



Research Article

Downregulation of DKK-1 and Upregulation of β -Catenin in Cancer Floating Spheroids is Associated with Chemoresistance to Wnt Signaling Pathway Inhibitors

 Vivek Kaushik,  Juan Sebastian Yakisich,  Yogesh Kulkarni,  Neelam Azad,  Anand Krishnan V. Iyer

Department of Pharmaceutical Sciences, School of Pharmacy, Hampton University, VA, USA

Abstract

Objectives: DKK-1, a negative regulator of β -catenin in the Wnt pathway is commonly upregulated in cancer cells but its expression and association to chemoresistance have not been investigated extensively. Our aim was to evaluate the expression of DKK-1 and β -catenin as well as the effect of the Wnt pathway modulators iCRT-14 (iCRT) and WAY626611 (WAY) alone or in combination with Nigericin (NIG) on the viability of cancer cells grown under routine conditions (RC) or as floating spheroids (FSs).

Methods: RC or FSs from human prostate (PC3 and LnCAP) and breast (MCF-7 and MDA-MB231) cells were treated for 72 hs with WAY or iCRT. Cell viability was determined by the MTT or the CCK-8 assays and protein expression was determined by western blot analysis.

Results: DKK-1 was expressed at different basal levels in RC but was undetectable in FSs. β -catenin expression was increased in FSs. RC of PC3 and MCF-7 were sensitive to both iCRT and WAY but FSs generated from these cell lines were resistant to both drugs. NIG potentiated the effect of both Wnt inhibitors in FSs.

Conclusion: Marked changes in the expression of Wnt pathway proteins, DKK-1 and β -catenin in FSs are associated with resistance to WAY and iCRT.

Keywords: Chemoresistance, DKK-1, tumorspheres, stemness, Wnt/ β -catenin

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The serum levels of Dickkopf-1 (DKK-1), a negative regulator of β -catenin in the Wnt signaling pathway has been reported to be elevated and positively correlated with a poor prognosis in patients with prostate cancer (PCa)^[1] and other types of cancers such as breast^[2] and bladder.^[3] Secreted DKK-1 binds to the LRP5/6 receptors and negatively regulates the Wnt pathway.^[4] DKK-1 was shown to stimulate prostate cancer growth and metastasis^[5] and therefore DKK-1 inhibitors may be useful anticancer drugs.

DKK-1 may have different roles in different systems: for instance, while in primary prostate cancer cells DKK-1

promote growth and metastasis it inhibits bone growth in PCa-induced osteoblastic metastases.^[5] Up-regulation of serum DKK-1 occurs as an early event in PCa development^[6] but decreases during progression from primary tumor to metastasis.^[7] DKK-1 tissue expression is also higher in PCa compared to benign disease.^[1]

In addition, solid tumors display intratumoral heterogeneity in part due to microenvironmental conditions that induce phenotypic changes such as stemness properties and chemoresistance. Due to the complex role of DKK-1 in primary tumor cells and normal tissue (e.g., the future site of

Address for correspondence: Juan Sebastian Yakisich, MD. Department of Pharmaceutical Sciences, School of Pharmacy, Hampton University, VA, USA

Phone: +1 (757) 995-4055 **E-mail:** juan.yakisich@hamptonu.edu

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metastasis) as well as its differential expression in normal vs neoplastic tissue it is of importance to evaluate the effect of DKK-1 inhibitors and Wnt pathway inhibitors in cancer cells displaying different phenotypes. We have previously shown that prostate, breast and lung cancer cells when growing under anchorage-independent conditions as floating spheroids (FSs) are highly resistant to conventional anticancer drugs compared to cell growing under routine culture conditions (RC) as adherent monolayers (Anchorage-dependent conditions).^[8]

