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Indole-2-amide based biochemical antagonist of Dishevelled PDZ domain interaction down-regulates Dishevelled-driven Tcf transcriptional activity

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Abstract—We designed and synthesized a series of indole-2-amide-based compounds that antagonize interaction between the Dishevelled (Dvl) PDZ domain and a peptide derived from the natural PDZ ligand Frizzled-7 (Fz7). These compounds inhibit Tcf-mediated transcription activated by exogenous Dvl via the biochemical antagonism. We confirmed tumor cell-selective activation of caspases by these compounds.

Wnt glycoproteins comprise a family of growth factors that interact with multiple components of the cellular machinery to stimulate diverse signaling pathways.[1] The most significant event in Wnt-promoted cellular growth is activation of transcription by β-catenin/Tcf complex (referred to hereafter as Tcf transcription) after nuclear translocation of hypo-phosphorylated β-catenin. Tcf activates the transcription of several tumor-related genes, including *cyclin-D1*,[2] *c-myc*,[3] and *survivin*.[4] Dishevelled (Dvl) promotes the Tcf transcriptional activation by suppressing the GSK3/APC tumor suppressor complex.[5] Several lines of evidence suggest that Dvl overexpression is sufficient to enhance tumor growth.[6,7] Expression of exogenous Dvl is well known to activate Tcf transcriptional activity without co-treatment with Wnt.[8] These findings suggest that Dvl can induce the Tcf transcription independently of the upstream components of the Wnt signaling pathway. APC is an up-regulator of caspases, which are crucial mediators of apoptosis induction.[9] Consistently, Wnt signaling prevents the caspase activation.[10] Taken together, the available evidence suggests that inhibition of the Dvl function in cancer cells may suppress their growth and trigger their apoptosis by up-regulating the caspases.

The Dvl-family proteins contain a PDZ domain that interacts with several proteins including Fz7,[11] Dapper,[12] and Idax.[13] The functional consequences of the Dvl PDZ interactions are compromised among these binding partners, whose expression level depends on the cellular context. RNAi approaches cannot elucidate Dvl functions specific to the PDZ domain because Dvl is a multi-domain, multi-functional molecule. We envisioned that a small molecule antagonist of the Dvl PDZ domain interactions would be a good tool to investigate the function of the Dvl PDZ domain in the Tcf transcription and the caspase activation.

We have reported chemical scaffolds based on indole-2-[14,15] and -3-carbinol[16,17] that bind to PDZ domains by mimicking a peptide sequence of β-strand ligand. Here, we report a series of new indole-2-amide-based compounds that are biochemical and functional antagonists of Dvl PDZ domain interactions.

Our indole scaffold adopts diversity at the 3- and 2-positions, which are likely to mimic the side chains of the second and fourth amino acid residues, respectively, of the extra-carboxyl terminus of the PDZ domain ligand.[14,16] The second amino acid residue does not make extensive contact to the PDZ domain.[18] We installed a 2-phenylethyl group at the indole-3-position, which we have successfully utilized to design a general scaffold for targeting of PDZ domains.[14–17] The fourth amino acid residue (mimicked by the indole-2-position)

