Antagonistic Effect of Small-molecule Inhibitors of Wnt/β-catenin in Multiple Myeloma

BHAGAVATHI A. NARAYANAN[1], NICOLE A. DOUDICAN[2], JEESUN PARK[3,6], DAZHONG XU[1], NARAYANAN K. NARAYANAN[1], RAMANUJ DASGUPTA[4,6] and AMITABHA MAZUMDER[5,6]

[1]Department of Environmental Medicine, [2]Department of Dermatology, [3]Division of Immunology, [4]Department of Pharmacology, [5]Department of Medicine and [6]NYU Cancer Institute, New York University School of Medicine, New York, NY, U.S.A.

Abstract. *Background: Development and progression of multiple myeloma is dependent on the bone marrow (BM) microenvironment, and within the BM, a number of factors are secreted, including the Wnt ligands. Bone marrow stromal cells (BMSC) secrete Wnt ligands that activate Wnt signaling in multiple myeloma. The canonical Wnt pathway which is mediated through the transcriptional effector β-catenin (β-cat) is commonly de-regulated in many cancers. Cells with active β-cat-regulated transcription (CRT) are protected against apoptosis; conversely, inhibition of CRT may prevent cell proliferation. Materials and Methods: In this study, we tested the efficacy of recently described inhibitors of CRT (iCRTs; oxazole and thiazole) for their selective antagonistic effect on Wnt-β-cat response in MM cells MM.1, U266, BMSC and primary BMMC obtained from patient samples (n=16). Results: We demonstrated that iCRTs we used, block Wnt/β-cat reporter activity, down regulate β-cat expression and inhibit cell proliferation in a dose-dependent manner with an optimal dose closer to 15 μM. Our data further indicate that iCRTs do not influence the expression of the upstream components of the Wnt pathway DKK1 at the optimal dose, suggesting that iCRTs may specifically target β-cat in MM cells. Additionally, iCRT-treatment of MM cells, co-cultured with BMSC, showed an inhibitory effect on VEGF and cell migration. Conclusion: This study provides the first in vitro data evaluation of newly-described iCRTs as potential Wnt-β-cat/VEGF pathway antagonists in multiple myeloma.*

Correspondence to: Bhagavathi A. Narayanan, New York University School of Medicine, Department of Environmental Medicine, Tuxedo, NY 10987, U.S.A. e-mail: Bhagavathi.Narayanan@nyumc.org; Ramanuj Dasgupta, New York University School of Medicine, New York, Department of Pharmacology and NYU Cancer Institute, New York, NY 10016, U.S.A. e-mail: Ramanuj Dasgupta@nyumc.org; Amitabha Mazumder, New York University School of Medicine, Department of Medicine and NYU Cancer Institute, New York, NY 10016, U.S.A. e-mail: Amitabha.Mazumder@nyumc.org

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Multiple Myeloma (MM) is the second most frequent hematological cancer in the US and is characterized by the clonal proliferation of neoplastic plasma cells in association with elevated serum monoclonal protein levels. Clinical manifestations of MM include lytic bone lesions, anemia, immunodeficiency, and renal impairment. It is evident from earlier studies that maintenance of MM within the bone marrow (BM) microenvironment largely depends on a number of factors including cytokines IL-6 and the family of Wnt ligands that are secreted by the BM stromal cells (BMSCs) (1-5). Wnt ligand specifically activates the canonical Wnt pathway which is mediated through the key transcriptional effector β-catenin (β-cat). In unstimulated cells, β-cat is phosphorylated by the Axin/APC/GSK3β-mediated destruction complex (DC), and is a target for ubiquitination and subsequent proteasome-mediated degradation. Wnt induction blocks the activity of the DC, thereby resulting in the cytosolic accumulation of non-phosphorylated, active β-cat, which translocates to the nucleus and together with T-cell factor/Lymphoid Enhancer Factor (TCF/LEF) transcriptional factors, activates the transcription of downstream target genes. The Wnt pathway has been shown to play an important role in the regulation of cancer cell proliferation and differentiation (2, 6-8). Previous studies have shown that malignant MM plasma cells overexpress β-cat, including its N-terminally non-phosphorylated form, suggesting that β-cat/TCF-mediated transcription may be active in MM cells (9). Studies using both *in vitro* and *in vivo* models have shown that Wnt-β-cat signaling mediates critical events in the development of MM and thus indicates related phenotypic changes in plasma cells (10). Although a recent study reports the therapeutic efficacy of bortezomib *via* Wnt-independent stabilization of β-cat (11), a role for Wnt signaling in MM remains unclear.

Dickkopf-1 (DKK1), a soluble inhibitor of Wnt/β catenin signaling, functions by binding to the Wnt co-receptor LRP5 to regulate its function on the cell surface in MM cells (12). However, de-regulation of CRT in cancer development makes the β-cat-TCF complex an ideal target for therapeutic approaches (13-15). Given the dual role of Wnt in normal bone formation and in myeloma disease, our interest was to test the chemosensitivity of recently identified small molecule inhibitors of β-cat-regulated transcription (iCRTs) that are designed to specifically target β-cat/TCF-regulated transcription (16). Using human MM cell lines and patient-derived BMMC that express nuclear β-cat, we report that iCRTs (oxazole and thiazole) are effective in down regulating nuclear β-cat and in reducing cell proliferation. Our findings further indicated a significant decrease in the level of vasculoendothelial growth factor (VEGF), in cells treated with iCRTs. Although our attempts to test the *in vivo* efficacy of iCRTs in pre-clinical models are in progress, we provide the first *in vitro* data evaluation of iCRTs as potential Wnt/β-cat/VEGF pathway antagonists in MM that could effectively block or decrease the disease progression at clinically relevant doses.

Materials and Methods

Compounds. The iCRT compounds (oxazole) iCRT-3 and thiazole (iCRT-5) were procured from "ChemDiv"; http://us.chemdiv.com. The concentrations used for this study were made in DMSO.

Patient samples. Human serum, BMMC (Bone marrow mononuclear cells) and BMSC (Bone marrow stromal cells) samples (n=16) were obtained from patients with early- and active late-stage multiple myeloma. Informed consent for the human samples was approved by New York University School of Medicine, Institutional Review Board to Dr. Mazumder (PI, Director of Myeloma Program) for research purpose.

