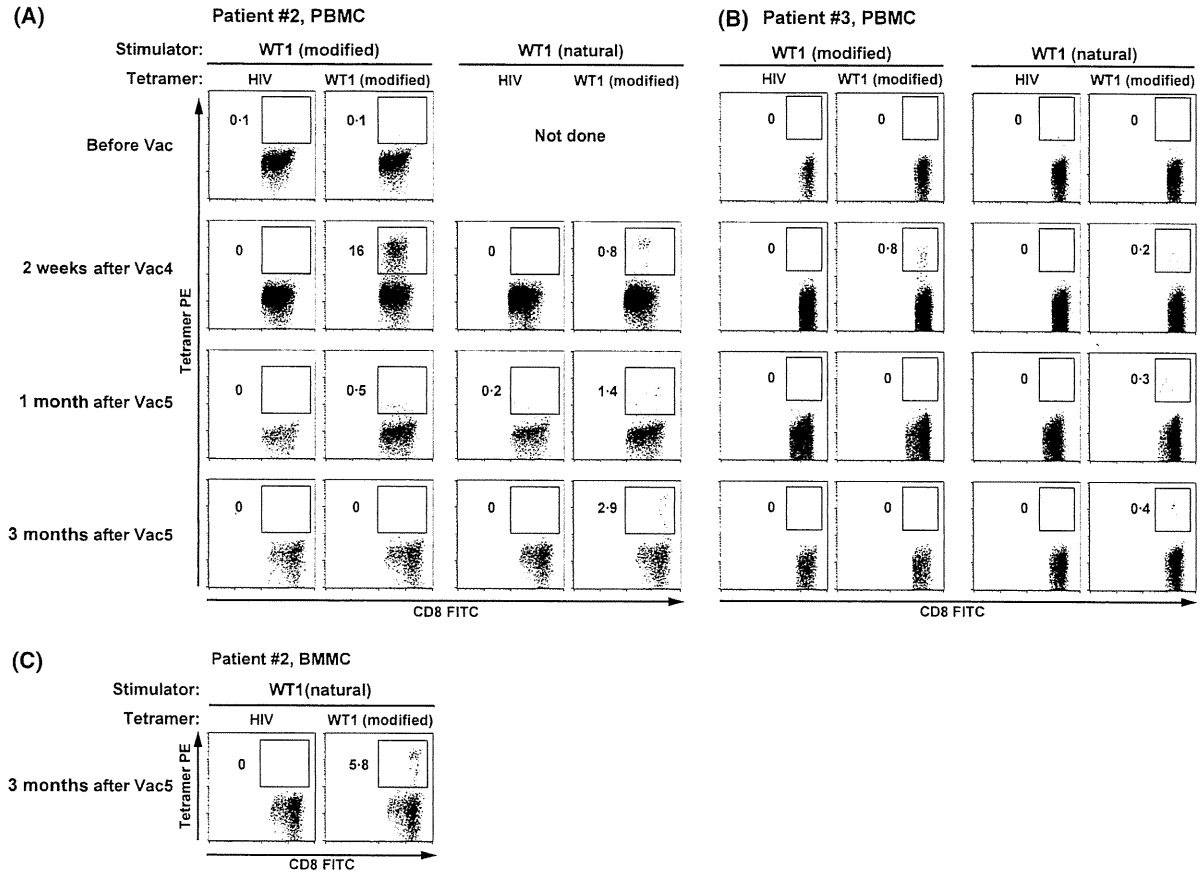

Fig 1. (A) HLA tetramer staining of PBMCs from Patient 2. At indicated time points, PBMCs were stimulated with DCs pulsed with the WT1 modified peptide [WT1 (modified)] or with the natural peptide [WT1 (natural)] for a week, then stained with PE-conjugated HIV/HLA-A*2402 tetramer as a negative control or PE-conjugated WT1 modified peptide/HLA-A*2402 tetramer and FITC-conjugated anti-CD8 mAb, and analysed by flow cytometry. Dead cells were excluded by propidium iodide staining. Numbers indicate percentages of tetramer-positive cells within the $CD8^+$ population. (B) HLA tetramer staining of PBMCs from Patient #3. At indicated time points, PBMCs were stimulated and analysed as in (A). (C) HLA tetramer staining of bone marrow mononuclear cells in Patient #2. Three months after the 5th vaccination, the cells were stimulated in vitro with DCs pulsed with the WT1 natural peptide for a week, and then stained and analysed as in (A).