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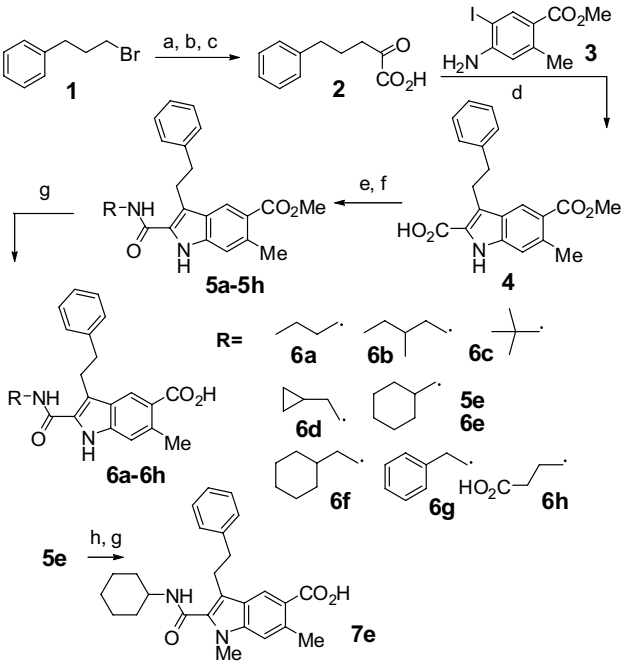
defines the preference and selectivity of canonical PDZ domain interactions.[19] However, the PDZ domain of Dvl has shown to bind to both the extra-carboxyl termini[12] and with internal[11,13] sequences, with no clear preference defined by the fourth amino acid region. Therefore, we postulated that screening of a chemical library having diversity at the indole-2-position would be feasible to investigate the structure–activity relationship of binding to this PDZ domain, rather than rational design from the ligand sequences.

We designed indole-2-carboxylic acid **4** as a key scaffold for library production (Scheme 1). A pyruvic acid derivative **2** that contains the 2-phenylethyl moiety was prepared from 3-phenylpropylmagnesium bromide and dimethyl oxalate. The preparation of **4** proceeded smoothly via palladium-catalyzed coupling[20] of **2** with iodoaniline **3**.[15] The indole-2-carboxylic acid **4** was coupled with various amines to prepare the amide library **6a**–**h**. To assess the significance of hydrogen donors on the indole scaffold, we also prepared methylated variants of **6e** (**5e** and **7e**, Scheme 1).

To assay these compounds' biochemical antagonism of the Dvl PDZ domain interactions, we utilized AlphaScreen energy transfer assays, because they were not affected by auto-fluorescence of the indole scaffold. As a probe for detecting the Dvl PDZ domain interaction, we designed a biotin-tagged peptide derived from the intracellular region of the Fz7 Wnt receptor, which contains the KTXXXW motif responsible for the direct binding[11] of Fz7 and its functional consequence as a Wnt receptor.[21] We chose GST-tagged PDZ domain of human Dvl for the assay, because it offers higher signal intensity than does His-tagged PDZ domain. The signal generated by the interaction of the biotinylated Fz7 peptide and the GST-fused human Dvl PDZ domain was titrated with the test compounds. As the concentration of the compound increased, we observed diminishing signal, signifying competitive antagonism. Representative competition curves are shown in Figure 1. The IC_{50} activity of biochemical antagonism is summarized in Table 1.

We began exploring structure–activity relationships (SAR) by changing the size of the indole-2-amide side chain of **6a**. **6a** showed only weak, incomplete antagonism (~30% of full inhibition) even at a concentration of 1 mM. However, the extension and incorporation of methyl groups on this side chain increased the biochemical potency of antagonism (compare **6a** to **6b**), suggesting the presence of a hydrophobic cavity in the PDZ domain that accommodates a small hydrophobic group. However, binding was decreased by too-close proximity of the methyl substitutions to the indole scaffold (compare **6c** to **6b**). Incorporation of carboxylic acid on this side chain did not increase the activity (**6h**). A cyclopropyl group at this position afforded incomplete antagonism (**6d**), similar to that of **6a**. Meanwhile, a larger carbocyclic ring increased the efficacy of antagonism, as compounds possessing a cyclohexyl group at this position (**6e**, **6f**) showed increased biochemical antagonism. These data clearly show the preference of the



Scheme 1. (a) Magnesium turnings, THF, reflux. (b) Dimethyl oxalate (3 equiv), THF, 0 °C, 1 h. (c) Sodium hydroxide (3 equiv), MeOH, room temp, 0.5 h. Yield of steps a–c = 52%. (d) **2** (5 equiv), **3**[15] (1 equiv), palladium acetate (0.15 equiv), DABCO (3 equiv), DMF, 100 °C, 18 h. Yield = 64% based on **3**. (e) HBTU (2.5 equiv), diisopropylethylamine (3 equiv), DMF, room temp, 1 h. (f) RNH_2 (3 equiv), room temp, 18 h. For preparation of **5h** benzyl 3-aminopropionate was used as the RNH_2. (g) 10% sodium hydroxide aq (3 equiv), MeOH, 1, 4-dioxane, 90 °C, 18 h. (h) MeI, K_2CO_3, DMF, microwave, 150 °C, 15 min. Yield = 95%.

