

fractory multiple myeloma who had been enrolled in a previous phase II study [8] and who achieved at least no change (NC) with thalidomide treatment as per the study protocol.

2. Patients and Methods

2.1. Eligibility

Patients were deemed eligible for enrollment in this study if they had responded and maintained at least NC assessed at the 16th week of the phase II study period with single-agent thalidomide treatment given for at least 4 weeks [8]. According to the phase II study protocol, patients who achieved at least a minimal response (MR) continued on thalidomide treatment at the dosage with which the response had been obtained until the cutoff of the study. Otherwise, the dose of thalidomide was escalated by 100 mg every 4 weeks until the cutoff of the study (16th week) to a maximum of 400 mg/d. In total, 19 patients had achieved and maintained a response of at least NC according to the European Group for Blood and Marrow Transplantation response criteria [9] by the cutoff of the phase II study and were studied.

All patients gave written informed consent and agreed to abide by strict contraception. The study and the written informed consent form were approved by the institutional review board of each participating hospital. The study was conducted in accordance with the Good Clinical Practice for Trials of Drugs and the Declaration of Helsinki.

2.2. Treatment Schedule

Single-agent thalidomide treatment was continued in the 19 patients at their individual final doses of the phase II study until disease progression or intolerance occurred, for a maximum of 3 years. Patients were evaluated every 4 weeks for response and drug toxicity. Thalidomide was supplied by the Fujimoto Pharmaceutical Corporation (Osaka, Japan) and was given orally before sleep. No anti-thrombotic prophylaxis was instituted because no patients experienced thromboembolic events during the phase II study.

2.3. Response, Progression-Free Survival, Time to Next Treatment, and Toxicity Criteria

Responses were assessed by the decrease in the monoclonal protein measured at the time of entry into the phase II study using the European Group for Blood and Marrow Transplantation response criteria [9]. Progression-free survival (PFS) was measured from the date of initiation of thalidomide treatment in the phase II study until death or disease progression, whichever was earlier. Time to next treatment (TTNT) was measured from the date of initia-

tion of thalidomide treatment until death or the date of initiation of the next treatment. The PFS and TTNT curves were constructed according to the Kaplan-Meier method. Toxicities were graded using the National Cancer Institute Common Toxicity criteria (version 3).

3. Results

3.1. Patient Characteristics

A total of 19 patients were enrolled between December 2005 and April 2006. Patients were followed until March 2009 and the median follow-up from the start of the phase II study was 3 years (156 weeks; range, 28 - 180 weeks). The characteristics of the 19 patients are shown in **Table 1**. The mean age was 60 years (range, 42 - 81 years). More than half of the patients had relapsed after HDT-ASCT.

Eleven patients (57.9%) were treated with thalidomide at a dosage of 100 mg/day, 2 patients (10.5%) were treated at a dosage of 200 mg/day, 2 patients (10.5%) were treated at a dosage of 300 mg/day, and 4 patients (21.1%) were treated at a dosage of 400 mg/day.

3.2. Response

At the time of entry to this study, 5 patients (26.3%) had partial response (PR), another 5 patients (26.3%) had MR,

Table 1. Patients characteristics.

Variables	Total
Number of cases	19
Mean age (yr)	60.0
Range (yr)	42 - 81
Time sinceDx(yr)	5.03
Range (yr)	0.17 - 17
Sex (male/female)	8/11
M protein type	
IgG	12
IgA	6
Light chain	1
PS (0/1/2)	15/3/1
ISS stage (I/II/III)	11/3/5
Prior therapy	
Chemotherapy	8
Lines (median, range)	1.5, 1 - 3
ASCT	11
β2M (mg/L) median, range	2.50, 1.0 - 10.24
LDH (IU/L) median, range	162.5, 105 - 346

Dx; diagnosis, PS; performance status, β2M; β2 microglobulin.

and the remaining 9 patients (47.4%) had shown NC to the latest thalidomide therapy.

The cumulative response rate is shown in **Figure 1**. The reduction of M-protein was continued at the 112th week. MR was obtained in 73.7% at the 24th week and in 78.9% at the 32nd week. PR was obtained 31.6% at the 24th week, 31.6% at the 32nd week, 42.1% at the 48th week, and 47.4% at the 112th week.

3.3. Progression-Free Survival

Figure 2 shows the PFS curve of the 19 patients studied; the median PFS was 104 weeks (1.99 years). The PFS rates were 73.0% at 1 year, 55.1% at 2 years, and 28.0% at 3 years. Only 1 patient has died on the 31st week.

3.4. Time to Next Treatment

Figure 3 shows the TTNT curve of the 19 patients. The median TTNT was 144 weeks (2.76 years). The TTNT rate was 73.7% at 1 year, 63.2% at 2 years, and 47.4% at 3 years. Thalidomide/high-dose dexamethasone treatment was conducted in 6 patients and melphalan/prednisolone/-thalidomide treatment was conducted in 3 patients after the discontinuance of single-agent thalidomide maintenance therapy. Eight patients continued single-dose thalidomide treatment over 40 months after progressive disease. Thalidomide was discontinued in only 2 patients.

3.5. Toxicities

All 19 patients experienced at least Grade 1 toxicity; however, no patient experienced Grade 4 or greater hema-

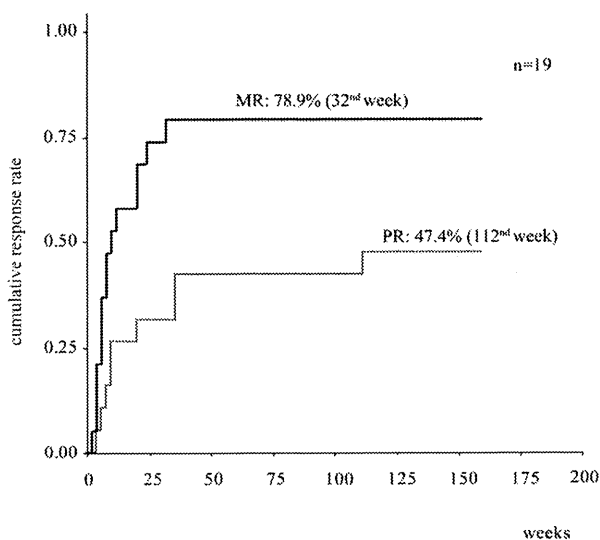


Figure 1. The cumulative response rate in patients treated with long-term thalidomide maintenance. The black line

shows minimal response rate and the gray line shows partial response rate.