responses to both KLH and the modified WT1 peptide as detected by skin delayed type hypersensitivity tests and/or IFN-γ ELISPOT assays (data not shown). WT1-specific immune responses were also assessed by HLA tetramer staining, using an HLA-A*2402/modified WT1 peptide tetramer (Fig. 1). In either of the two patients, tetramer-positive T cells were not detected in peripheral blood mononuclear cells (PBMCs) without *in vitro* expansion at any time points. In both patients, however, tetramer-positive $CD8^+$ T cells became detectable 2 weeks after the 4th vaccination in PBMCs stimulated with the modified peptide for a week *in vitro*. In addition, albeit at much lower frequencies, tetramer-positive T cells were also detected in natural peptide-stimulated PBMCs. Interestingly, whereas tetramer-positive T cells rapidly declined thereafter in modified peptide-stimulated PBMC, tetramer-positive T cells persisted until 3 months after the 5th vaccination in natural peptide-stimulated PBMCs. Stimulation with the natural peptide also expanded tetramer-positive T cells from bone marrow (Fig. 1C). We assessed numbers of γδ T cells in peripheral blood and bone marrow and serum IFN-γ levels to determine whether the vaccinations induced activation of γδ T cells *in vivo*. However, we could not detect it using these assays (data not shown). In association with the inductions of an immune response to WT1, either transient decrease in leukemic cells (Patient #2) or disease stabilization (Patient #3) was observed (Table 1), suggesting that the vaccinations may have impacted on the clinical courses of these patients.

These data indicate that vaccination with the modified WT1 peptide expands modified peptide-reactive T cells, a small fraction of which cross-reacts with the natural peptide. Notably, whereas modified peptide-specific T cells rapidly declined after the termination of vaccination, natural peptide-reactive T cells survived longer *in vivo*. Although the reason is not clear, it might be due to persistent stimulation with the endogenous cognate antigen derived from residual leukemic cells, as observed in

$CD8^+$ T cell memory maintained by persistent antigenic stimulation in chronic viral infection (Shin *et al*, 2007). Thus, although vaccination with a modified peptide may expand many 'useless' T cells that do not recognize the natural peptide, a small fraction of T cells that cross-react to the natural peptide may preferentially survive and can be exploited as effector cells upon boosting. A recent report on immunotherapy using WT1 mRNA-electroporated DCs indicates the promise of DC-based immunotherapy for AML after reducing a tumour burden by chemotherapy (Van Tendeloo *et al*, 2010). Further strengthening of vaccination, such as a combination of other treatments to block immunosuppressive pathways, may lead to an improved clinical outcome.

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Conflict of interest

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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).

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, Sandhoferstaβ-e, 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′- GCGGCGATCAAGAAGCTG TC-3′, and rev, 5′- CCGGTTGCTGCTACATGAAC-3′. The specific primers for β-catenin were fwd, 5′-GCCGGCTATTGTA-GAAGCTG-3′, and rev, 5′-GAGTCCCAAGGAGACCTTCC-3′. The specific primers for actin were fwd, 5′-CATGTACGTTGC TATCCAGGC-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 h 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 three healthy individual donors. CML cells obtained from two 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 ± 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 ± standard error of the individuals tested. These observations indicate that AV65 was approximately five 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 form 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 CML cells, co-staining of cells by PI and TUNEL was performed to detect DNA fragmentation. Double staining revealed that both K562 and BV173 arrested at the G1 to S phase transition in response to AV65 treatment at 30 nM for 12 h (Fig. 3A). Cell cycle analysis also showed that AV65 treatment increased the G1 phase population in these two cell lines in a time- and dose-dependent manner, coincident with an increase in the number of cells in subG1 phase (Fig. 3B). To further identify the mechanism of cell cycle-dependent cell death, the transcript levels of cyclin-dependent kinase inhibitors (CKIs) were assessed during the G1 phase in CML cell lines. Real time PCR analysis showed that the transcripts of p21, p27, and p57 were increased by AV65 treatment in K562, BV173, and KBM5 cells. However, p53 transcript levels in these cell lines were not altered by AV65 treatment (Fig. 3C). These data indicate that AV65 induced cell cycle arrest in a p53-independent manner.