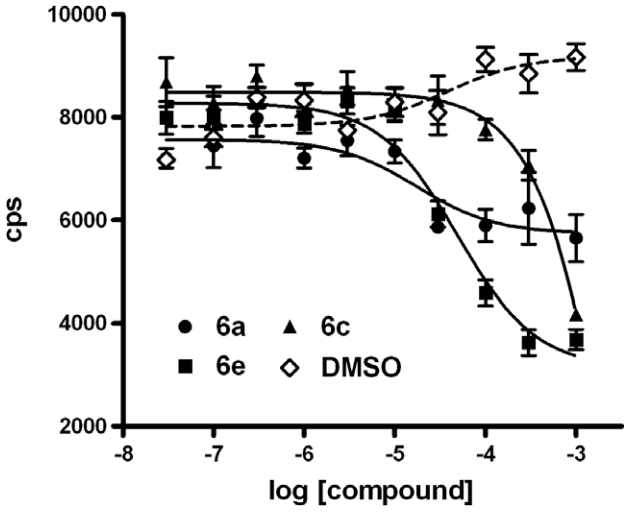


Figure 1. Representative competition curves measured by AlphaScreen. The GST-fused Dvl1 PDZ domain protein and biotin-Fz7 peptide were allowed to equilibrate and were then incubated with test compound to titrate competitive inhibition of the peptide binding. The binding curve was obtained as the mean of three independent experiments, and the IC_{50} (Table 1) was calculated by Prism (GraphPad software). Compound **6a** has shown incomplete antagonism. Corresponding % of DMSO control has shown no activity.

Table 1. Biochemical antagonism (IC_{50}) of the interaction of the Dvl PDZ domain and the Fz7 PDZ binding region in a competitive AlphaScreen assay; and inhibitory effect on Dvl1-activated Tcf reporter activity in HEK293T cells, normalized by cellular viability and shown as DMSO = 0%

Compound	Dvl1 PDZ IC_{50} (μM)	Dvl3 PDZ IC_{50} (μM)	Tcf reporter inhibition% at 100 μM
6a	>500	>500	5>
6b	159	160	26
6c	>500	>500	5>
6d	>500	>500	ND
6e	49	23	27
6f	157	309	16
6g	197	291	23
6h	>500	>500	5>
5e	97	57	35
7e	78	92	39

ND, no data.

Dvl PDZ domain to a mid-sized hydrophobe in the indole-2-position.

The indole-5-carboxylic acid mimics the extra carboxyl terminus of ligand of the PDZ domain,[14,16] that is an essential motif in interacting to canonical PDZ domains through hydrogen-bond network.[18] However, Dvl PDZ domain binds to both the extra carboxy-terminal ligand (Dapper[12]) and internal sequence (Fz7[11]), thus it is expected that the carboxylic acid is not essential in binding to this PDZ domain. Consistently, methylation at this position (**5e**) only modestly diminished the potency. Methylation at the indole-1-position also modestly but not dramatically diminished the potency (**7e**), suggesting that the Dvl PDZ domain may have small hydrophobic cavities in the region that fit the indole-1-methyl. It is noteworthy that those three indole-2-amide compounds (**5e**, **6e**, **7e**) maintain the activity in spite of lacking a hydroxyl group on the indole,[14–17] that mimics essential hydrogen-bond interaction of S/T(−2) residue of class1 PDZ domain ligands. Taken together, these SAR data suggest that ligand binding of the Dvl PDZ domain is controlled by hydrophobic interaction, rather than hydrogen-bond formation.