Cell lines and cell culture. MM.1 and U266 cells were kindly provided by Dr. Hearn Cho (Cancer Institute at the Mount Sinai Medical Center, New York). The cells were cultured at 37˚C, 5% CO2 in RPMI-1640 (Mediatech-Cellgro) containing 10% heat inactivated fetal bovine serum and 1M HEPES buffer with 20 μg/ml gentamycin (Invitrogen), as described earlier (17). Primary myeloma cells (BMMC) from patient samples were prepared and cultured as described earlier (18). The primary BMSCs used in this study were cultured in Iscove's modified Dulbecco's medium containing 20% FBS, 2 mM L-glutamine and 100 g/ml penicillin/streptomycin. Cell culture medium and adherent BMSCs grown in 6-well plates were used for co-culture studies with MM cells and for assays including VEGF analysis and cell migration.

STF16 luciferase reporter assay. To perform the Wnt-β-cat responsive STF16 luciferase reporter assays, MM.1 and U266 cells were transfected with 50 ng each of the Wnt responsive STF16 luciferase reporter and pCMV-RL normalization reporter using Lipofectamine LTX (Invitrogen) in 96-well plates. Description of the Wnt response STF16 reporter constructs are presented in earlier publications (16). Transfected cells were then maintained in RPMI with 10% FBS at 37˚C for 24 h and subsequently treated with indicated concentrations of iCRT-3 and iCRT-5 (5-50 μM). Luciferase reporter activity was then measured by Dual-Glo system using L-MaxII^{384} (Molecular Devices), as directed by the manufacturer (Promega). Normalized relative luciferase activity in response to treatments was compared with that obtained from cells treated with DMSO (control). For experiments involving specific Wnt activation MM cells were pre-treated with 20 μM LiCl, for a period of 24 h.

Transfection with siRNAs. The β-cat gene-specific siRNA duplexes along with HiPerFect transfection reagents were used to transfect MM cells (siRNA-1 and siRNA-2). siRNA sequences of the human β-cat were resuspended in the suspension buffer provided by the manufacturer (Qiagen). Next, 30 nmol siRNA were gently introduced into MM cells by mixing with HiPerFect transfection reagent, as described by us in our earlier publications (19). To achieve >85% knockdown, the transfection was continued for up to 72 h. Effective reduction in β-cat protein expression was determined by immunofluorescence detection and western blot analysis.

Immunofluorescence detection of β-catenin. Control, treated or transfected MM cells were seeded into the 2-well chamber slides (LAB-TEK-Nalge Nunc) and grown for 48 h. To view the nuclear β-cat localization, cells collected after cytospin preparations were then fixed with 10% neutral-buffered formalin (NBF) at RT, for 30 min, and washed gently with 1× PBS, premeabilized in 1% triton-X and followed by incubation in 5% FBS, at RT, for 30 min. After gently removing the blocking solution, the cells were incubated with mouse anti-β-cat antibody (Invitrogen, lot # 940535A) for 1 h followed by staining with phylloidin dye Alexaflour-488 goat anti-rabbit *vs*. isotype control. Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) was performed before the cells were imaged for localization of β-cat. Green fluorescence signal for β-cat over DAPI was viewed at 40× using an Olympus AX-70 epi-fluorescence microscope (Olympus America, Melville, NY USA), equipped with a computer-controlled digital camera (Spot) for imaging. The positively-stained cells were quantified with Image Pro plus software (Media Cybernetics, Silver Spring, MD), as described earlier (19). Phase-contrast images of unstained cells were captured using a Leitz-LABOVERT microscope.

Cell proliferation analysis. Actively growing cells were plated in triplicate in 96-well plates at a density of 5×10^5 per well with 100 μl medium containing 5-50 μM iCRT-3 or iCRT-5, and cells treated with 1% DMSO served as the control. After 48 h of treatment, cell proliferation analysis was performed using the MTS kit (Promega). Absorbance was read after at 490 nm using a 96-well plate reader (Spectra Max-M2-Molecular devices). Cells pre-treated with LiCl (20 μM) a GSK3β inhibitor served as the control for Wnt activation. Cell growth was calculated from the mean relative decrease or increase in the optical density at 490 nm compared to the DMSO-treated cells. Inhibition of cell proliferation was calculated based on the mean values of three repeated assays.

Transmigration migration assay. Rate of migration of MM cells was assessed using a 24-well BD FluoroBlok Transwell Inserts (BD Biosciences, Bedford, MA, USA) with 8 μM pore size. Briefly, MM cells (50,000) pre-treated with the iCRTs-3 and iCRTs-5 (15 μM) were seeded (in 200 μl) into the inserts with RPMI medium,

containing 0.25% serum. The bottom well contained RPMI with 10% FBS. After 48 h of incubation the bottom well was filled with 500 μl of Calcein fluorescent dye, prepared according to the directions of the manufacturer (BD Biosciences). Calcein AM is the most suitable indicator for staining viable cells due to its low cytotoxicity. The fluorescence intensity emitted by the migrated cells was measured at 540 nm using a plate reader Max-M2 (Molecular Devices, Sunnyvale, CA). The experiments were repeated three times.

Western blot analysis. Total protein lysates (30 μg/lane) of MM cells treated without or with iCRTs (15 μM) for 48 h were prepared in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) containing a protease inhibitor cocktail (cØmplete™ , Boehringer Mannheim, Germany), as described earlier (19). Immunobloting was done by standard SDS-PAGE (12%) using antibodies against β-catenin (Invitrogen, lot # 138400) and DKK1 (Cell signaling, lot 1, 4687S). Secondary antibodies conjugated to horseradish peroxide (Thrermoscientific, # 32430) and the loading control α-tubulin was obtained from Santa Cruz Biotechnology. In addition, protein samples from HEK 293 cells were used to confirm β-cat in non-myeloma cells. Reactive protein bands for β-cat were developed using an enhanced ECL chemiluminescence detection kit (Amersham Biosciences). All blots were stripped and re-probed with α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) to normalize protein loading. Each experiment was repeated three times using the same sets of samples. Quantification of reactive protein bands were performed by densitometric analysis and the fold change was calculated by normalization with α-tubulin.