Assessment of apoptosis by PI/Annexin V double staining revealed that AV65 induced apoptosis in K562 and BV173 cell lines in a time- and dose-dependent manner (Fig. 4A). Treatment with zVAD partially suppressed AV65-induced apoptosis in BV173 cells, which indicates that AV65 induced caspase-dependent apoptosis in BV173 cells (Fig. 4B). In K562 cells, however, the effect of AV65 on the induction of apoptosis did not change with zVAD treatment (Fig. 4B), despite the inhibition of caspase-3 by zVAD (data not shown). These results show that AV65 inhibited β-catenin/T cell factor (TCF) transcription signaling, which result in cell cycle arrest in the G1 phase through the upregulation of CKIs and the induction of apoptosis in CML cells.

3.3. AV65 enhanced the effect of imatinib

Bcr-Abl is reported to stabilize β-catenin in CML cells through tyrosine phosphorylation [17]. The expression pattern of Bcr-Abl and its downstream effector proteins was therefore examined (Supplementary Fig. S1). Interestingly, the expression of Bcr-Abl and its phosphorylated form was downregulated by AV65, and the levels of phosphorylated Erk1/2, Akt, and Stat5 were also decreased. To investigate the combined effects of AV65 and IM on K562 cells, cell proliferation was assessed using a modified MTT assay with 6 concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the IC_{50}) of each agent or a combination using a constant ratio of one of the drugs. The IC_{50} value for AV65, which was obtained as described above, was 10 nM, and that of IM was 100 μM. The CIs and the Fa values at each dilution were calculated using the CalcuSyn software. Dose–effects and CI–Fa plots describing the effects of AV65 and IM combinations are shown in Fig. 5. As shown in Fig. 5A, combination treatment with AV65 and IM caused 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 four 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 IC_{50} values ranging from 21.6 to 46.5 nM (Supplementary Fig. S2).

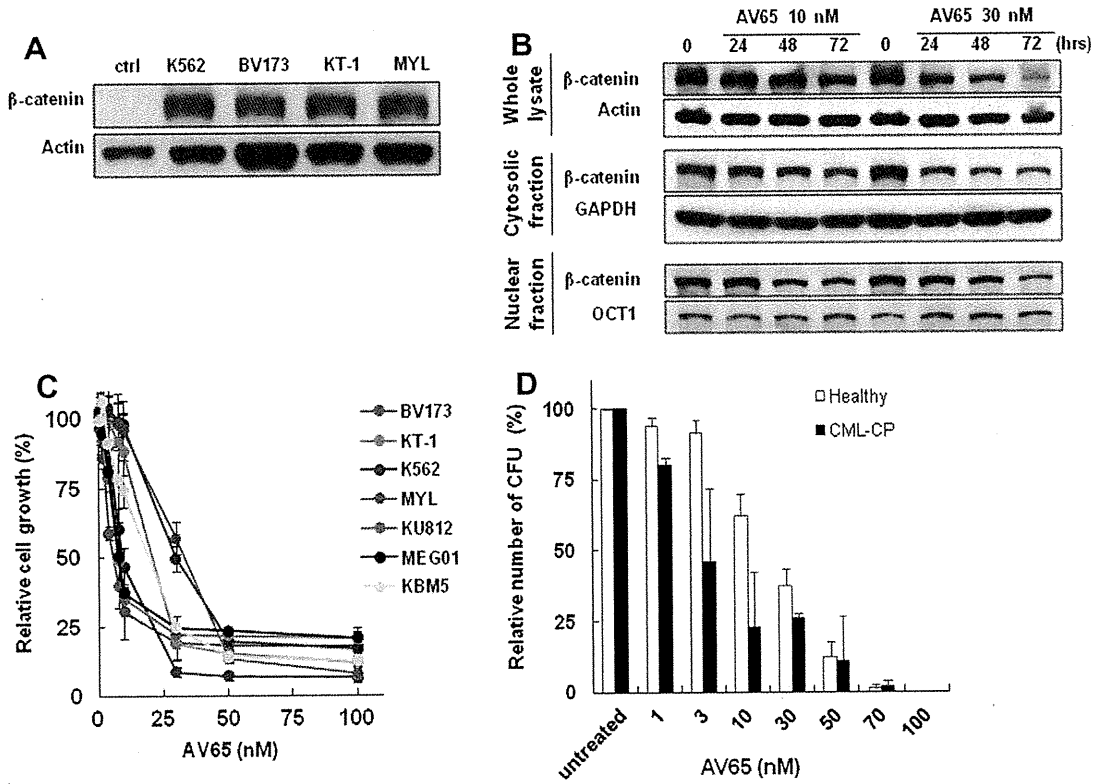

Fig. 1. Growth inhibitory effect of AV65 in human CML cells. (A) Expression of β-catenin in the K562, BV173, KT-1, and MYL CML cell lines and total BM cells from a healthy volunteer (ctrl) as a control. (B) K562 cells were treated with AV65 and the expression of β-catenin was detected by Western blotting. Results represent the means of three independent experiments. (C) Seven imatinib-sensitive human CML cell lines were exposed to AV65 for 72 h 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 ± SD of three independent experiments.

