VIP Antitumor Agents

DOI: 10.1002/anie.200902981

Sulindac Inhibits Canonical Wnt Signaling by Blocking the PDZ Domain of the Protein Dishevelled**

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The protective anticancer effect of nonsteroidal anti-inflammatory drugs (NSAIDs) has attracted much attention, and clinical trials of several NSAIDs are under way for treatment or prevention of various cancers.[1] As NSAIDs are best known for their inhibition of cyclooxygenase 1 and 2 (COX-1/2), they are hypothesized to suppress tumor growth by blocking prostaglandin synthesis.[2] However, this hypothesis does not explain all of the available data.[3–5] Accumulated evidence suggests that some NSAIDs also target the Wnt/β-catenin signaling pathway, a key regulatory pathway for cell development and growth, in human cancer cells.[4,5a–c] For example, sulindac (Clinoril) has been shown to suppress canonical β-catenin-related Wnt signaling in breast cancer, lung cancer, and colon cancer cell lines.[4e] However, the molecular mechanism of this effect is not clear.

Wnt signaling plays crucial roles in embryonic development and in tissue maintenance in adults.[5] Abnormal activation of Wnt signaling is observed in several types of cancers.[5,6] Dishevelled (Dvl) is a key molecule in the Wnt pathways that, through its PDZ domain, relays Wnt signals from membrane-bound Wnt receptors to downstream components.[5,7] We and other research groups have worked to develop small-molecule inhibitors of the Dvl PDZ protein–protein interaction for use in the elucidation of biological processes and as potential cancer-treatment and prevention agents.[8] Herein, we show that both sulindac and sulindac sulfone bind to the PDZ domain of Dvl and sulindac suppresses Wnt3 A induced β-catenin signaling at the level of Dvl. Our results suggest that the anticancer protective effect of sulindac (and its metabolite) reflect not only COX-1/2 inhibition but also the inhibition of abnormal canonical Wnt signaling by blockade of the Dvl PDZ domain.

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[**] This work was supported by NIH grants CA21765 (Cancer Center Support Grant) and GM081492, by the American Lebanese Syrian Associated Charities (ALSAC; J.J.Z.), and to ARC and LNCC (D.-L.S.). We thank Sharon Naron for editing the manuscript; the Hartwell Center for Bioinformatics and Biotechnology at St. Jude for computational time; S. Malone, M. Zhou, and Dr. C. Ross for computer-related technical support; Dr. W. Zhang for assistance with the NMR experiments; and Y. Shao for providing proteins.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200902981.

To test the binding of sulindac and sulindac sulfone to the Dvl PDZ domain, we conducted chemical shift perturbation experiments with NMR spectroscopy; this method is widely used to characterize protein–ligand interactions.[9] When added to a solution of ^{15}N-labeled Dvl PDZ domain, both compounds generated chemical shift perturbations that indicated binding to the same region of the Dvl PDZ domain (Figure 1 a and Figure S1 in the Supporting Information). The structure of the Dvl PDZ domain comprises six β strands (βA–βF) and two α helices (αA and αB). The chemical shift perturbations induced by binding indicated that both compounds bind to the Dvl PDZ domain between its αB and βB structures, as do the native ligands of the domain.[7,10]

We next examined whether sulindac and sulindac sulfone bind exclusively to the Dvl PDZ domain, as there are many PDZ domains in the human proteome.[8c,11] We tested three other PDZ domains that are representative of most human PDZ domains: the first and second PDZ domains of PSD-95 protein (PDZ1 and PDZ2, respectively; class 1 PDZ domains) and the seventh PDZ domain of GRIP1 protein (PDZ7; a class 2 PDZ domain). The Dvl PDZ domain belongs to neither class 1 nor class 2.[7,11] Remarkably, NMR titration experiments showed little or no interaction between sulindac or sulindac sulfone and these three PDZ domains (see Figure S2 and Table S1 in the Supporting Information), this result indicates that sulindac and sulindac sulfone bind specifically to the Dvl PDZ domain.