VEGF analysis by Enzyme-linked Immunosorbent Assay (ELISA). MM cells (U266) were grown as described in the earlier section. The primary BMSCs used in this study were obtained from patients and cultured in Iscove's modified Dulbecco's medium containing 20% FBS, 2 mM L-glutamine and 5 mg/ml penicillin/streptomycin. Cell culture medium collected from U266 cells and co-cultured with adherent BMSCs (grown in 6-well plates) and treated with iCRTs were used for VEGF analysis. ELISA assays were performed using Human VEGF Quantikine ELISA Kits (R&D systems, Minneapolis, MN) by following the manufacturer's protocol. These assays employ the quantitative sandwich enzyme immunoassay technique. The resultant color was read at 450 nm using an ELISA plate reader Max-M2 (Molecular Devices, Sunnyvale, CA). The concentrations of VEGF in the samples were determined by interpolation from a standard curve made from the standard provided by the manufacturer. The experiments were performed in triplicate, and repeated at least twice.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from the MM cells treated with iCRTs (15 μM) using Trizol reagent (Life Technologies/GibcoBRL), as described earlier (20, 21). A two-step RT-PCR carried out with total RNA (5 μg) extracted from U266 and BMSC, treated with 50 μM iCRT-3, was used for initial denaturing for 2 min at 95˚C and continued the amplification with an extension at 72˚C, 7 min for 33 cycles using VEGF gene-specific primer sequences upper 5'atttacaacgtctgc gcatctt 3' lower, 5'ctcgccttgctgctctacctc3' along with the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), upper 5' GGATGACCTTGCCCACAGCCT 3' lower, 5'CATCTCTG CCCCCTCTGCTGA 3' as the internal control (IDT). Real-time quantitative PCR was performed in triplicate with a Smart Cycler (Cepheid, Sunnyvale, CA), using the SYBR-Green mix (Applied Biosystems, Branchburg, NJ), as described earlier by us (19, 20). Results were normalized to amplification of GAPDH and to determination of the fold change based on $2^{\Delta\Delta Ct}$.

Statistical analysis. Data from all the experiments are presented as the mean±SD, from at least three independent experiments. Measurements of statistical differences were determined using two-way ANOVA followed by Tukey's multiple comparison procedure (30). Differences between the treatment and control groups were analyzed using the Student's *t*-test. Statistical analyses were performed using GraphPad Prism 4 software (San Diego, CA, USA).

Results

β-catenin expression in MM cells. Although earlier studies have documented Wnt signaling in human MM cells exhibiting nuclear β-cat and its effect on the downstream target genes of the Wnt pathway (2, 21, 22), in order to test the chemosensitivity of iCRTs in myeloma cells, we first confirmed the expression of β-cat in human the MM cell types U266 and MM.1, in addition to using patient-derived BMMCs. As shown in Figure 1A, immunofluorescent detection of β-cat in U266 cells showed nuclear localization (FITC-merged) in >70 to 80 % of the cells, a similar trend was observed in MM.1 and BMMC. This observation is consistent with the western blot analysis of β-cat in the total protein lysate, thus indicating a consistently similar level of expression among the examined cell lines (Figure 1B-C). The above data on the expression of β-cat in MM cell lines and cells from patient samples provide the rationale for using these cells to test the efficacy of iCRTs that are specifically designed to target nuclear β-cat signaling (16).

Inhibition of Wnt/β-catenin response by iCRTs in myeloma cells. To determine the efficacy of iCRT-3 and iCRT-5 (Figure 2A) in antagonizing Wnt signaling activity in MM cells, we transfected U266 and MM.1 cells with Wnt/β-cat-responsive reporter plasmids (STF16), as described earlier (16). Our data confirmed that both U266 and MM.1 cells showed >85% transfection efficiency (determined by immunoflourescence detection) after 24 h. Treatment of STF16-transfected MM cells with iCRT-3 and iCRT-5 showed a dose-dependent decrease (2-3 fold) in the reporter activity respectively, with an optimal inhibitory effect at 15 μM, followed by a significant decline at a dose of 50 μM (Figure 2B). To further confirm the specific effect of iCRTs, in separate assays, MM cells were pre-treated for 24 h with LiCl (20 μM), (a GSK3β inhibitor known to activate Wnt signaling by stabilizing β-cat), and then subsequently treated with different doses of iCRTs. As shown in Figure 3A and B, iCRT-3 significantly inhibited both the LiCl-induced reporter activity and cell proliferation, as determined by