Our hypothesis is that changes in DKK-1 expression induced by microenvironmental conditions with concomitant modulation of the β -catenin levels may affect chemoresistance of cancer cells to Wnt inhibitors. The aim of this study was to investigate the expression of DKK-1 and the anticancer activity of the DKK-1 inhibitor WAY262611 (WAY) ^[9] and the β -catenin inhibitor iCRT-14 (iCRT),^[10] alone or in combination with Nigericin (NIG) in cells growing under RC as well as cells growing as FSs. The rationale for the use of these drugs in combination is based on studies that demonstrated that NIG exerts anticancer activity in H460 lung cancer cells^[11] and colorectal cancer^[12] by inhibiting Wnt/ β -catenin signaling pathway and potentiated the effect of the cardiac glycoside Digitoxin that is also a Wnt signaling pathway inhibitor.^[11]

Methods

Drugs

WAY-262611, iCRT-14 and Nigericin were purchased from Fisher (Hampton, NH) and were prepared as stock solutions (10 mM, 10 mM and 25 mM, respectively) in DMSO. Final dilutions were freshly prepared in culture media before use. Matched DMSO concentration were (equivalent to the highest concentration, below 1% was used in all wells.

Cell culture

The cell human cancer cell lines PC3 (Prostate), LNCaP (Prostate), MCF-7 (Breast) and MD-MBA-231 (Breast) were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (PC3, LNCaP) or DMEM high glucose media (MCF-7, MD-MBA-231) supplemented with 10 FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. All cells were cultured in a 5% CO₂ environment at 37 °C.

Short-Term Antiproliferative Effects (MTT assay)

For routine culture conditions (RC), cells (~2,000 cells per well) were plated in 96 well cell-culture microplates (Costar, USA) and incubated overnight in the appropriated culture media to allow them to adhere. Cells were then exposed

to the appropriate concentration of drug or vehicle for 72 hours. Cell viability was evaluated by the MTT assay. The absorbance of solubilized formazan was read at 570 nm using ELISA reader (Bio-TEK, Synergy-1). In all cases, the highest concentration of DMSO was used in the control and this concentration was maintained below 1 % (v/v). This DMSO concentration did not show any significant antiproliferative effect on the cell line in a short-term assay.

Tumor Spheroids Assay

Cells growing in ultralow attachment plates (ULAP) in the presence of serum as floating spheroids (FSs) were obtained as previously described.^[8] For viability assays, FSs were collected in 15 mL Falcon tubes, centrifuged at 700 rpm \times 3 min, and resuspended in fresh media. In order to plate the same number of cells, this cell suspension was split in 1 mL aliquots. Vehicle or drugs were added to each aliquot and then 150 μ L cell suspension was loaded into each microwell (in a 96-well plate) and incubated for 72 hours. Cell viability was evaluated by the CCK-8 assay (Dojingo Laboratories).

Western Blotting

Preparation of cell lysates and Western blotting were performed as described previously.^[8] Antibodies for DKK-1, β -catenin, and GAPDH were purchased from Cell Signal Inc. (Danvers, MA). Peroxidase-conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). The immune complexes were detected by chemiluminescence and quantified using analyst/PC densitometry software (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

The IC₅₀ (drug concentrations inhibiting cell growth by 50%) were determined by interpolation from the dose-response curves using a sigmoidal logistic three-parameter equation using SigmaPlot (V. 11.0) software. IC₅₀ values (not shown), calculated from MTT assays using adherent cells, were used to select three relevant concentrations (one above, one below and one close to the calculated IC₅₀) for FSs experiments. Each experimental point represents the mean \pm standard deviation (SD) of quadruplicate or sextuplicate wells (see figures for details).

Results and Discussion

DKK Expression is Downregulated FSs

We first evaluated the expression DKK-1 and β -catenin that are key upstream and downstream proteins of the Wnt signaling pathway in cells growing under RC as well as in cells growing as FSs. These two systems offer a simple model to

study chemoresistance of cancer cells: cells growing as FSs have been shown to display high degree of chemoresistance to a plethora of conventional anticancer drugs compared to cells growing under RC.^[8]

Figure 1 shows that all cell lines (LNCaP, MCF-7, PC3 and MDA-MB-231) growing under RC conditions express “basal” but different levels of both DKK-1 and β -catenin. Cells grown as FSs showed reduced or undetectable level of DKK-1 concomitant with an expected increased expression of β -catenin. These data are in agreement with the current literature that demonstrates a negative regulation of the Wnt signaling pathway by DKK-1.^[13] It also suggests that in FSs the activity of the Wnt pathway becomes largely independent of DKK-1.