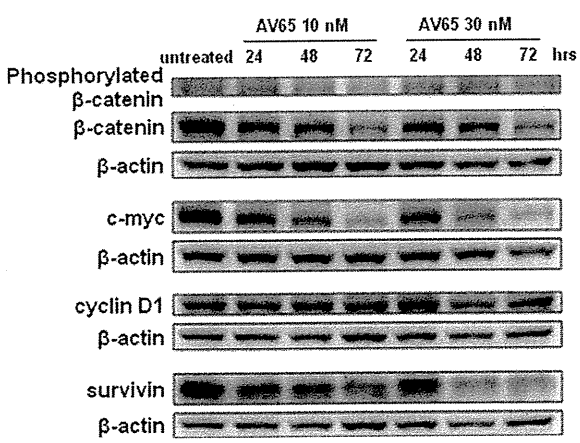

Fig. 2. Effect of AV65 on the expression of β-catenin and its downstream targets. K562 CML cells were treated with AV65 at the indicated concentrations for 72 h. Changes in the expression of β-catenin and its downstream effector proteins were evaluated.

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

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

4. Discussion

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

β-Catenin is a downstream effector of the canonical Wnt signaling pathway. The activation of the Wnt pathway is closely linked to carcinogenesis [18,36]. While the N-terminal phosphorylation of β-catenin triggers its ubiquitination and degradation by the 26S proteasome [37], the stabilized form of β-catenin translocates into the nucleus and activates the transcription of Wnt target genes including c-myc, survivin, and cyclin D1, resulting in the prolifer-