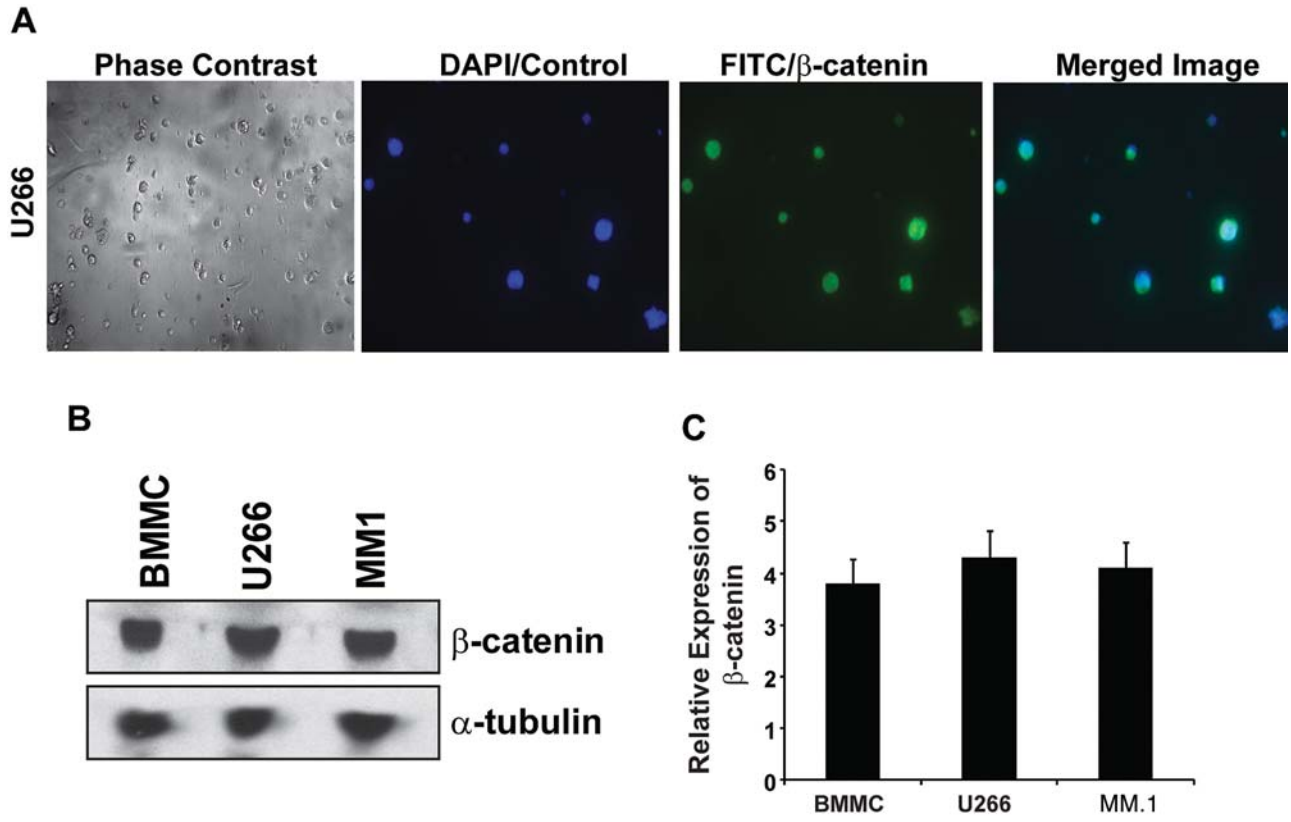

Figure 1. *β-Catenin expression in MM. (A) Immunofluorescence detection of nuclear β-cat in the cytospin preparation of U266 cells. Approximately 5000 cells/slide were fixed in 10% NBF, followed by staining for β-cat and counter staining with DAPI to label the nuclei, as described in Methods. (B) Immunoblotting of total β-cat expression in MM and BMMC cell lysates. Western blot analysis using the respective protein lysate (30 μg/lane) was performed as described in Methods. The antibody for α-tubulin was used as loading control. (C) Bar graph represents the relative mean pixel density of the reactive protein bands of β-cat with reference to the loading control α-tubulin from three independent blots. The data presented in each bar represents mean±SD.*

MTS assays in U266 and MM.1 cell lines. Although the effect of LiCl on the Wnt reporter activity compared to DMSO was not greatly enhanced, the inhibitory effect induced by iCRT-3 is significant ($p<0.001$). A similar effect was observed in cells treated with iCRT-5 in three repeated assays. The iCRTs-induced cytotoxic effect at 15 μM concentration compared to the DMSO control was significant in all the patient-derived BMMC samples (n=16) (Figure 3C) (**$p<0.01$).

Effect of iCRTs on total β-cat protein vs. DKK1. In this study, iCRT-treated cells revealed an inhibitory effect on total β-cat protein (Figure 4A-B), this was typically not observed in other examined cancer cell types, including colon cancers (HT29, HCT116, SW480) or human breast adenocarcinoma cells (MCF7) (16). As a positive control, protein samples from HEK 293 cells were used to confirm β-cat expression. Given the role of Wnt and the up-stream antagonist DKK1 in normal bone formation and MM development, we examined whether inhibition of Wnt-β-cat response by iCRTs indirectly affects the expression of DKK1. Our findings from western blot analysis of MM cells treated with iCRTs at the optimal or higher doses (15 μM or 50 μM) showed no significant change in the expression of DKK1 protein (Figure 4C). Taken together these data reveal a possibility that in MM cells iCRTs may indirectly influence the levels of β-cat protein.

Effect of iCRTs on vascular endothelial growth factor (VEGF). While bone marrow angiogenesis is a hallmark, indicating the progression of multiple myeloma, it is also correlated with the severity of the disease. We therefore tested the potential effect of iCRTs on Wnt/β-cat-mediated angiogenic processess. With this focus, first, we measured the VEGF level, a transcriptional target of Wnt/β-cat signaling, in the serum samples of MM patients presented with early and late stage disease. As shown in Figure 5A, we observed elevated levels (ranging from 300 to 500 pg/ml) of VEGF in patients with late-stage MM.