To further investigate the specificity with which sulindac and sulindac sulfone recognize the Dvl PDZ domain, we determined the structure of Dvl PDZ domain in complex with sulindac (Figure 1 b). 2D (^{15}N,^{13}C)-double-filtered nuclear Overhauser effect (NOE) spectroscopy experiments showed that sulindac bound to Dvl PDZ has a *cis* (*Z*) conformation (Figure S3 and Table S2 in the Supporting Information).[12a] The complex structure was determined from 45 intermolecular NOE signals (obtained from 3D ^{13}C-F1-half-filtered, F2-edited NOE–HSQC experiments) between bound sulindac and the PDZ domain, and seven intramolecular NOE signals of sulindac (Figure S4 and Table S3 in the Supporting Information).[12] An ensemble of the 20 lowest-energy calculated structures of the complex is shown in Figure 1 b. In the complex, sulindac binds to the peptide pocket of the PDZ domain (Figure 2, and Table S4 in the Supporting Information), in which the sulindac carboxylate group forms hydrogen bonds with the amide groups (L262, G263, and I264) in the PDZ-domain loop region (Figure 2 b). The sulindac methyl-

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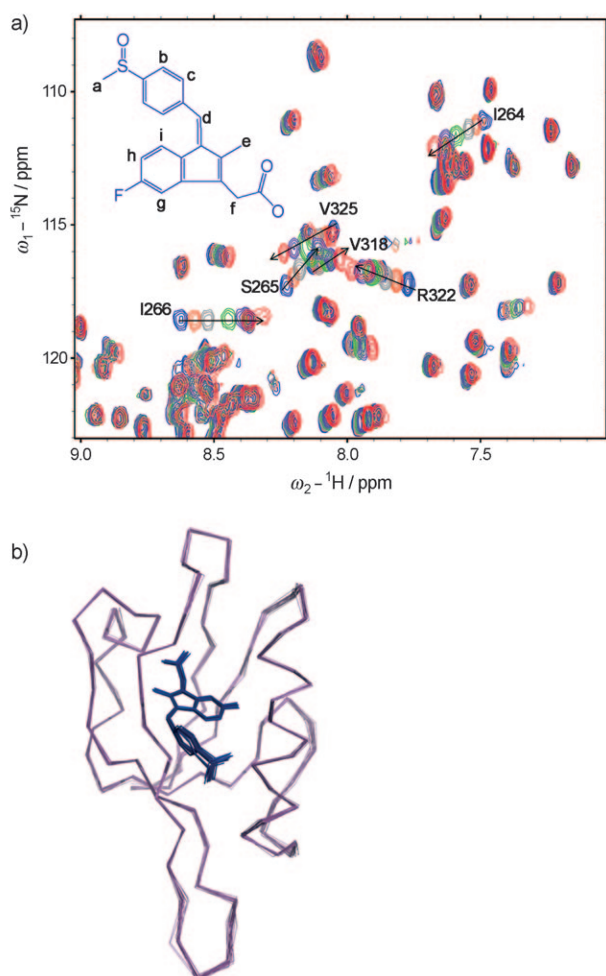

Figure 1. Sulindac directly binds to the PDZ domain of Dishevelled. a) The extended ^{15}N-HSQC spectra of ^{15}N-labeled Dvl PDZ domain during the titration of sulindac (blue: free state; red: bound state). b) Ensemble of the 20 lowest-energy conformations of the PDZ–sulindac complex. Magenta: backbone of PDZ domain; blue stick model: sulindac.

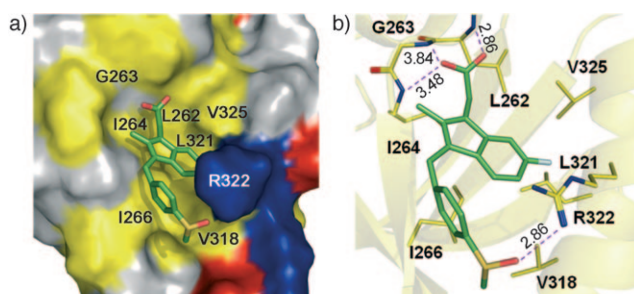

Figure 2. Three-dimensional structure of the Dvl PDZ domain in complex with sulindac. a) Surface representation showing the binding interface between the Dvl PDZ domain and sulindac (stick model). Yellow: hydrophobic amino acid residues in Dvl PDZ; blue: positively charged residues; red: negatively charged residues; gray: uncharged polar residues. b) Sulindac (stick model) and Dvl PDZ (cartoon model). Yellow: Dvl PDZ domain residues in contact with sulindac. The hydrogen bond distances (Å) between oxygen (red) and nitrogen (blue) are indicated.