FSs are Highly Resistant to WAY and iCRT

We next tested the effect of the DKK-1 inhibitor WAY^[9, 14] and the Wnt pathway inhibitor iCRT (10) on the viability of

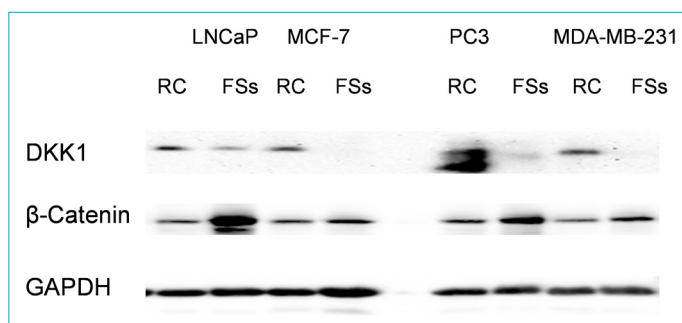


Figure 1. DKK1 expression is downregulated in cell growing FSs. DKK-1 expression in human cancer cells lines growing under RC or as FSs (7 days old).

PC3 and MCF-7 cells growing under RC as well as in cells growing as FSs. Figure 2 shows that cells growing under RC were more sensitive to both inhibitors when compared to cells growing as FSs. At the mechanistic level WAY prevents DKK-1-mediated Kr2/LRP5/DKK-1 complex formation and internalization^[9] and our results can be explained as follows: Under RC PC-3 cells express very high levels of DKK-1 compared to MCF-7 (Fig. 1) and therefore, they are relatively more resistant to WAY compared to MCF-7 cells (Fig. 2). In FSs, where the levels of DKK-1 are undetectable (MCF-7 cells) or almost undetectable (PC-3 cells) WAY has little or no effect at all because the Wnt pathway became largely independent of DKK-1. iCRT was identified as a Wnt inhibitor through an RNAi genetic screen and it was shown to decrease the expression of β -catenin and β -Dvl2, another protein of the Wnt pathway.^[15] Thus, the exact target of iCRT is not known and therefore, its cellular effects are more difficult to understand. The chemoresistance of FSs to iCRT could be partially attributed to increased expression/activation of β -catenin (FSs express higher levels of β -catenin as compared to cell growing under RC, Fig. 1) as it has been demonstrated in other systems. For instance overexpression of β -catenin was demonstrated to drive chemoresistance to Cisplatin in oral squamous cell carcinoma^[16] and activation of β -catenin induced temozolomide resistance in glioma cells.^[17]

NIG Potentiates the Anticancer Effects of WAY and iCRT in FSs

Our *in vitro* data suggest that inhibitors of DKK-1 or β -catenin alone will have limited effect when tested *in vivo* in highly