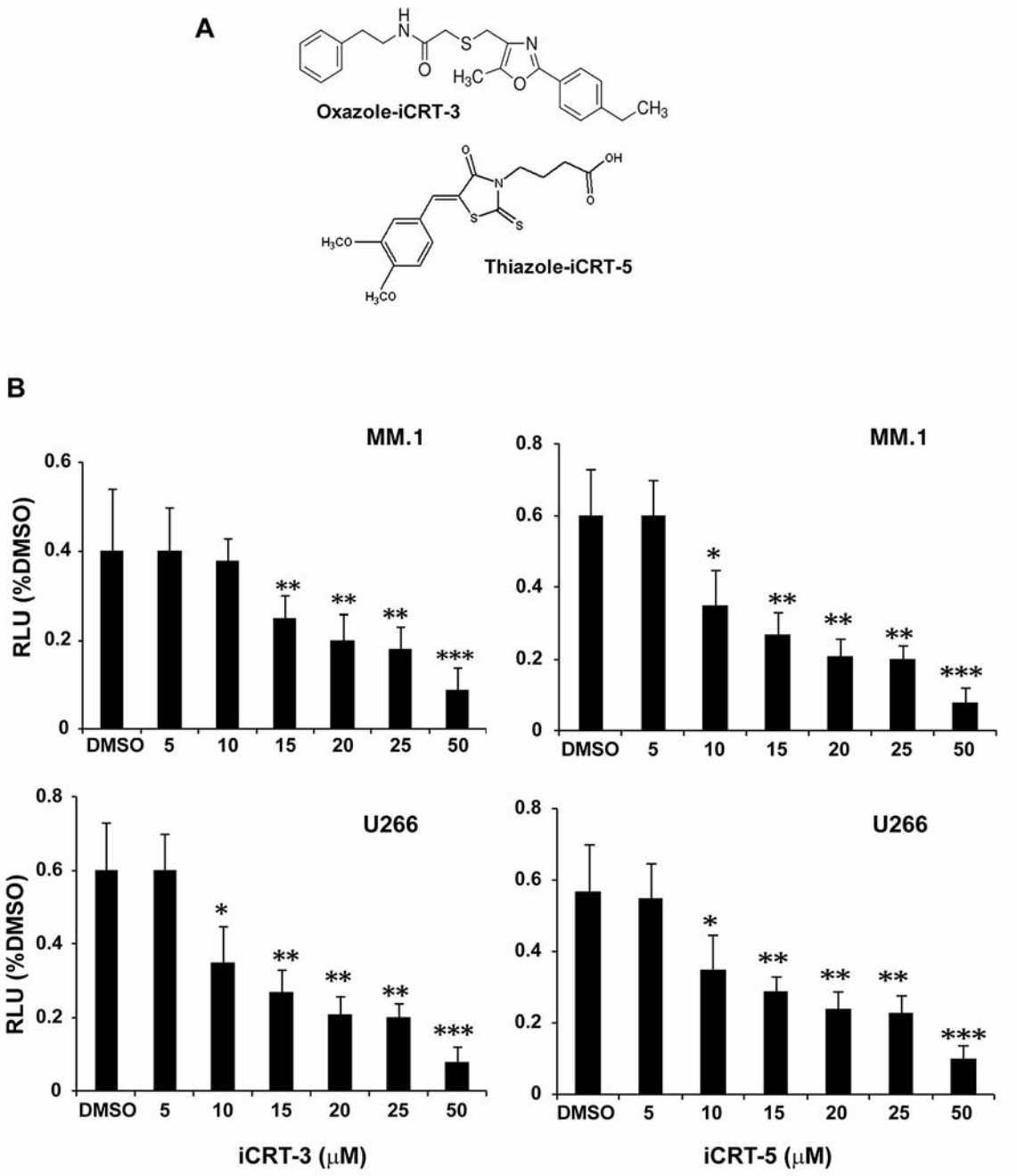

Figure 2. *Inhibition of Wnt/β-cat response STF16 luciferase reporter activity by iCRTs in myeloma cells. (A) Chemical structure of iCRTs used in this study. (B) Luciferase reporter activity. The assay was conducted in MM cells transfected with 50 ng each of the Wnt-responsive STF16 luciferase reporter and pCMV-RL normalization reporter using Lipofectamine LTX (Invitrogen) as the transfection agent in 96-well plates. After 24 h of transfection, cells were subsequently treated with iCRT-3 or iCRT-5 (5-50 μM). Luciferase reporter activity was then measured using the Dual-Glo system (Promega), as described in Methods. Normalized relative luciferase activity (% RLU) in response to the treatments for 24 h was compared to cells treated with DMSO (control). The data presented in each bar represent mean±SD. Statistical differences at *p<0.05, **p<0.01, and ***p<0.001.*

To test the efficacy of iCRTs on the VEGF level in MM cells, the cell culture medium obtained from the co-culture of bone marrow stromal cells (BMSC) with human U266 cells were used. This is based on the findings from earlier studies that binding of MM cells to BMSCs triggers the production of VEGF (23). Treatment of both BMSC and U266 cells with iCRT-3 or iCRT-5 resulted in a marked reduction in VEGF (ranging from 200-400 pg/ml) expression, compared to