ene group (labeled f in Figure 1 a) contacts the side chains of PDZ-domain residues L262 and V325 and the sulindac aromatic rings (g, h, i in Figure 1 a) contact the hydrophobic pocket formed by PDZ-domain residues L262, I266, V325, L321, and V318. The sulindac benzyl group (b and c in Figure 1 a) and methyl group (a) contact PDZ-domain residues I266 and V318. Notably, the side chain of R322 on the PDZ-domain α helix (αB-5′) forms a hydrogen bond with the sulfonyl oxygen atom of sulindac (S=O···H−N$_\eta$; $d_{O-N\eta} \approx 2.9$ Å; Figure 2 b). Consistent with this result, the R322 A mutant PDZ domain interacted negligibly with sulindac (Figure S5 in the Supporting Information). Therefore, residue R322 on the αB helix of Dvl PDZ appears to play a crucial role in determining the specificity of binding. The residue Arg in the αB-5′ position (R322) occurs in no PDZ domain except that of Dvl (Figure S7 in the Supporting Information). Therefore, this interaction is likely to explain the specific binding of sulindac and sulindac sulfone to the Dvl PDZ domain. Indeed, sulindac sulfide, which is the most prominent metabolite of sulindac, bears the COX-1/2 inhibition, and has a thioether instead of a sulfonyl group, binds to the PDZ domain with a much weaker affinity (Figure S6 in the Supporting Information).

To obtain further insight into the binding mechanism of sulindac and sulindac sulfone to the Dvl PDZ domain, we performed a competitive binding assay using a peptide derived from the C-terminus of Dapper (Rox-SGSLKLMTTV$_{COOH}$, Rox-DprC). Dapper protein is known to antagonize Wnt signaling by binding to the PDZ domain of Dvl.[7,10] We first measured the binding affinity between this peptide and the PDZ domain of Dvl by monitoring fluorescence polarization during titration of unlabeled Dvl PDZ into a solution of fluorescence-labeled Rox-DprC ($K_D \approx (8.0 \pm 1.0)$ μM).[7] We then added either sulindac or sulindac sulfone to the solution of the labeled peptide and repeated the same binding assay; we found that both compounds inhibited interaction of the peptide with Dvl PDZ (K_I values, (10.7 ± 1.2) μM and (8.0 ± 0.2) μM, respectively; Figure 3). As the binding affinities of Dapper, sulindac, and sulindac sulfone to the Dvl PDZ domain are similar, sulindac and sulindac sulfone, like Dapper, effectively block cellular Wnt signaling by disrupting the interactions between Dvl and the Wnt receptors Frizzled (Fz).[7]

To confirm that sulindac inhibits canonical Wnt signaling, we co-injected Wnt3A mRNA (1 pg) and the *Siamois* reporter construct[13] into the animal pole region of *Xenopus* embryos at the two-cell stage and cultured them with different concentrations of sulindac. A luciferase assay was performed on ectodermal explants dissected at the blastula stage. As shown in Figure 4 a, and consistent with our previous results,[8a] sulindac inhibited *Siamois* promoter-driven luciferase activity induced by Wnt3A. To establish whether inhibition of Wnt signaling by sulindac occurs upstream or downstream of Dvl, we also co-injected the animal pole region of *Xenopus* embryos with the *Siamois* reporter construct and Dvl mRNA (100 pg) or β-catenin mRNA (500 pg), treated the embryos with sulindac (3.5 μg mL^{-1}, 9.8 μM), and assayed luciferase activity (Figure 4 b). As expected, sulindac attenuated Dvl-mediated Wnt signaling

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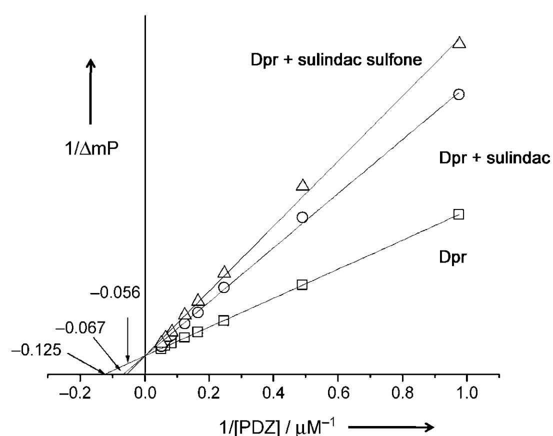
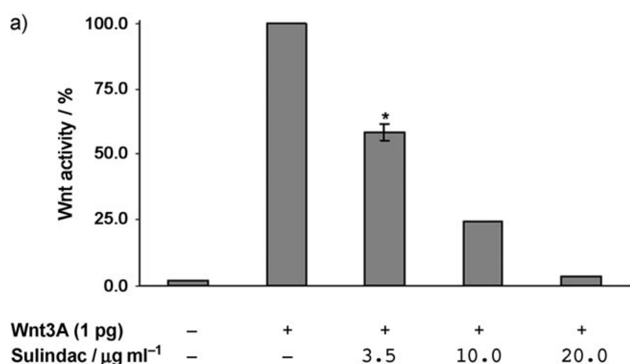