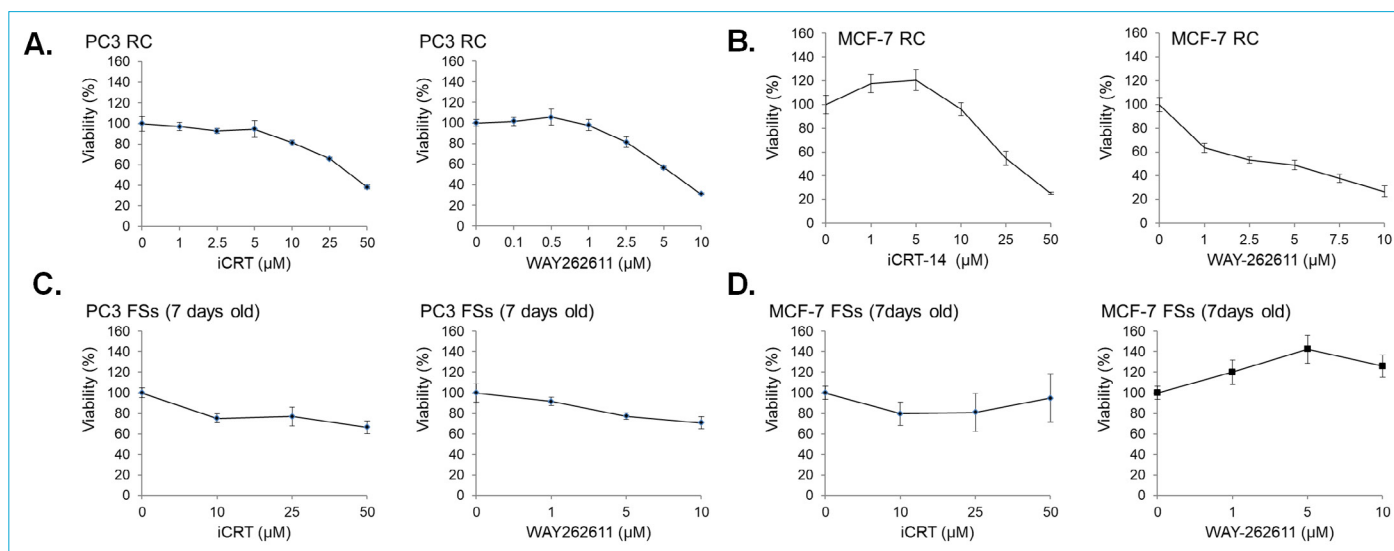


Figure 2. Effect of iCRT and WAY on the viability of human PC3 (Prostate) and MCF-7 (Breast) cancer cells. Cells grown under RC (a, b) or FSs (c, d) were incubated with the indicated concentration of iCRT or WAY for 72 hs. Cell viability was measured by the MTT or CCK-8 assay. Data (mean \pm SD) is representative of three independent experiments.

heterogeneous tumors where cells some cells may express low levels of DKK-1 and/or increased levels of β -catenin. For these reasons, we tested the effect of NIG in combination with WAY or iCRT. Figure 3 shows that NIG alone was able to decrease the viability of PC-3 and MCF-7 FSs and when used in combination with WAY or iCRT it potentiated the effect of both drugs. Western blot analysis (Fig. 4) showed that combination treatment (WAY+NIG) clearly decreased the expression of β -catenin in MCF-7 and PC-3. However, the combination (iCRT + NIG) decreased the expression of β -catenin in MCF-7 cells but not in PC-3 cells. This discrepancy could be due to several factor such as: a) cell type dependent effect of NIG (e.g., NIG induced the expression of DKK-1 in PC-3 cells but not in MCF-7 cells) or b) cell type dependent basal expression of DKK-1 (e.g., PC3 cells show higher basal expression of DKK-1 in PC-3 cells compared to MCF-7 cells). At present, the exact mechanism by which NIG downregulates Wnt/ β -catenin signaling pathway is poorly understood. Recent studies demonstrated that NIG at concentrations $<50 \mu\text{M}$ inhibit the proliferation of pancreatic cancer cells in a time and concentration dependent manner and upregulated and down-regulated (562 \uparrow ; 296 \downarrow) the expression of circular RNAs (circRNAs) after 8 h of treat-

ment.^[18] circRNAs are a type of non-coding RNA molecules that lack a 5'-terminal cap and 3'-terminal poly A tail that regulate cancer development via multiple mechanisms^[19] including by modulating/disrupting the Wnt/ β -catenin pathway.^[20] We recently found that NIG inhibited the viability of human H460 lung cancer cells growing under different culture conditions. Under RC NIG altered the expression of several key proteins of the Wnt pathway in a concentration dependent manner (e.g., decreased the expression of Wnt5a/b, LRP6 and β -catenin but increased the expression of Naked and Axin 1).^[11] Both iCRT^[15] and NIG^[11] were reported to decrease the expression of β -catenin but as shown in Figure 4 neither of them have significant decrease β -catenin expression on FSs obtained from MCF-7 and PC-3 cells. Moreover, in PC-3 cells iCRT + NIG slightly increased β -catenin expression. The latter is a paradoxical response that we have observed with other drugs in other systems and can be explained as the result of extensive re-wiring of signaling pathways when cells are grown under different culture conditions. We previously reported that in cell growing under prolonged periods of serum starvation (PPSS) the protein expression in response to drugs are different and sometimes paradoxical when compared to cells