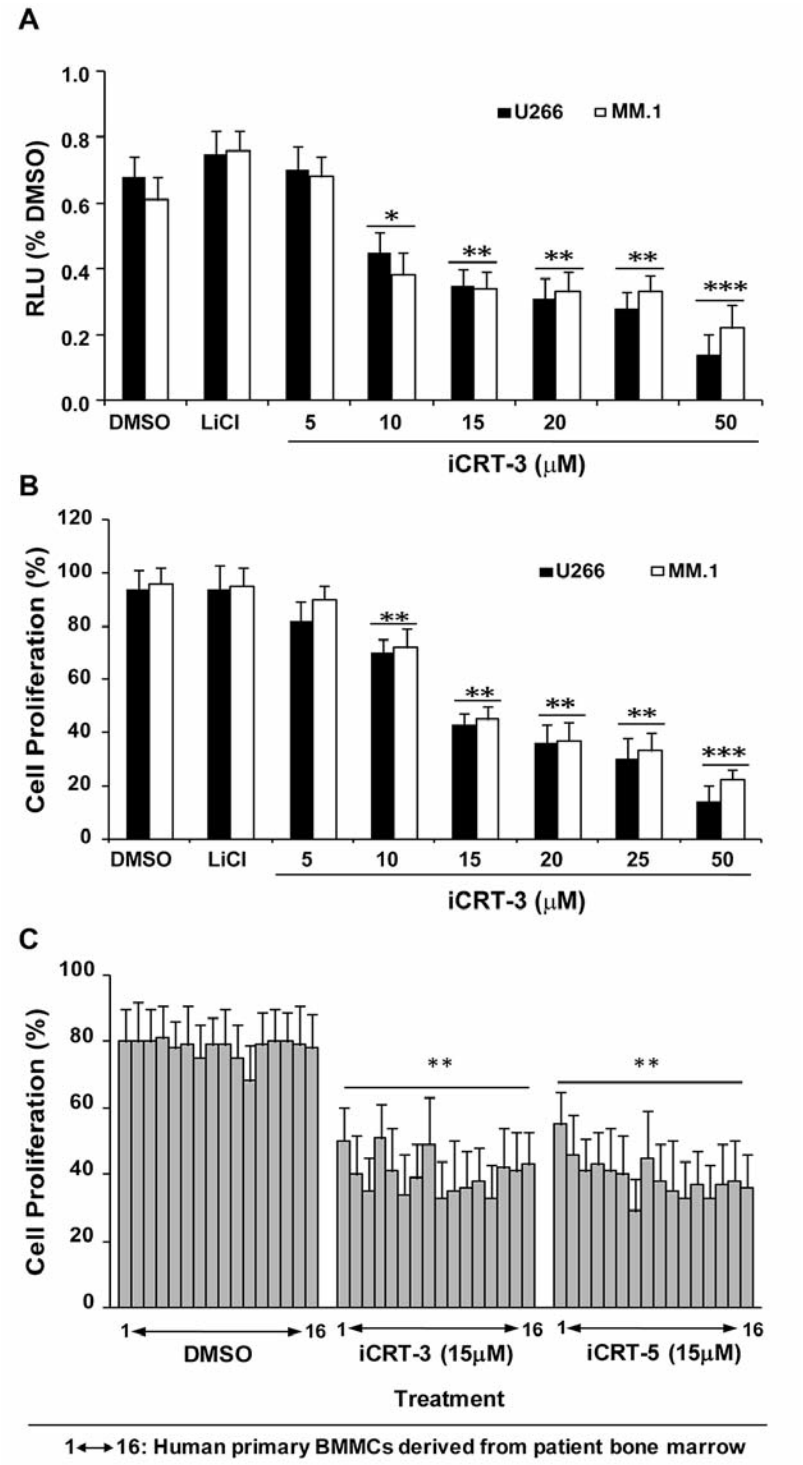

Figure 3. *Specific effect of iCRT-3 in MM cells on the STF16 luciferase reporter activity. (A) MM cells were pre-treated with 20 μM lithium chloride (LiCl) for 24 h before transfection with the STF16 reporter. Luciferase reporter activity was measured after treatment with iCRTs using the Dual-Glo system (Promega), as described in Methods. Normalized relative luciferase activity (% RLU) in response to the treatments after 24 h was compared to cells treated with DMSO (control). (B) Cell growth inhibition. MM cells pre-treated with 20 μM LiCl were analyzed for cell growth inhibition in response to iCRTs treatment using the MTS assay, as described in Methods. The data presented are the mean from three independent assays with error bars reporting standard deviation. Statistical differences at *p<0.05, **p<0.01, and ***p<0.001. (C) Human primary BMMCs derived from bone marrow aspirants were used to test the efficacy of iCRT-3 and iCRT-5 at a dose of 15 μM for 48 h; 1-16 represents the number of patient samples. The data presented in each bar represents mean±SD. **Statistical difference at p<0.01.*

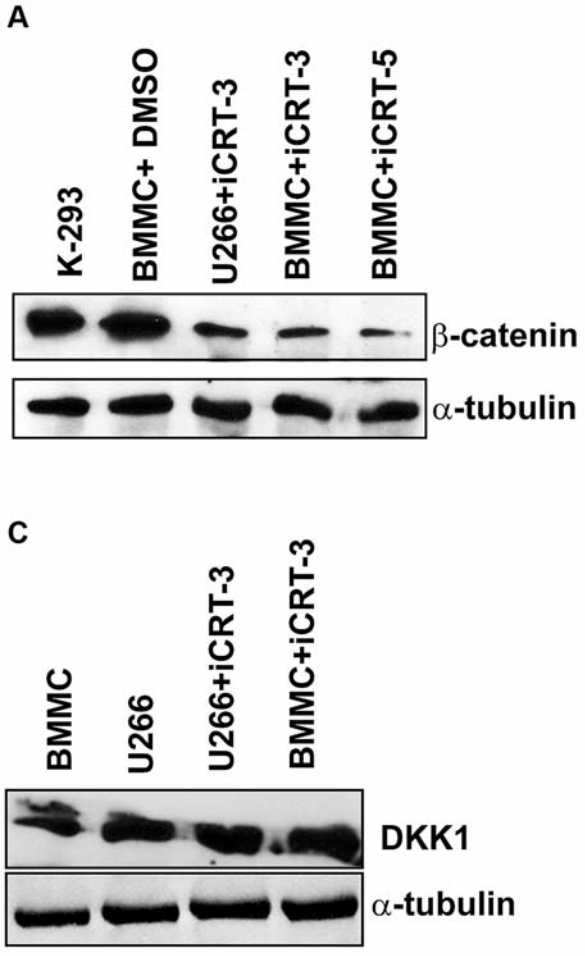

Figure 4. *Effect of iCRTs on β-catenin and DKK1 expression in MM cells. (A) Standard western blot analysis was performed as described in Methods, using 30 μg/lane of total protein lysate from BMMC and U266 cells treated with 15 μM of indicated iCRT for 48 h. Protein lysates from untreated HEK-293 cells and MM cells treated with DMSO served as the positive and experimental controls. (B) Quantification of the reactive protein bands, in terms of pixel density for total β-cat expression normalized to α-tubulin in BMMC and U266 cells, is presented in the bar graph representing the mean±SD. (C) Immunoblotting was performed using DKK1-specific antibody, as described in Methods. Total protein lysates were obtained from BMMC and U266 cells treated only with iCRT-3 (15 μM). The antibody for α-tublin served as the loading control. As there was no change in the levels of DKK1 in the untreated or treated cells, data on a quantification of the reactive protein bands are not presented.*

untreated cells (650-900 pg/ml). To determine whether the effect of iCRTs on the VEGF activity is specific to its ability to antagonize β-cat activity, we used culture medium collected from cells transiently transfected with siRNA for β-cat. As shown in Figure 5B, siRNA-mediated down-regulation of β-cat showed a significant decrease in the VEGF levels (200-400 pg/ml compared to siRNA control (800 pg/ml), thereby confirming that the effect of iCRTs is indeed mediated by their inhibitory activity on β-cat. In addition, qPCR analysis for VEGF revealed that the iCRT-mediated inhibition (>2 fold, $p<0.05$) is at the transcription level (Figure 5C), and these findings are consistent with the notion of VEGF being a Wnt/β-cat target gene.

Effect of iCRTs on MM cell migration. Since our findings revealed a negative effect of iCRTs on VEGF levels, we next determined the effect on Wnt/β-cat/VEGF-mediated cell migration. U266 cells transfected with reporter plasmid (STF16) followed by treatment with iCRTs (15 μM) and/or with an exogenous source of VEGF (30 ng) as positive control, were assessed for the rate of migration. Our findings after 24-h treatments showed a significant decrease in cell migration compared to untreated controls (by 2-3-fold decrease, $p<0.05$), determined based on the Calcein fluorescence readings of the migrated cells (Figure 6A). Parallel experiments conducted to determine any binding of U266 with BMSC revealed that the cells pre-treated with VEGF showed an inhibitory effect by iCRTs on the binding or clustering of cells, as shown in the images (arrow) by iCRTs (Figure 6B) compared to the untreated cells or cells supplemented with VEGF-alone.