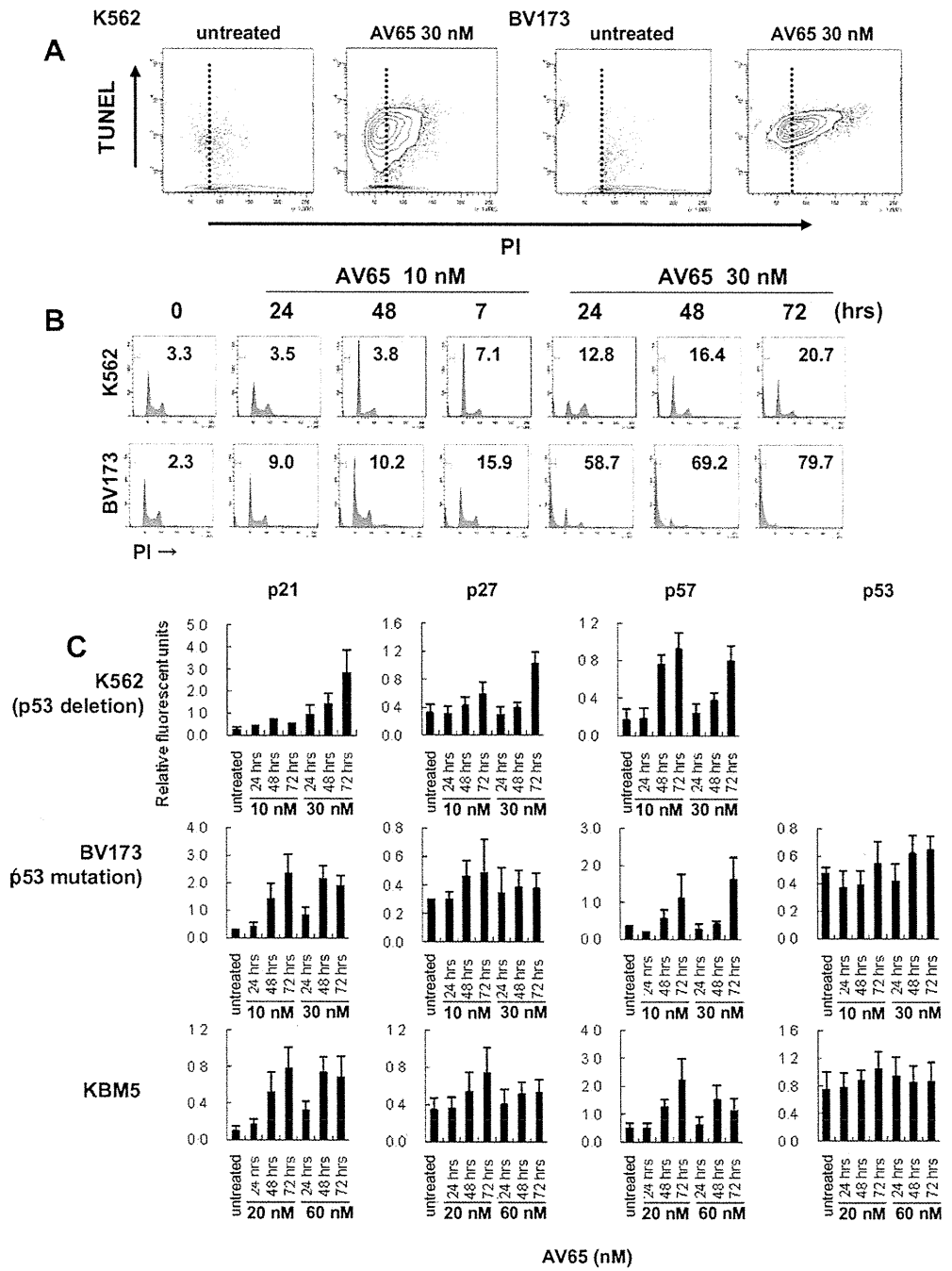

Fig. 3. Cell cycle analysis and transcript levels of CDK inhibitors in AV65-treated CML cells. (A) K562 and BV173 were exposed to AV65 at a concentration of 10 nM for 12 h. Apoptosis induced by AV65 was analyzed by PI and TUNEL double staining. Each dot line in the scattergram indicates G1 phase. (B) 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 Fig. 2a and b are representative of three independent experiments. (C) Transcript levels of CKIs in CML cells detected by real time PCR analysis. The results represent the means + SD of three independent experiments.

ation of cancer cells. β-catenin therefore is considered a therapeutic target for the development of anticancer drugs [32,38–40], In prior studies, AV65 was identified as a novel inhibitor of Wnt/β-catenin signaling using transcriptional profiling and HTS technology [21,22], gene expression profiles before and after siRNA-mediated knockdown of β-catenin were compared, and candidate genes that were modulated by the inhibition of the pathway were identified. A consensus set of candidate genes was identified and their transcriptional profiles were validated using a series of small molecule probes capable of inhibiting the Wnt/β-catenin pathway. An early stage series of com-