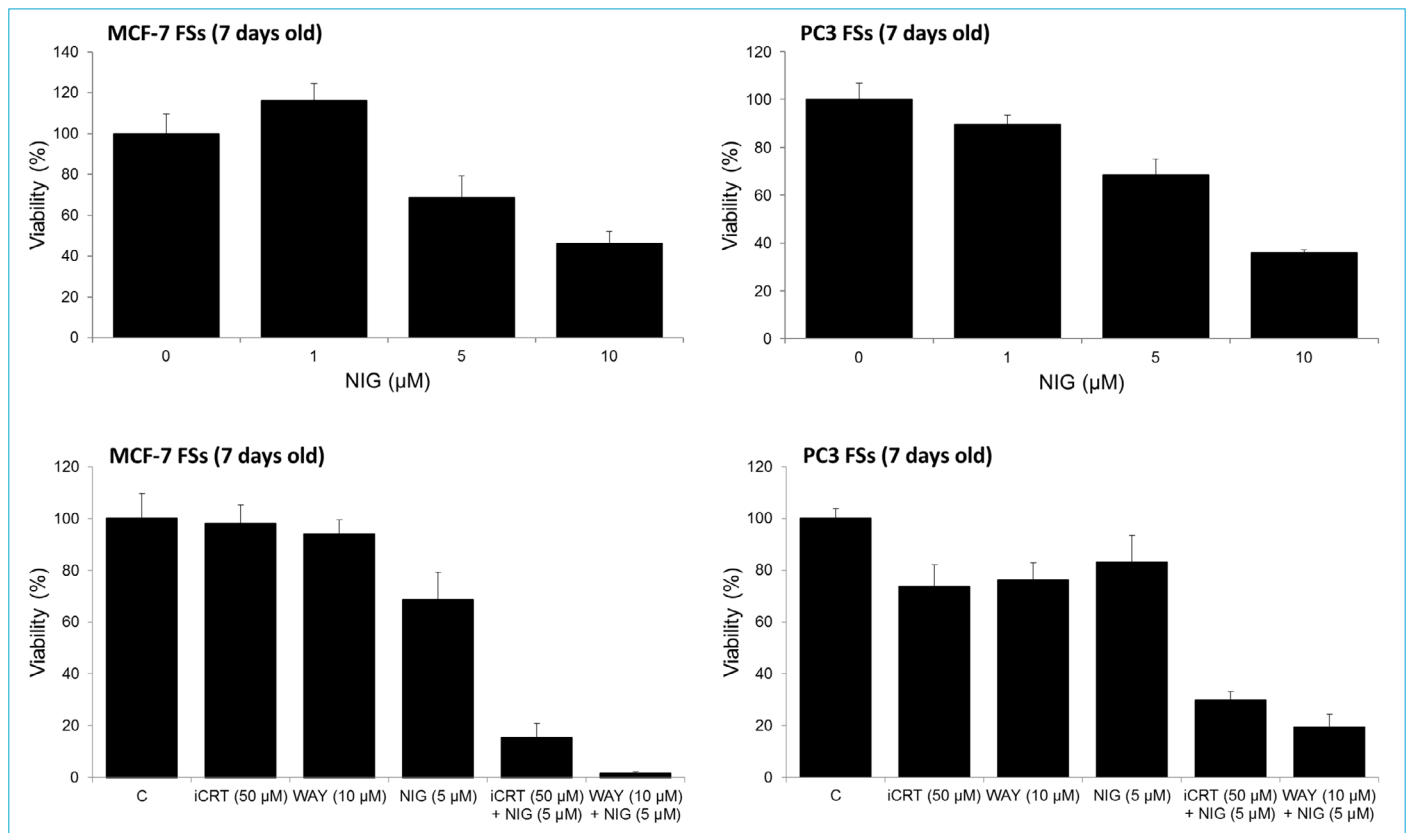


Figure 3. NIG potentiates the anticancer effect of iCRT and WAY on FSs. Cells grown as FSs were incubated with the indicated concentrations of iCRT, WAY or NIG alone or in combination for 72 hs. Cell viability was measured by the CCK-8 assay. Data (mean \pm SD) is representative of three independent experiments.