Discussion

Wnt signaling plays a key role the in the regulation of bone mass and in the development of multiple myeloma (24). Interestingly, overexpression of β-cat in osteoblasts has been demonstrated to induce a high bone mass phenotype

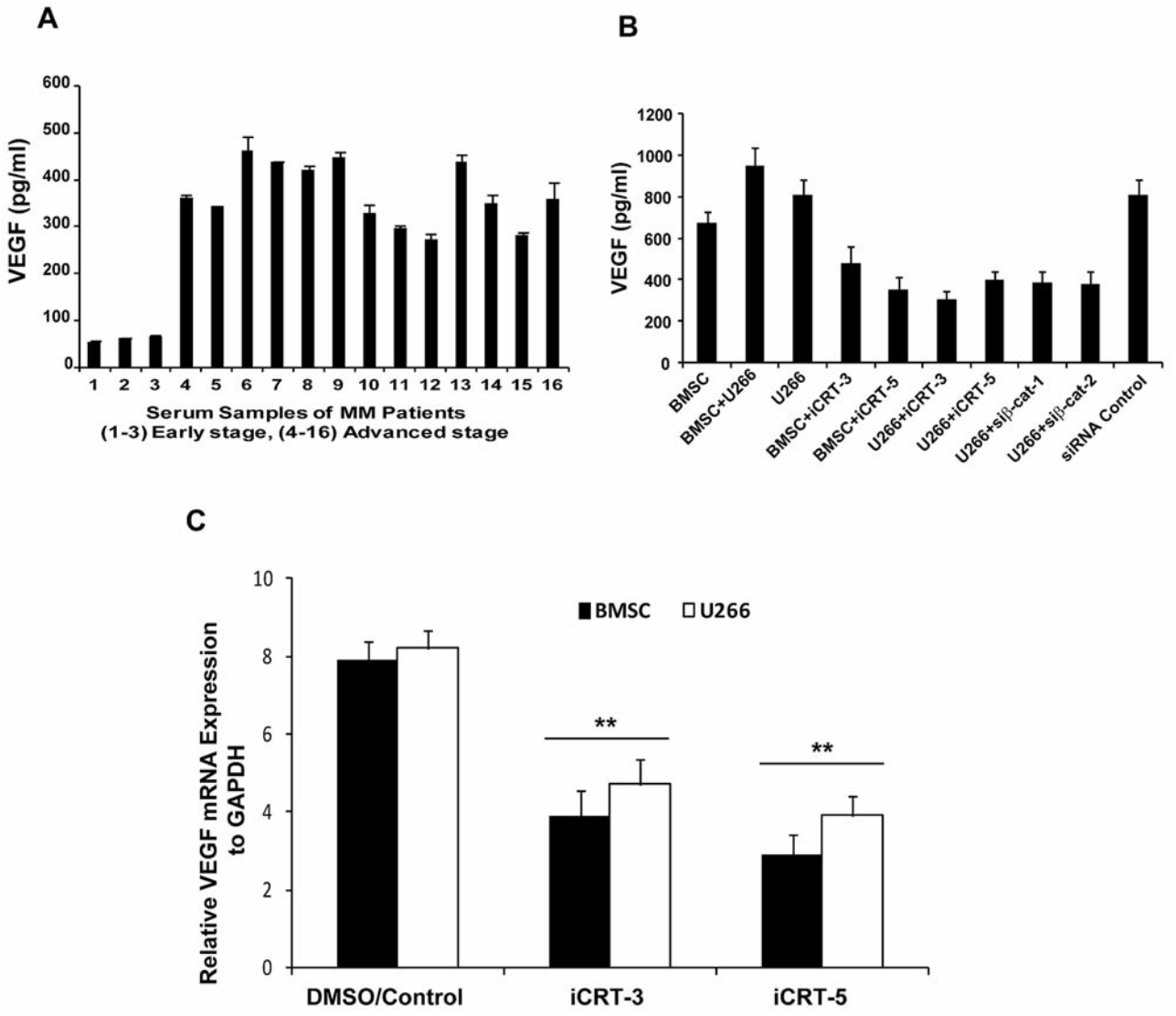

Figure 5. *Inhibitory effect of iCRTs on VEGF. (A) Serum analysis for VEGF levels in MM patients (n=16) was performed using an ELISA-based assay, as described in Methods. The data presented are the mean from three independent assays with error bars reporting standard deviation. (B) Human primary BMSCs derived from bone marrow were used in co-culture with U266 cells. Secretion of VEGF into the medium by adherent BMSC co-cultured with U266 in the presence or absence of iCRTs (15 μM) and/or transfected with β-cat siRNA was measured after 24 h. C. RT-PCR was performed as described in Methods using total RNA (5 μg) isolated from BMSC and U266 cells with or without exposure to iCRTS. Relative expression of VEGF-mRNA to GAPDH is presented in the bar graph with the mean±SD. ***Statistical difference at p<0.01.*

(25). However the precise functions of specific antagonists of Wnt/βcat signaling and its importance against growth and survival of myeloma cells are unclear. Findings from this study indicate that β-cat is predominantly present in the nucleus of MM cells. This is consistent with earlier observations in myeloma cells (9). We further demonstrate that the iCRTs as antagonists of Wnt signaling block Wnt/β-cat reporter activity, down regulate β-cat expression and inhibit cell proliferation in different myeloma cell types in addition to human primary BMMC cells from 16 patients with either advanced- or early-stage of the disease. We observed a dose dependent effect with an optimum effect at 15 μM, however; the clinical relevance of this dose is being still confirmed in *in vivo* studies. In this context, it is also important to recall earlier *in vivo* studies using yet another small-interfering RNA (siRNA) targeting β-cat which has been shown to suppress the progression of MM in mouse models (26). Interestingly, iCRT-treated cells revealed an inhibitory effect on the total β-cat protein as shown in Figure 4A-B. This was typically not observed in