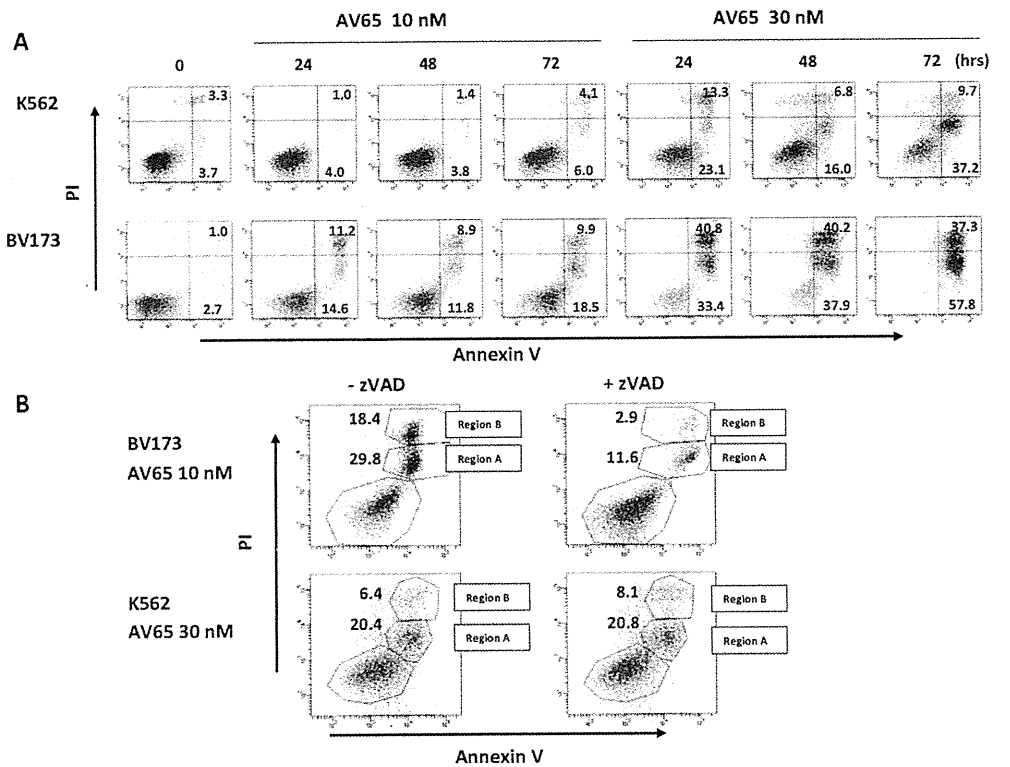

Fig. 4. Induction of apoptosis by AV65 treatment in CML cells. (A) K562 and BV173 cells were exposed to AV65 for 24, 48, and 72 h at concentrations of 10 nM and 30 nM. Cell were stained with PI and Annexin V-FITC and subjected to flow cytometric analysis for the determination of apoptosis. The numbers 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. Cell were stained with PI and Annexin V-FITC. The numbers inside each histogram indicate the percentage of early apoptotic cells (Region A) and late apoptotic/necrotic cells (Region B). Results are representative of three independent experiments.

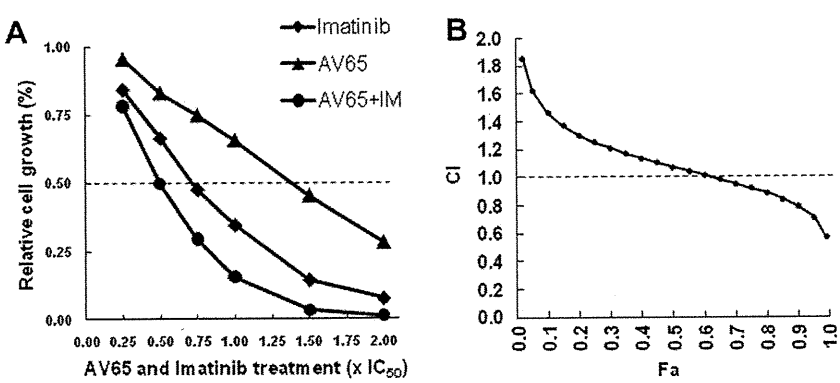

Fig. 5. The inhibitory effects of AV65 in combination with IM on K562 cells. (A) K562 cells were incubated for 72 h with six concentrations (0.25, 0.5, 0.75, 1.0, 1.5, or 2.0 times the IC_{50}) of AV65 and IM or a combination of the two agents using the constant ratio design of a modified MTT assay.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

pounds was selected for further analysis as inhibitors of the Wnt/β-catenin pathway. Ultimately, AV-65 was generated (Yao, in revision).

In the present study, the effect of AV65 on the suppression of the proliferation of IM-sensitive and -resistant CML cells was demonstrated. AV65 decreased β-catenin protein levels in CML cell lines in a time- and dose-dependent manner (Fig. 1B). Work from our group reveled that AV65 promotes the degradation of β-catenin via the ubiquitin–proteasome pathway (Yao, in revision). β-catenin translocation to the nucleus decreased in response to AV65 treatment (Fig. 1B). As we have demonstrated that AV65 decreased TCF transcriptional activity (Yao, in revision), the expression of its downstream proteins including c-myc and survivin were also reduced (Fig. 2A), resulting in cell growth inhibition. As expected, the proteasome inhib-