Figure 3. Competitive binding experiments. The K_D value of the fluorescently labeled Dapper-derived peptide Rox-DprC was obtained by plotting $1/\Delta mP$ against $1/\Delta$[PDZ], where ΔmP is the fluorescence polarization change (×1000) of Rox-DprC and Δ[PDZ] is the concentration change of the PDZ domain. The K_I values of sulindac and sulindac sulfone were obtained by using the equation $K_D^{app} = (K_D/(1+[I]/K_I)$.

a)



Wnt3A (1 pg)	–	+	+	+	+
Sulindac / μg ml−1	–	–	3.5	10.0	20.0

b)

Average of Wnt activity / %	
Wnt3A/sulindac	58.6 (±3.1)[a]
Dsh/sulindac	55.3 (±22.1)[a]
β-catenin/sulindac	108.2 (±65.3)[a]

[a] Four independent experiments

Figure 4. Effect of sulindac on canonical Wnt signaling in *Xenopus* embryos. a) Sulindac inhibits canonical Wnt signaling induced by Wnt3A in a concentration-dependent manner. Luciferase activity was measured in ectodermal explants dissected at the late blastula stage. Activity in the untreated control was assigned a value of 100%. Four embryos were treated with 3.5 μg mL^{-1} of sulindac; the mean ± standard deviation (SD) is shown. The remaining data represent the mean value from three independent experiments. b) Sulindac attenuates canonical Wnt signaling at the level of Dvl. Values are mean luciferase activity ± SD from four independent experiments.

but not β-catenin-mediated signaling. Therefore, sulindac blocks canonical Wnt signaling at the level of Dvl.

Dvl is a critical regulator of Wnt signaling pathways.[5] In the canonical Wnt signaling pathway, the Wnt signal is passed from the membrane Wnt receptor Fz to Dvl, which then relays the signal to downstream components.[5,7] The direct recognition between Dvl PDZ domain and a conserved sequence (KTXXXW) in Fz, localized two residues after the seventh transmembrane domain, is the key interaction in the pathway.[7] Indeed, Dvl PDZ-binding peptides as well as small molecules that target the Dvl PDZ domain can effectively block Wnt signaling.[8] We show that sulindac and sulindac sulfone bind the peptide-binding site of the Dvl PDZ domain with an affinity comparable to that of the native binding partners[7] and that, like other Dvl PDZ inhibitors, sulindac blocks Wnt signaling at the Dvl level in *Xenopus*. Therefore, we propose that sulindac exerts its chemoprotective anticancer effect not only by inhibiting COX enzymes[2] but also by inhibiting Wnt signaling by Dvl.

The abnormal activation of Dvl has been linked to several types of cancer, including mesothelioma, breast cancer, prostate cancer, cervical cancer, and non-small-cell lung cancer.[6] In many cases, over-expression of Dvl is implicated in the activation of Wnt signaling. Therefore, inhibition of Dvl PDZ-domain interactions by sulindac or sulindac sulfone should counteract this cancer-promoting effect. However, Wnt signaling may be activated in various ways in cancer[14a] and tumor development, and progression often result from cumulative events that abnormally activate Wnt signaling.[15,16] For example, most cases of colorectal cancer are initiated by activation of the Wnt pathway in the presence of a mutant adenomatous polyposis coli (APC) tumor suppressor, but in many cases, the level of Wnt signaling increases during tumor progression.[14b] Epigenetic inactivation of Wnt antagonist genes, such as *dikkopf* and *sfrp*, often occurs early in colorectal cancer progression and could induce the constitutive Wnt signaling that is a necessary complement to APC mutations in the evolution of colorectal cancer.[15] Therefore, even though APC is downstream of Dvl in the canonical Wnt signaling pathway, inhibition of Wnt signaling through sulindac blockade of the Dvl PDZ domain should help to suppress the progression and evolution of colorectal cancer. For example, in a recent study, restoration of expression of *dact3* (Dapper/Frodo), a protein antagonist of Dvl PDZ domain that is epigenetically repressed in colorectal cancer, resulted in attenuation of Wnt signaling and apoptosis of colorectal cancer cells.[16]