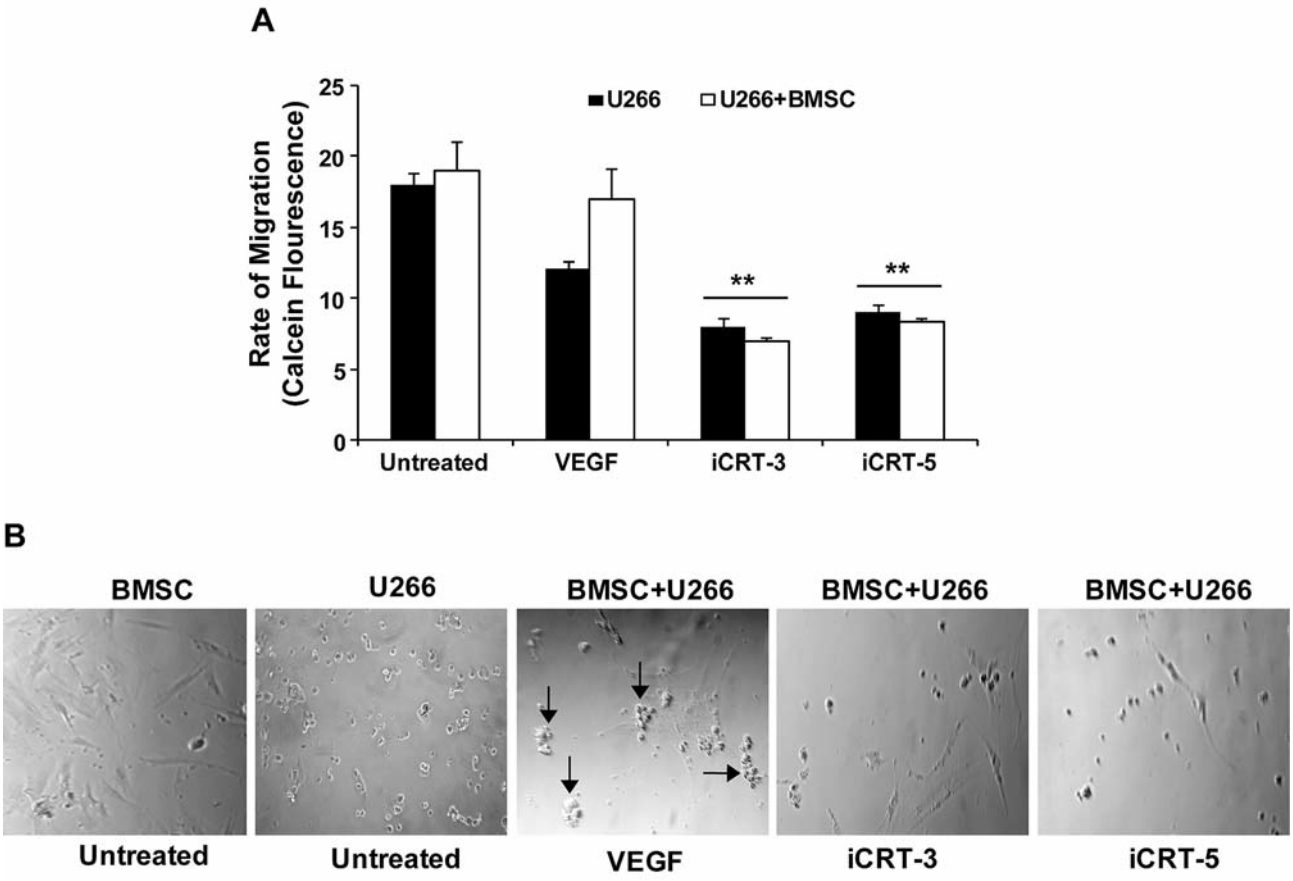

Figure 6. *iCRTs inhibit MM cell migration and adhesion of BMSC with U266 cells. (A) U266 cells in the presence of absence of BMSCs were grown in 24-well transwell migration plates as described in Methods. Migratory potential of cells in the presence of iCRTs (15 μM each) was measured after 24 h using Calcein fluorescent intensity of the migrated cells. Cells treated with VEGF showing clustering of cells (arrow) in untreated and stimulated with VEGF serve as controls. Arbitrary relative fluorescent units were used to represent the rate of migration based on the fluorescence intensity. The data presented in each bar represent the mean±SD. **Statistical difference at p<0.01. (B) Phase-contrast images of untreated or iCRT-treated U266 and BMSC cells were taken after 24 h of treatment.*

few other cancer cell types examined, including colon cancers (HT29, HCT116, SW480) or human breast adenocarcinoma cells (MCF7) (16) and thus suggests a possibility of inducing specific effects in MM cells. The canonical Wnt pathway is regulated by large number of antagonists, including the DKK family of proteins, among which DKK1 and DKK2 have been well-characterized (12, 27, 28). Given the role of Wnt and DKK1 in normal bone formation and MM development, we examined whether inhibition of Wnt-β-catenin response by iCRTs affects the DKK1 protein expression. Our data from western blot analysis of MM cells treated with iCRTs showed no change in the expression of the DKK1 protein even at a higher dose of 50 μm for 48h, suggesting that the iCRTs-mediated negative regulation of the Wnt pathway, is likely to act at the level of regulating downstream components of the Wnt pathway.

Another important aspect of our findings is the possible inhibition of VEGF by iCRTs. VEGF is a crucial cytokine that directs and promotes tumorigenesis and potentiation in the bone marrow. Our findings on demonstrating elevated serum levels of VEGF in patients with advanced MM are consistent with earlier reports (29) and are correlated with the risk for angiogenesis (4, 30-33). Although few studies have shown that binding of MM cells to BMSC up-regulates VEGF secretion and also triggers IL-6 production (34), our findings on the impact of iCRTs on VEGF-mediated migration are significant. Migration is one of the fundamental processes involved in myeloma cell invasion and dissemination, and extravasation of myeloma cells from blood vessels into the BM is likely to be controlled by several chemoattractants including VEGF (35). Our data on the elevated levels of VEGF in advanced MM patient samples indicate that VEGF could be a potent effector of

myeloma cell transmigration through vascular endothelium and BMSC stromal cells. Consistently, our findings on the co-culture of BMSC cells with U666 cell showed an increase in migration and clustering of cells that was significantly inhibited by iCRTs. Although our findings from cytotoxic assays reflect a similar inhibitory effect on cell proliferation in most of the cell types, including the examined human primary cells, the clinical relevance of the optimal dose of 15 μM or less has yet to be confirmed in murine models for MM. Taken together, these observations suggest that VEGF produced by bone marrow stromal cells may form a gradient leading to attraction of myeloma cells into the BM cavity where higher concentrations of VEGF may promote cell survival and proliferation. Overall, our findings from this study indicate that the Wnt/β-cat/VEGF pathway-dependent increase in migration and proliferation of MM cells can be antagonized by specific inhibitors of nuclear β-cat activity, thereby underscoring the importance of developing iCRTs as a novel class of Wnt-directed therapeutics in human MM.

Acknowledegements

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