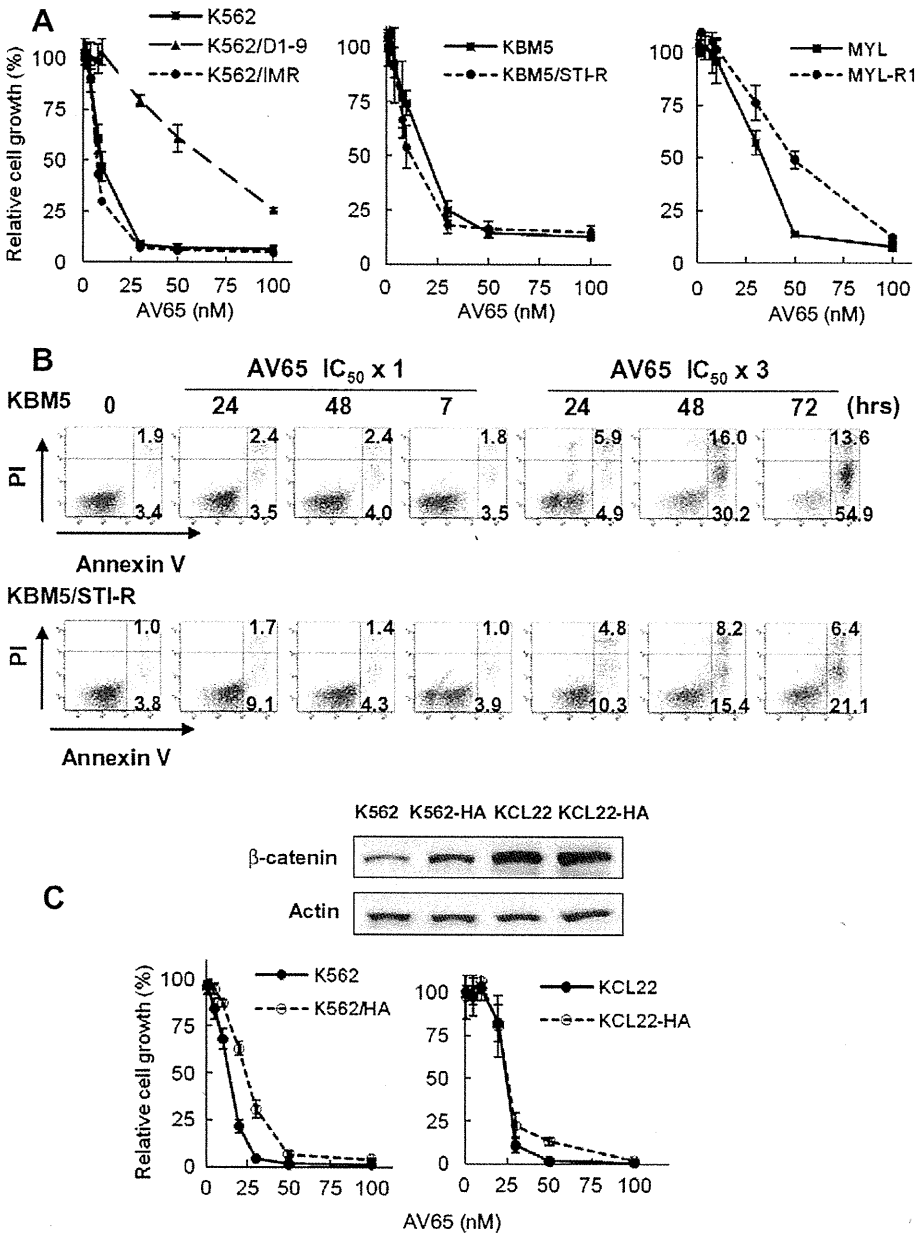

Fig. 6. Inhibitory effect of AV65 on IM-resistant CML cells. (A) Four IM-resistant CML cell lines; K562/IMR (Bcr-Abl amplification), MYL-R1 (Lyn overexpressing), KBM5/STI-R (harboring the T315I mutation), and K562/D1-9 (P-gp overexpressing), were exposed to AV65 for 72 h and its anti-proliferative effects were examined by a modified MTT assay. The results represent the means ± SD of three independent experiments. (B) KBM5 and KBM5/STI-R cells were exposed to AV65 for 24, 48, and 72 h at concentration of 1 × IC_{50} (20 nM, 15 nM, respectively) and 3 × IC_{50} (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 three 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 ± SD of three independent experiments (lower panel).

itor suppressed the degradation of β-catenin by AV65 (Supplementary Fig. S3). Flow cytometric analysis showed that AV65 induced caspase-dependent apoptosis in BV173 cells, but apoptosis was caspase-independent in K562 cells. These results suggest that the effect of AV65 on induction of apoptosis in CML cells may or may not be accompanied by the activation of caspases and that apoptosis with caspase activation varies depending on the CML cell type, as previously observed [29]. The present results, together with other data from our group (Yao, in revision), showed that AV65 inhibited TCF transcriptional activity by promoting the degradation of β-catenin, which resulted in the induction of cell death. The inhibition of TCF transcriptional activity by AV65 caused the downregulation of the

expression of survivin and c-myc, which are downstream effectors of β-catenin. Interestingly, AV65 also decreased the expression of the phosphorylated forms of Bcr-Abl, Erk1/2, Akt, and Stat5 (Supplementary Fig. S1). Although this mechanism remains still unclear, these observations suggest that Wnt/β-catenin signaling might play a role in the stabilization of Bcr-Abl. Further investigations are necessary to clarify this issue.