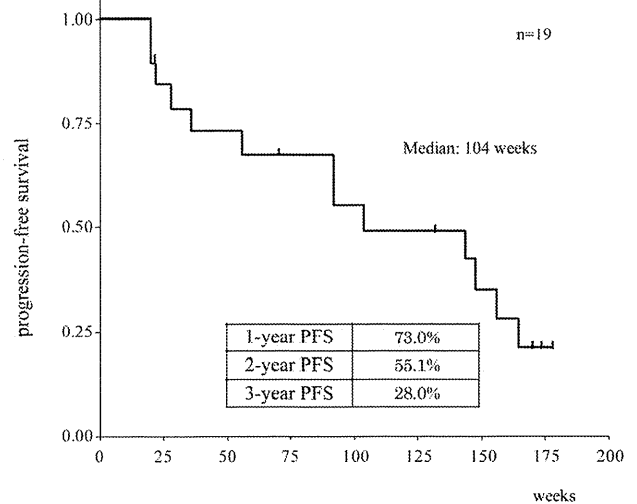


Figure 2. Progression-free survival in patients treated with long-term thalidomide maintenance. The curve was constructed according to the Kaplan-Meier method.

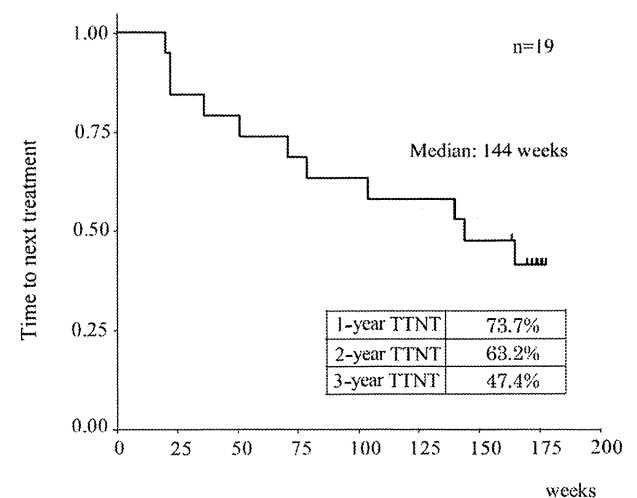


Figure 3. Time to next treatment in patients treated with long-term thalidomide maintenance. The curve was constructed according to the Kaplan-Meier method.

atologic toxicity or Grade 3 or greater non-hematologic toxicity. The toxicity profile observed in at least 10 patients is shown in **Table 2**. The most common Grade 3 hematologic toxicities were neutropenia in 9 patients (47.4%), lymphopenia in 5 (26.3%), and leucopenia in 4 (21.1%). The most common Grade 2 non-hematologic toxicities were peripheral neuropathy in 3 patients (15.8%), constipation in 2 (10.5%), and skin rash in 2 (10.5%). One patient receiving 200 mg/d thalidomide but no thromboprophylaxis experienced deep vein thrombosis on the 71st week.

Table 2. Toxicity profile.

Toxicity	n (%)			
	Total	Grade 1	Grade 2	Grade 3
Hematological				
Neutropeni	14 (73.7)	1 (5.3)	4 (21.1)	9 (47.4)
Lymphopenia	10 (52.6)	1 (5.3)	4 (21.1)	5 (26.3)
Leucopenia	10 (52.6)	2 (10.5)	4 (21.1)	4 (21.1)
Basophilia	11 (57.9)	11 (57.9)	0	0
Non-hematological				
Constipation	16 (84.2)	14 (73.7)	2 (10.5)	0
Peripheral neuropathy	15 (78.9)	12 (63.2)	3 (15.8)	0
Somnolence	14 (73.7)	13 (68.4)	1 (5.3)	0
Dry mouth	11 (57.9)	11 (57.9)	0	0
Edema	10 (52.6)	10 (52.6)	0	0
Tremor	10 (52.6)	10 (52.6)	0	0
Skin rash	10 (52.6)	8 (42.1)	2 (10.5)	0

Toxicity or intolerance that resulted in the discontinuation or dose reduction of thalidomide occurred in 4 of the 19 patients (21.1%). These 4 patients were treated with thalidomide at a dosage of over 200 mg/day. Thalidomide treatment was discontinued in 2 patients: deep vein thrombosis occurred in 1 patient treated with 200 mg/d on the 71st week, and nephrotic syndrome occurred in 1 patient on the 132nd week. Thalidomide dosage was reduced in another 2 patients because of peripheral neuropathy and neutropenia. No patients treated with 100 mg/d thalidomide discontinued treatment due to toxicity.

4. Discussion

This study was conducted prospectively to evaluate the efficacy and safety of long-term treatment with single-agent thalidomide in patients who had been enrolled and obtained at least NC in a phase II trial of the use of thalidomide in patients with relapsed/refractory multiple myeloma [8]. The dosage of thalidomide in this continuous treatment study was determined by the one with which each patient had responded by achieving at least NC in the preceding phase II trial.

Nineteen patients were enrolled. Eleven patients (57.9%) were treated with continuous thalidomide treatment at a dosage of 100 mg/day, 2 patients (10.5%) were treated at a dosage of 200 mg/day, 2 patients (10.5%) were treated at a dosage of 300 mg/day, and 4 patients (21.1%) were

treated at a dosage of 400 mg/day. The discontinuation or dose reduction of thalidomide occurred in 4 patients (21.1%). These 4 patients were treated with thalidomide at a dosage of over 200 mg/day. In contrast, no patients treated with 100 mg/d discontinued thalidomide due to toxicity. According to these findings, low-dose thalidomide might be adequate for the maintenance treatment for multiple myeloma.