Next, we assayed inhibition of the up-regulation of Tcf transcription by exogenous Dvl in HEK293T cells, which are believed to respond normally to the Wnt signaling pathways. As these cells have no mutation or overexpression that upregulates Tcf transcription, their intrinsic Tcf transcription activity is at background level. However, their Tcf activity significantly increases upon transient expression of Dvl, signifying Dvl-driven up-regulation of Tcf transcription. We have transiently transfected HEK293T cells with superTOPflash plasmid,[22] an optimized reporter for Tcf activation of transcriptional activity, and an expression vector encoding Dvl1. The cells were then incubated with the test compound for 1 day. Tcf reporter activity was measured as firefly luciferase and was normalized by the cellular viability to express activity of Tcf transcription. As expected, **6a**, **6c**, and **6h**, whose activity in the AlphaScreen assay was very weak, showed no inhibition. Other compounds with biochemical activity inhibited the Tcf transcription at 100 μM (Table 1) but not at 50 μM. Some discrepancy observed in between the biochemical antagonism potency and transcriptional down-regulation activity may be due to difference of cellular permeability or stability in the cell cultures. These results suggest that inhibition of the Tcf transcription reflects the biochemical antagonism to the Dvl PDZ domain. No compounds have significantly reduced the viability signal, suggesting that the down-regulation of the Tcf transcription is not cytotoxic effect.

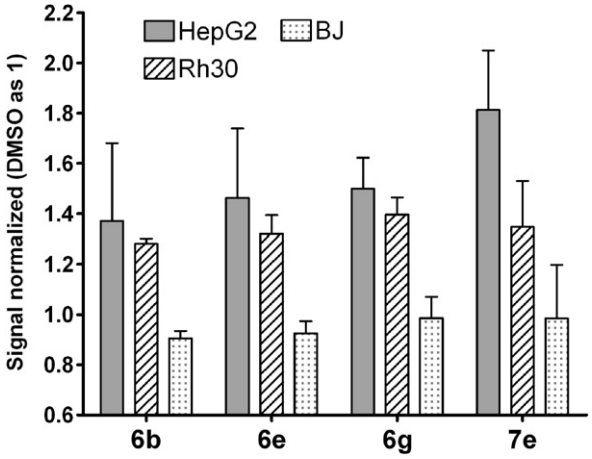


Figure 2. Caspase activation in HepG2, Rh30, and BJ cells. The cells were treated with test compound (100 μM) for 2 days, and caspase activity was measured by Caspase3/7-Glo (Promega) reagent. The activity signal is represented as the mean proportion of that of DMSO control (considered equivalent to 1) in three independent experiments. Error bars represent standard deviation.

To confirm the apoptosis effect of the indole-2-amide compounds, we assayed caspase activity in cells after treatment with the compounds (Fig. 2). In HepG2 hepatocellular carcinoma cells and Rh30 rhabdomyosarcoma cells, treatment with all compounds except **5e** significantly increased caspase-3/7 activity. In contrast, such activation was not observed in *hTERT*-immortalized BJ cells[23] normal human foreskin fibroblasts, except those treated with **6f**. These results suggest that Tcf activation of transcriptional down-regulation can induce apoptosis selectively in cancer cells.

In summary, we have demonstrated that the indole-2-amide scaffold can be utilized to functionally antagonize interactions with the PDZ domain of Dvl, thereby down-regulating Dvl-driven Tcf activation of transcription. The biochemical potency of this antagonism is enhanced by incorporating a small hydrophobic group in the substituent on the 2-amide moiety. Compounds derived from this scaffold can cause apoptosis selectively in cancer cells.

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Supplementary data

Synthetic procedure, analysis data, protocols for biochemical assay, and cell-based assay. Supplementary data associated with this article can be found, in the online version. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.039.

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