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

In the present study, the effects of AV65 were shown to be independent from Bcr-Abl expression levels, Lyn overexpression, or the Abl T315I mutation in human CML cell lines. AV65 is also effective against Ba/F3 cells expressing different mutant forms of Bcr-Abl, including T315I. These observations indicate that AV65 is effective against IM-resistant CML cells. Importantly, AV65 inhibited the growth of CML cells harboring the T315I mutation. To overcome IM-resistance in CML, a second generation of TKIs has been developed. However, the T315I mutation confers resistance to all known TKIs [9,42]. Moreover, studies have demonstrated that patients with the T315I mutation have a poor prognosis [43,44]. The development of novel agents directed against the T315I clone is therefore important, and several multi-targeted kinase inhibitors have recently been shown to be effective against the T315I mutation [45–47]. The present data demonstrate the potential of the AV65 compound as a novel agent against CML with the T315I mutation. However, the IC_{50} value of AV65 in K562/D1–9 cells (P-gp overexpressing) was approximately 60.1 nM, which was higher than the IC_{50} in the parental cell line and in other IM-resistant cells. This observation suggests that AV65 is a substrate of P-gp. As IM is currently the drug of choice for the treatment of CML, the effects of combination treatment with AV65 and IM were investigated and the results showed that AV65 enhanced the inhibitory effects of IM (Fig. 5).

AV65 also inhibited the growth of hypoxia-adapted CML cell lines at concentrations comparable with those shown to be effective in the respective parental cell lines. Although a definite CML stem cell niche has not been identified, leukemic stem cells are located in an osteoblastic niche [48,49], which is a hypoxic region of the BM [27], The self-renewal of normal hematopoietic stem cells favors hypoxia [50] and resistance to hypoxia is one of the defining features of leukemic stem cells [51]. HA cells survive long-term under hypoxic conditions (1% O_2) and include a large number of cells in a dormant state and resistant to Abl TKIs. Furthermore, these cells exhibit a 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 Kathyrn Strand are employees of PGx Health, A Division of Clinical Data, Inc.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2011.08.002.

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Rapid automated detection of ABL kinase domain mutations in imatinib-resistant patients

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ABSTRACT

ABL tyrosine kinase inhibitor (TKI), imatinib is used for BCR–ABL$^+$ leukemias. We developed an automatic method utilizing guanine-quenching probes (QP) to detect 17 kinds of mutations frequently observed in imatinib-resistance. Results were obtained from 100 μL of whole blood within 90 min by this method. Detected mutations were almost identical between QP method and direct sequencing. Furthermore, the mutation-biased PCR (MBP) was added to the QP method to increase sensitivity, resulting earlier detection of T315I mutation which was insensitive to any ABL TKIs. Thus, the QP and MBP-QP may become useful methods for the management of ABL TKI-treated patients.

1. Introduction

The ABL tyrosine kinase inhibitor (TKI), imatinib mesylate (imatinib) is the standard treatment for patients with chronic myeloid leukemia (CML) [1]. Imatinib is also used in combination with chemotherapy for Philadelphia chromosome positive (Ph$^+$) acute lymphocytic leukemia (ALL) [2]. Most newly diagnosed CML patients in chronic phase (CML-CP) respond well to imatinib. However, there is a high relapse rate among advanced phase (AP) and blast crisis (BC) patients owing to the development of mutations in the ABL kinase domain (KD) that cause drug resistance [3].

More than 90 different *bcr–abl* mutations encoding distinct single amino-acid substitutions within the ABL KD have been isolated from imatinib-resistant CML and Ph$^+$ALL patients. However, the number of frequent mutations is limited and only a relative small proportion is clinically very relevant [4]. To overcome imatinib-resistance, four second generation ABL TKIs (nilotinib, dasatinib, bosutinib and bafetinib) were developed, which inhibit the phosphorylation of most mutated BCR–ABL, except T315I [5]. Recently, several differences in their inhibitory mechanisms were identified [6–8]. Thus, it is very important to

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