The beneficial effect of long-term treatment with thalidomide was observed at least until week 112 (2.15 years). The PR rate was 26.3% at the time of initiation of maintenance treatment (16th week) and continuously increased up to 47.4% at the 112th week. The MR rate also increased from 26.3% at the initiation of maintenance treatment to 78.9% at the 32nd week. Singhal *et al.* reported that the response of thalidomide treatment was obtained within 4 months in patients with refractory myeloma [10]; however, our study revealed the response of long-term thalidomide maintenance treatment gradually increased over 4 months after initiation of thalidomide.

The PFS was fairly long in this study. It is difficult to compare this result with those in the published reports on single-agent thalidomide treatment in cases of relapsed/refractory multiple myeloma, because most trials used a starting thalidomide dose of 200 mg/d and utilized a dose escalation up to 800 mg/d, and also because the follow-up period is not as long as that in our study [6,11]. The

case series studied may be mostly composed of low-risk patients in terms of age, International Staging System stages, β 2-microglobulin levels, and lactate dehydrogenase levels. Recent studies have shown the importance of obtaining complete response (CR) not only in patients with newly diagnosed multiple myeloma but also relapsed/refractory multiple myeloma [12,13]. However, in patients in the low-risk category, survival is not significantly different between patients with CR and those with PR [14]. Another recent study has disclosed that maintaining CR is more important than obtaining CR in terms of longer survival duration [15]. It is also noted that patients with low-risk disease can survive longer with PR status [14]. In our patients, long-term maintenance treatment with thalidomide upgraded the initial response status and sustained the upgraded response status, which resulted in prolonged PFS.

The TTNT was extremely long compared with the PFS. Eight patients continued single-dose thalidomide treatment after progressive disease because of the absence of progression to active myeloma, namely clinical relapse [16]. According to this finding, one of the reasons for the long TTNT might depend on the slow progression to active myeloma during thalidomide maintenance treatment. Our results are in agreement with the comment of Stewart [17] that, in a slower-tempo relapse, sequencing of drugs may offer superior overall survival results.

With regard to a long-term treatment with thalidomide, there has been a concern of the late development of neuropathy if given sufficient length of time with low-dose thalidomide [18]. However, long-term treatment with low-dose thalidomide for as long as 3 years in the present study did not result in delayed development of adverse events. Furthermore, because of the lower toxicity of low-dose thalidomide, the patients could stay on the treatment and enjoyed a long-term survival with good activities of daily living.

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ORIGINAL ARTICLE

AV-65, a novel Wnt/ β -catenin signal inhibitor, successfully suppresses progression of multiple myeloma in a mouse model

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Multiple myeloma (MM) is a malignant neoplasm of plasma cells. Although new molecular targeting agents against MM have been developed based on the better understanding of the underlying pathogenesis, MM still remains an incurable disease. We previously demonstrated that β -catenin, a downstream effector in the Wnt pathway, is a potential target in MM using RNA interference in an *in vivo* experimental mouse model. In this study, we have screened a library of more than 100 000 small-molecule chemical compounds for novel Wnt/ β -catenin signaling inhibitors using a high-throughput transcriptional screening technology. We identified AV-65, which diminished β -catenin protein levels and T-cell factor transcriptional activity. AV-65 then decreased *c-myc*, cyclin D1 and survivin expression, resulting in the inhibition of MM cell proliferation through the apoptotic pathway. AV-65 treatment prolonged the survival of MM-bearing mice. These findings indicate that this compound represents a novel and attractive therapeutic agent against MM. This study also illustrates the potential of high-throughput transcriptional screening to identify candidates for anticancer drug discovery.

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Keywords: multiple myeloma; Wnt; β -catenin; high-throughput screening

Introduction

Multiple myeloma (MM) is a malignant neoplasm of plasma cells characterized by frequent chromosomal translocations and gene mutations.¹ High-dose chemotherapeutics in conjunction with hematopoietic stem cell transplantation has been one standard clinical course of the treatment of MM.^{2–4} As we gain a better understanding of the pathogenesis underlying MM, new molecular targeting agents can be developed.^{5,6} Nevertheless, MM remains incurable at present.⁷ Thus, it is important to continue to investigate new therapeutic agents that focus on targeting the biology of MM cells.

β -Catenin is a downstream effector in the canonical Wnt signaling pathway; thus, it is involved in the regulation

of cell fate, proliferation and the self-renewal of stem cells. In the absence of Wnt ligands, cytosolic β -catenin undergoes proteasomal degradation through sequential phosphorylation and ubiquitination.^{8,9} In the process, β -catenin that is phosphorylated by casein kinase I, and subsequently glycogen synthase kinase-3 β , forms a degradation complex comprised of glycogen synthase kinase-3 β , casein kinase I, axin and adenomatous polyposis coli. Phosphorylated β -catenin is ubiquitinated by cellular β -transducin repeat-containing proteins (β -TrCP), and subsequently degraded by the 26S proteasome.¹⁰ Upon Wnt ligand binding to Frizzled receptors and the co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6, the dishevelled phosphoprotein inactivates glycogen synthase kinase-3 β , and the β -catenin degradation complex dissociates, resulting in the stabilization of β -catenin. Stabilized β -catenin translocates to the nucleus, where it binds to lymphoid enhancer-binding factor (LEF) and T-cell factor (TCF) proteins to activate the transcription of target genes such as *c-myc* and *cyclin D1*, which are involved in cell proliferation and survival.^{11,12} Activation of Wnt signaling is closely linked to the process of carcinogenesis.¹³ Human MM-derived cell lines and primary MM cells overexpress β -catenin, whereas in normal plasma cells, β -catenin expression is undetectable.^{14–16} The recent study demonstrated that genes of soluble Wnt signal antagonists were methylated in MM cells, resulting in repression of their transcripts, and consequently, the increased expression of β -catenin.¹⁷ Overexpression of β -catenin promotes transcriptional activity of target genes related to the proliferation of MM cells, whereas inhibition of Wnt/ β -catenin signaling suppresses MM growth.^{15,16} These results suggest that β -catenin might be an attractive therapeutic target for MM.