growing under RC. For instance, the drug Obatoclox when tested under RC decreased the expression of ABCG2 and Bcl2 but when tested in cell growing under PPSS paradoxically increased the expression these two proteins. Aberrant and paradoxical results in the expression of these proteins and other were observed with other drugs such as Verapamil and Sorafenib (alone or in combination) (for detail see Figure 5 in^[21]). In summary, it is likely that FSs undergo extensive rewiring of the Wnt pathway that result in a) a different relation between DKK1 and β -catenin expression (e.g., the expression of β -catenin did not significantly decrease in NIG-treated FSs despite a very high levels of DKK-1) and, b) a paradoxical increased expression of β -catenin in iCRT+NIG-treated FSs obtained from PC-3 cells (Fig. 4).

Conclusion

Our data demonstrated that FSs undergo profound changes in the expression of the Wnt signaling proteins DKK-1 and β -catenin that are likely associated with resistance to specific Wnt pathway inhibitors when used as single agents. While the chemoresistance of FSs to WAY is associated to the downregulation of DKK-1 to undetectable levels, the chemoresistance of FSs to iCRT is likely due to increased expression of β -catenin. Collectively, our results support our hypothesis that changes in DKK-1 expression induced by microenvironmental conditions with concomitant upregulation of β -catenin expression affect chemore-

sistance of cancer cells to Wnt inhibitors. In addition, our results provide additional evidence that under different culture conditions cells undergo extensive rewiring of signaling pathways that in turn produces aberrant and sometimes paradoxical protein expression in response to certain drugs. These findings indicate that monotherapy using small molecules targeting specific proteins of the Wnt signaling pathway will be of limited use in highly heterogeneous tumors. Combination chemotherapy with other Wnt inhibitors as NIG constitutes a promising approach to overcome chemoresistance due to cancer cell plasticity.

Disclosures

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

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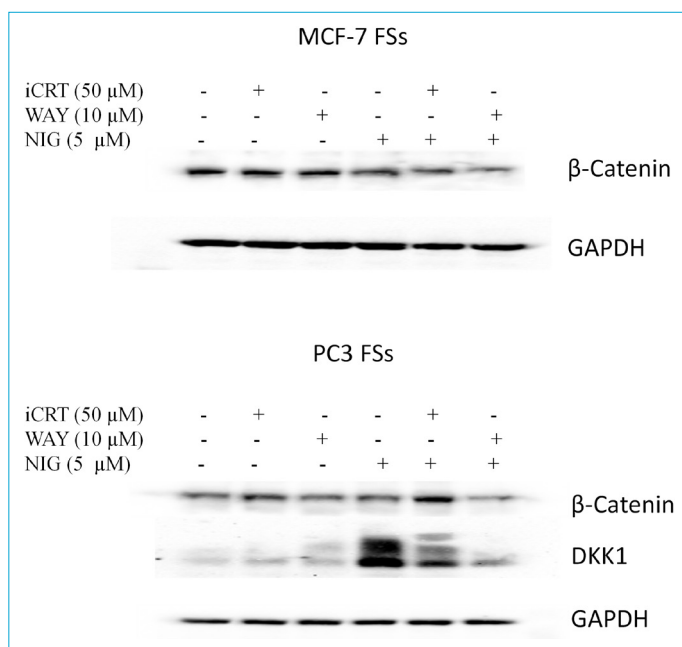


Figure 4. Combined treatment with NIG + WAY decreases the expression β -catenin in FSs. FSs (7 days old) were prepared from MCF-7 and PC-3 cells and then treated with the indicated concentrations of iCRT, WAY or NIG alone or in combination for 24 h.

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