The testing of NSAIDs as anticancer chemopreventive agents is an excellent example of current efforts to “repurpose” old drugs to new applications.[4c,17] However, unresolved questions such as the mechanism of action of NSAIDs limit their clinical application to the cancer prevention or treatment.[1a] Our findings suggest that at least one of the chemopreventive mechanisms of sulindac is the blockade of canonical Wnt signaling downstream of the Dvl PDZ domain. This conclusion is consistent with the fact that sulindac and sulindac sulfone exert similar chemopreventive effects although they have very different effects on the COX enzymes (sulindac is a nonspecific COX inhibitor, whereas sulindac sulfone does not inhibit COX).[4f] It also provides a rationale to explore other NSAIDs and their analogues and metabolites as potential anticancer therapeutic and preventive agents. Moreover, since sulindac interacts with Dvl PDZ

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domain at low micromolar affinity, further development of more potent inhibitors is clearly needed.

Experimental Section

Structure determination of the Dvl PDZ-sulindac complex: Distance restraints were obtained by conducting several types of NOE spectroscopy experiments, including 2D (^{13}C,^{15}N)-double filtered ^{1}H-NOE spectroscopy, 2D ^{13}C F1-half-filtered ^{1}H–^{1}H NOE spectroscopy, and 3D ^{13}C-F1-half-filtered and F2-edited HSQC–NOE spectroscopy, on a sample of ^{13}C/^{15}N-double-labeled Dvl PDZ domain bound to unlabeled sulindac in a PDZ/sulindac ratio of 1:10.[13b] NOE restraints were grouped into distance ranges according to their relative intensity (Tables S2 and S3 in the Supporting Information). Topology and parameter files for sulindac were generated by using xplo2d,[18a] and the charge parameters of sulindac were calculated by using the antechamber module in the AMBER8 software package.[18b] To calculate the structure of the complex, we used the simulated annealing protocol in the program CNS[18c] with the modified X-ray structure of *Xenopus* PDZ domain (1 L6O)[10] as the starting point; all calculations were carried out using the program HADDOCK.[18d] In the final calculation, two different restraints were used to elucidate the complex structure: 1) NOE-based unambiguous distance restraints and 2) the hydrogen-bond restraints, which were set at $1.6\,Å < d_{NH-O} < 2.4\,Å$ and $2.4\,Å < d_{N-O} < 3.5\,Å$ by using the donor–acceptor pairs identified in the initial rounds of structural calculation. 2000 initial structures of the Dvl1 PDZ/sulindac complex were generated. The 20 lowest-energy conformations among the final 100 water-refinement structures of the complex were selected (Table S4). The refined structures were analyzed using the programs PROCHECK[18e] and MOLMOL[18f] and have been deposited into the Protein Data Bank (PDB code 2KAW).

Fluorescence binding studies: A Fluorolog-3 spectrofluorometer (Jobin–Yvon Inc.) and a 10×4 mm quartz cell (Hellma Inc.) with magnetic stirring were used to conduct the competition binding experiments (see the Supporting Information for details).

Luciferase assay: *Xenopus* eggs were obtained from females that had received injections of 500 IU of human chorionic gonadotropin (Sigma)and had been artificially fertilized. Synthesis and microinjection of capped mRNAs were carried out as previously described.[19] The mRNA and the *Siamois* reporter DNA[13] were coinjected into the animal pole region at the two-cell stage. Injected embryos were cultured in the presence of sulindac at different concentrations. Animal cap explants from untreated and treated embryos were dissected at the late blastula stage and a luciferase assay (Promega) was performed by preparing cell lysates from 10 explants and measuring the luciferase activity in a Lumat LB 9507 luminometer.[13]

Received: June 2, 2009
Published online: July 27, 2009

Keywords: antitumor agents · hydrogen bonds · signal inhibition · signal transduction

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