Progress in molecular biology has enabled significant advances in drug discovery strategies. For example, RNA interference is a technique that can be used to knockdown the expression of specific genes in treated cells. An altered transcriptional profile can then be used as a biomarker for a specific molecular targeting, and quantitative evaluation can be applied to high-throughput technology.^{18,19} Using high-throughput transcriptional screening technology (Supplementary Figure 1), we identified effective inhibitors of the Wnt/ β -catenin signaling from a library of more than 100 000 chemical compounds (Supplementary Figure 2). From this initial series, we optimized a novel Wnt/ β -catenin signal inhibitor, AV-65, that significantly suppressed the growth of MM cells and prolonged survival in a mouse model of orthotopic MM. These results demonstrate that AV-65 is a novel and attractive therapeutic agent for the treatment of MM.

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Materials and methods

Cell lines, reagents and animals

Human AMO-1, KMS-12-BM, RPMI8226, NCI-H929, U226 and OPM-2 myeloma cell lines, and the IM-9 Epstein-Barr virus-transformed cell line derived from an MM patient, were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). RPMI8226/LR5 melphalan-resistant cells were kindly provided by Dr William S Dalton (University of South Florida, Tampa, FL, USA).²⁰ HCT-15 colorectal adenocarcinoma cells were purchased from American Tissue Culture Collection (Manassas, VA, USA). The 293T cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. IM-9, RPMI8226, NCI-H929 and U266 cells were cultured in RPMI1640 (Gibco, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Tokyo, Japan), 2 mM L-glutamine (Gibco) and 1% penicillin-streptomycin (Gibco). The AMO-1 and KMS-12-BM cell lines were cultured in RPMI1640 containing 20% FCS, 2 mM L-glutamine and 1% penicillin-streptomycin. The RPMI8226/LR5 cell line was cultured in media containing 10% FCS, 2 mM L-glutamine, 1% penicillin-streptomycin and 5 μ M melphalan (Sigma-Aldrich, Tokyo, Japan). Hepatocytes were cultured using CS-C complete medium kit (Cell Systems, Kirkland, WA, USA). HCT-15 cells were cultured in RPMI1640 containing 5% FCS, 2 mM L-glutamine and 1% penicillin-streptomycin. The 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin-streptomycin. All cell lines were maintained at 37°C in a fully humidified atmosphere of 5% CO₂ in air.

Peripheral blood mononuclear cells from three healthy volunteers were isolated by using Ficoll-Hypaque (GE Healthcare Japan, Tokyo, Japan), and cultured with in Iscove's modified Dulbecco's medium (Gibco) containing 20% FCS, 2 mM L-glutamine and 1% penicillin-streptomycin. Bone marrow (BM) samples were obtained from MM patients who were hospitalized in the Hospital of Kyoto Prefectural University of Medicine. In accordance with the Declaration of Helsinki recommendations, all procedures were approved by the institutional review board at Kyoto Prefectural University of Medicine, and written informed consent was obtained from every participant. Ficoll-Hypaque density centrifugation was used to separate mononuclear cells, followed by magnetic cell sorting (Miltenyi, Gladbach, Germany) using an anti-CD138 antibody (Ab) (Miltenyi) to enrich BM-derived MM cells. Cells were analyzed by FACS Canto II using the Diva software (BD Bioscience, Tokyo, Japan). The purity of the enriched CD138-positive cell populations was \geq 95%. Isolated MM cells were cultured in RPMI1640 containing 10% FCS, 2 mM L-glutamine and 20 ng/ml human interleukin-6 (R&D Systems, Minneapolis, MN, USA).

Specific pathogen-free 6- to 8-week old female nonobese diabetic-severe combined immune deficiency (NOD/SCID) mice (Clea Japan, Tokyo, Japan) were used for the *in vivo* experiments. Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

Growth inhibitory effects on myeloma cells

Cell proliferation was evaluated by the modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay using Cell-Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan), as described previously.^{21,22} Cells were seeded in a

flat-bottomed 96-well plate (BD Bioscience) at a density of 3×10^3 cells in 100 μ l of medium per well, and then incubated with serial dilutions of AV-65 for 72 h. The mean of four samples at each concentration was evaluated. Half maximal inhibitory concentration values were obtained using the nonlinear regression program CalcuSyn (Biosoft, Cambridge, UK).

Western blot analysis

Following treatment with AV-65, more than 1×10^6 cells were collected by centrifugation, and then the cells were washed with ice-cold phosphate-buffered saline (–) twice. Ice-cold radio-immunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 5 mM EDTA, 20 mM NaF, 1% NP-40) containing fresh phenylmethylsulfonyl (1 mM) and protease inhibitor (10 μ g/ml) was added to the cells. The suspension was transferred into a centrifuge tube and placed on ice for 15 min (min) with occasional vortexing to ensure complete lysis of the cells. The cell suspension was cleared by centrifugation at 14 000 g for 30 min at 4°C. Nuclear and cytoplasmic protein fractions were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. The supernatants (total cell lysate, nuclear and cytoplasmic protein fractions) were either used immediately or stored at –80°C. Protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories, Osaka, Japan). Immunoblotting was performed as described previously.^{16,21} Samples (20 μ g of protein) were analyzed using the following primary Abs, as indicated: anti- β -catenin (BD Pharmingen, San Jose, CA, USA), -Bad (Stressgen, Victoria, BC, Canada), -Bid (a kind gift from Dr David CS Huang, The Walter and Eliza Hall Institute of Medical Research (WEHI), Parkville, VIC, Australia),²³ -Bim (clone 3C5, produced by Dr LA O'Reilly (WEHI)), -Bcl-2 (Bcl-2-100; Upstate, Lake Placid, NY, USA), -Bcl-xL (Stressgen), -Puma (ProSci, Poway, CA, USA), -Noxa (Alexis Biochemicals, Lausen, Switzerland), -Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), -c-myc (Santa Cruz Biotechnology), -cyclin D1 (BD Pharmingen), -Oct-1 (Santa Cruz Biotechnology), survivin (Cell Signaling Technology, Danvers, MA, USA) and -actin (Sigma-Aldrich). Horseradish peroxidase-coupled immunoglobulin G (Amersham Biosciences, Tokyo, Japan) was used as a secondary Ab, and immunoreactive proteins were detected by enhanced chemiluminescence or ECL-plus kits (Amersham Biosciences).

Ubiquitination of β -catenin

At 12 h after AV-65 treatment, whole-cell lysates were obtained as described above. Lysates were subjected to immunoprecipitation using an anti- β -catenin monoclonal Ab (BD Pharmingen) and Dynabeads Protein A (Invitrogen), according to the manufacturer's instructions. Ubiquitination of β -catenin was detected with anti-mono- and anti-poly-ubiquitinyl conjugates (Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA).

TCF/LEF dual luciferase reporter assay

The activity of TCF/LEF transcription in HCT-15 cells was evaluated with the Wnt Signal Reporter Assay (SABioscience, Fredrick, MD, USA). HCT-15 colorectal cancer cell line expresses high levels of β -catenin²⁴ and is easily transfectable with plasmids. For each sample, 3×10^4 HCT-15 cells were reverse-transfected with 100 ng of a TCF/LEF firefly luciferase reporter plasmid and a constitutively expressing CMV-driven

Renilla luciferase reporter with SureFECT Transfection Reagent (SABioscience, Fredrick, MD, USA), according to the manufacturer's instructions. At 16 h post-transfection, media were changed to assay media (Opti-MEM containing 0.5% FBS and 1% non-essential amino acids) for 8 h, followed by AV-65 treatment for 14 h. Relative luciferase activity of cells was detected using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and a Wallac Victor 1420 Plate Reader (Perkin-Elmer, Waltham, MA, USA). The relative luciferase activities generated by each well were calculated by determining the ratio of the luciferase (*firefly/Renilla*) values to normalize the signal. Three independent transfections were carried out in duplicate for each of the concentrations tested.

Induction of apoptosis and cell cycle analysis

Cell cycle analysis using propidium iodide (PI) was performed as described previously.²² Apoptosis induced by AV-65 was determined using Annexin-V-FITC Apoptosis Detection Kit I (BD Bioscience), according to the manufacturer's instructions. Cells were analyzed by FACS Canto II using the Diva software (BD Bioscience). Caspase-3, -8 and -9 activities in the presence of AV-65 were evaluated using a fluorometric protease assay kit (MBL, Aichi, Japan), according to the manufacturer's instructions.

Lentiviral transduction

We produced β -TrCP knockdown IM-9 cells using a lentivirus vector expressing short hairpin RNA against β -TrCP. Plasmids expressing short hairpin RNA against β -TrCP and green fluorescent protein were provided by Dr Kenzo Tokunaga (International Medical Center of Japan, Tokyo, Japan). The lentiviral packaging plasmid psPAX2 was provided by Addgene (Addgene plasmid 12260, D Trono, Lausanne, Switzerland).

The pHIT/G plasmid²⁵ expressing the vesicular stomatitis virus-G protein was kindly provided by M Malim (University of Pennsylvania, Philadelphia, PA, USA). In all, 10 μ g of β -TrCP short hairpin RNA, 10 μ g of psPAX2 and 10 μ g of pHIT/G were added to a mixture of BES (*N,N*-bis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid)-buffered saline and calcium chloride. After incubation for 20 min, the solution was added dropwise to a 10 cm dish of 70% confluent 293T cells. The cells were incubated for 12 h at 37 °C in a humidified incubator with an atmosphere of 3% CO₂. The medium was replaced with 10 μ M forskolin-containing medium and incubated at 37 °C and 10% CO₂. After 48 h, the supernatants were harvested. In total, 1.0 \times 10⁶ IM-9 cells were transduced with 3 ml of the supernatant and polybrene (Sigma-Aldrich). We isolated GFP-positive cells by using a limiting dilution method.

Real-time polymerase chain reaction

RNA was purified with the QIAamp RNA Blood Mini Kit (Qiagen, Tokyo, Japan) and subjected to reverse transcription. Human β -TrCP gene expression levels were measured by a real-time polymerase chain reaction. A real-time polymerase chain reaction mixture contained 4 μ l of Taqman master mix (Roche Diagnostics GmbH, Mannheim, Germany), cDNA, pairs of primers and Taqman probe (Universal Probe Library; Roche Diagnostics GmbH). The cDNA was amplified with a Light Cycler 3.5 (Roche Diagnostics GmbH) using the following parameters: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Glyceraldehyde-3-phosphate was used as an internal to normalize the loading difference. The primers for β -TrCP were as follows: forward primer, CCAGATAAATAA CCATACACTGACCTC and reverse primer, CAGATACGTTAAA TACCGCAACTTT. The primers for glyceraldehyde-3-phosphate were as follows: forward primer, AGCCACATCGCTCAGACAC and reverse primer, GCCCAATACGCCAAATCC.

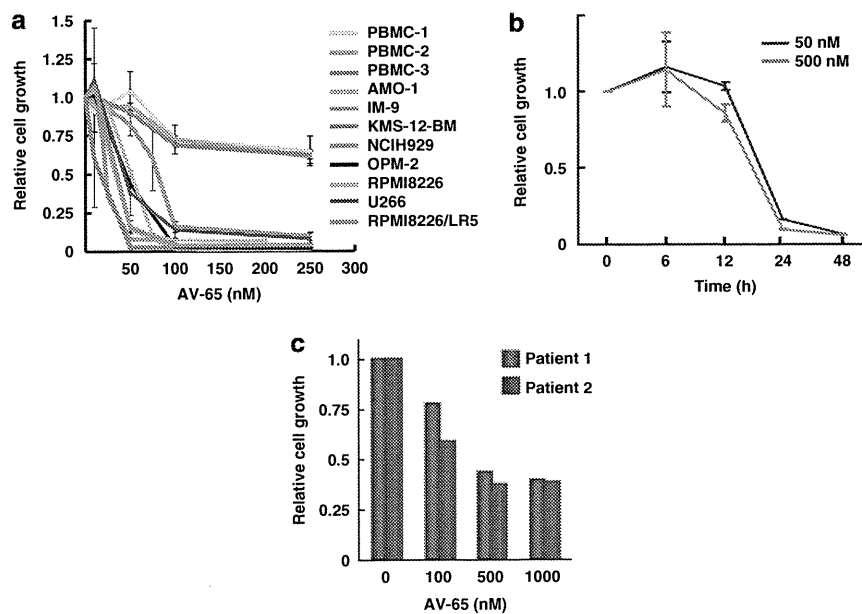


Figure 1 Inhibition of myeloma cell proliferation. (a) The dose-dependent effect of compounds on the proliferation of myeloma cell lines was evaluated by the modified MTT assay. Cells were incubated with serial dilutions of compounds for 72 h. Data represent the means \pm s.d. of three independent experiments, and four replicate experiments. (b) Time-dependent inhibition of proliferation of IM-9 cells. Cells were incubated with AV-65 for 6, 12, 24 and 48 h. Data represent the means \pm s.d. of three independent experiments, and four replicate experiments. (c) Effect of compounds on CD138-positive myeloma cells and CD138-positive normal plasma cells. CD138-positive myeloma cells and CD138-normal plasma cells were purified from BM mononuclear cells obtained from myeloma patients and healthy volunteers, respectively. Data represent the mean of four replicates for each concentration.

In vivo effects of AV-65 on myeloma cells

The *in vivo* inhibitory effects of AV-65 on MM cells were investigated using an orthotopic mouse model. After exposure to 2 Gy of irradiation, specific pathogen-free 7- to 8-week old female NOD/SCID mice (Japan Clea, Osaka, Japan) were inoculated intravenously with 5×10^6 IM-9 cells in 100 μ l of phosphate-buffered saline through the tail vein. IM-9 cells were successfully engrafted into the BM of all mice and IM-9-bearing mice died of MM approximately 32 days after inoculation. For the *in vivo* analysis of AV-65, the compound was formulated as a solid dispersion of 20%, the active drug ingredient with 80% of polyvinylpyrrolidone as an excipient to improve solubility. The *in vivo* formulation was administered intravenously through the tail vein in four cycles of 4 days on/1 day off, starting on the day following inoculation of IM-9 cells.

To investigate whether AV-65 treatment induced myelosuppression, we performed the colony-forming assay. After four cycles of AV treatment, NOD/SCID mice were killed and BM cells were obtained. Mononuclear cells were obtained by density centrifugation, and a colony-forming assay was performed using MethoCult GF M3434 (Stem Cell Technologies Inc., Vancouver, BC, Canada), according to the manufacturer's instructions. The complete blood counts of the mice treated with vehicle or AV-65 were measured using the MEK-6450 Celltac α cell counter (Nihon Kohden, Tokyo, Japan).

Statistical analysis

The *in vivo* effects of AV-65 treatment were analyzed using the log-rank test; the effects of AV-65 on colony-forming ability and complete blood counts were analyzed using the Student's *t*-test. A *P*-value of 0.05 was considered statistically significant.

Results

Inhibitory effects of AV-65 on myeloma cells in vitro

AV-65 inhibited growth of seven MM cell lines and the melphalan-resistant RMPMI8226/LR5 cell line in a dose-dependent manner (Figure 1a), with IC_{50} values after 72 h exposures in the range of 11.7–82.1 nM. However, AV-65 had lesser effects on normal peripheral blood mononuclear cells compared with MM cell lines (Figure 1a). AV-65 also inhibited the growth of IM-9 cells in a time-dependent manner (Figure 1b). CD138-positive myeloma cells obtained from MM patients were growth-inhibited by AV-65 in a dose-dependent manner (Figure 2c). Compared with the cell lines, higher doses of AV-65 were required for killing primary MM cells, presumably because the primary cells are quiescent and less sensitive to cytotoxic stimuli *ex vivo*.

Alteration of β -catenin expression and TCF transcriptional activity by AV-65

We next investigated alterations in β -catenin expression in MM cells. The levels of β -catenin in both the cytoplasmic and the nuclear fractions were diminished after 18 h by AV-65 treatment (Figure 2a). We also examined the ubiquitination of β -catenin in IM-9 cells following 12 h of exposure to AV-65. β -Catenin complexes were captured by immunoprecipitation with an anti- β -catenin Ab, and then analyzed by western blot using an Ab specific for ubiquitin–protein conjugates. Higher molecular weight ubiquitinated proteins were more prominent immunoprecipitates from AV-65-treated cells (Figure 2b), which

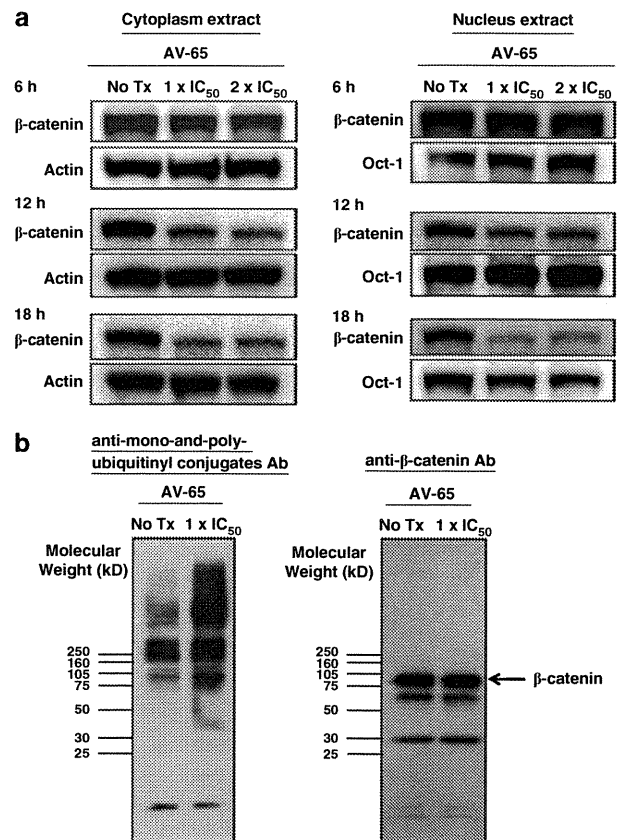


Figure 2 Alteration of β -catenin expression in myeloma cells by AV-65 treatment. (a) Cells were incubated with serial dilutions of AV-65 and harvested after 6, 12 and 18 h. Nuclear and cytoplasmic protein fractions were obtained and analyzed by western blot using an anti- β -catenin Ab. Results are representative of three independent experiments. (b) Ubiquitination of β -catenin by AV-65 treatment. Cells were incubated with the indicated concentration of AV-65 ($1 \times IC_{50}$), and then harvested after 12 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 μ l) was analyzed by immunoblot using anti-mono- and anti-poly-ubiquitinyl conjugates to detect ubiquitination of β -catenin (left panel). The bands of about 60 and 30 kDa detected in the membrane using anti- β -catenin Ab are nonspecific (right panel).

suggested that AV-65 induces the degradation of β -catenin through the ubiquitin–proteasome pathway.

We next investigated the modulation of TCF transcriptional activity in AV-65-treated HCT-15 colorectal adenocarcinoma cells using a TCF/LEF dual luciferase reporter assay. The cells were transfected with a TCF/LEF firefly luciferase reporter plasmid along with a constitutively active CMV-driven *Renilla* luciferase reporter plasmid to ensure transfection efficiency. Transcriptional activity in HCT-15 cells was decreased by AV-65 treatment in a dose-dependent manner (Figure 3a). Moreover, the expression of cyclin D1, c-myc and survivin, which are downstream targets of TCF,^{11,12,26–28} were suppressed in IM-9 and U266 cells (Figure 3b). Taken together, these results suggest that AV-65 enhances the ubiquitination and subsequent proteasomal degradation of β -catenin, and that β -catenin/TCF target gene promoter activity is decreased.

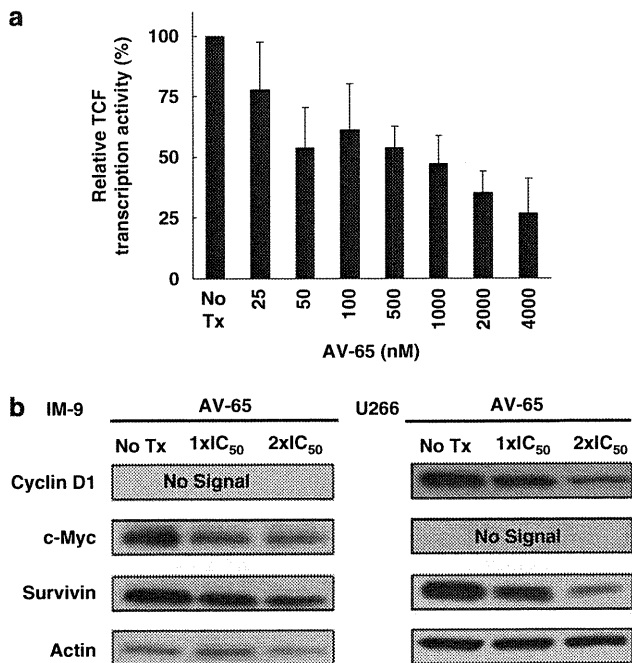


Figure 3 Inhibition of β -catenin-TCF signaling by AV-65 treatment. (a) Inhibition of TCF transcriptional activity in HCT-15 colorectal cancer cells. Cells were co-transfected with a TCF/LEF firefly luciferase reporter, and a CMV-driven *Renilla* luciferase reporter. To normalize transfection efficiency, cells were incubated with AV-65 for 14 h, and then luciferase activity was measured. Data represent the mean \pm 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 μ g of protein) were analyzed by western blot using the indicated Abs. Cyclin D1 and c-myc were not expressed in IM-9 and U266 cells, respectively.

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.

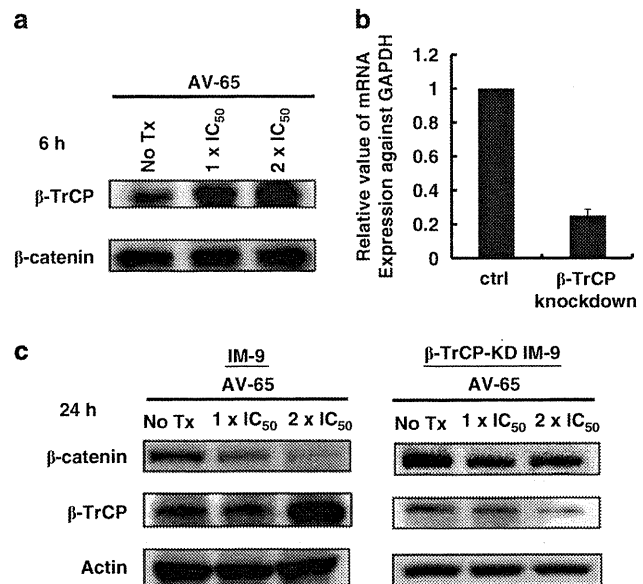


Figure 4 Enhancement of the interaction between β -catenin and β -TrCP by AV-65 treatment. (a) Cells were incubated with serial dilutions of AV-65 and then harvested after 6 h. Whole lysate was subjected to immunoprecipitation using an anti- β -catenin Ab, and immune complexes were captured using protein A beads. The protein A slurry (30 μ l) 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 \pm 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 μ g of protein) were analyzed by western blot using an anti- β -catenin Ab and an anti- β -TrCP Ab.

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.

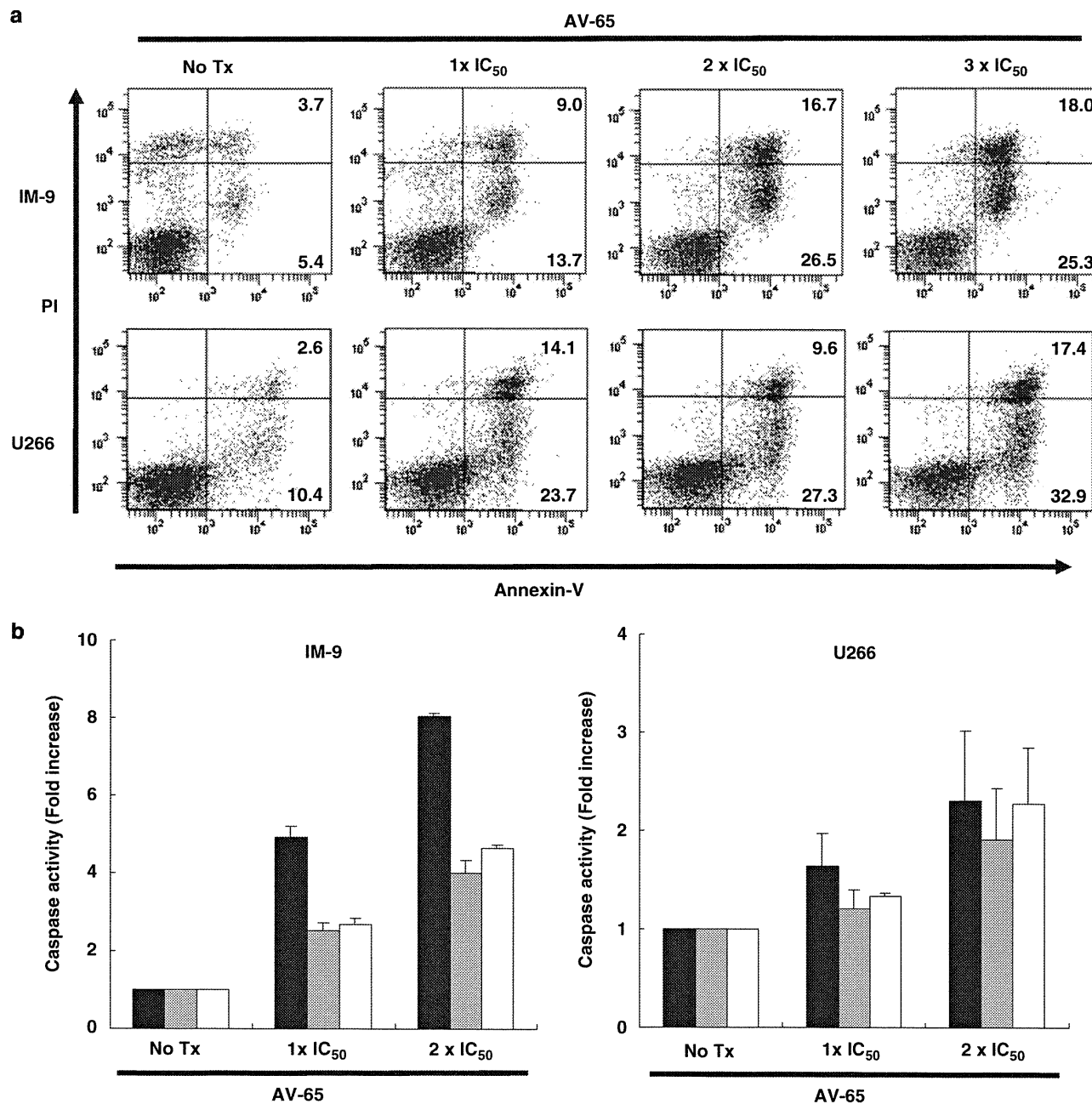


Figure 5 Induction of apoptosis in myeloma cells by AV-65. (a) Cells were incubated with serial dilutions of AV-65 and harvested after 24 h. Data are representative of three independent experiments. The numbers inside each quadrant indicate the percentage of the cell population with the quadrant characteristic. (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 \pm 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

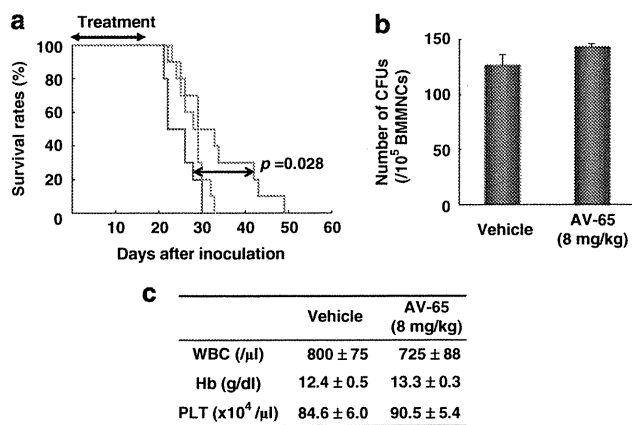


Figure 6 The *in vivo* inhibitory effects of AV-65 on MM cells in an orthotopic mouse model. Following irradiation (2 Gy), specific pathogen-free 7- to 8-week old female NOD/SCID mice were inoculated intravenously with 5×10^6 IM-9 cells in 100 μl phosphate-buffered saline through the tail vein. The day 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 \pm 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 \pm 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; *CEA-CAM6*, 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, Kathryn Strand and Janak Padia are employees of PGx Health, a Division of Clinical Data Inc.

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