Bioorganic & Medicinal Chemistry Letters 19 (2009) 2282–2284

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Novel small molecule activators of β-catenin-mediated signaling pathway: structure–activity relationships of indirubins

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ARTICLE INFO

Article history:
Received 30 December 2008
Revised 18 February 2009
Accepted 21 February 2009
Available online 26 February 2009

Keywords:
Wnt signaling
Novel activator of Wnt signaling
Indirubin
5-Nitro-3′-monoxime
β-Catenin

ABSTRACT

Based on the β-catenin-drived Wnt activator of bromoindirubin-3′-oxime (BIO), indirubin analogs were evaluated for β-catenin-mediated gene expression. A novel indirubin analog, indirubin-5-nitro-3′-oxime (INO), was considered a potential activator, and structure–activity studies were conducted with indirubins. These data suggest that INO might be a novel Wnt activator and has a potential of signaling regulator in β-catenin-mediated signaling pathways.

β-Catenin protein is an essential component of cell–cell adhesion and the canonical Wnt signaling pathway.[1] In the cell-cell interactions β-catenin associates with the cytoplasmic end of E-cadherin and β-catenin, and these complex at the adherence junctions forms a dynamic link to the cytoskeleton.[2,3] In the Wnt signaling pathway, β-catenin plays a role as a transcriptional activator with T-cell factor (TCF)/lymphoid enhancer factor (LEF) DNA binding proteins.[4,5] Generally, in the absence of Wnt signaling, the level of cytoplasmic β-catenin is maintained low through the degradation of β-catenin by the machinery of destruction complex. β-Catenin is phosphorylated by the serine/threonine kinases casein kinase I (CKI) and glycogen synthase kinase-3β (GSK-3β) bound to a scaffolding complex of axin and adenomatous polyposis coli (APC). The phosphorylated β-catenin is recognized by β-transducin repeat-containing protein (β-TrCP), targeted for ubiquitination, and degraded by the 26S proteosome. Activation of Wnt signaling leads to inhibition of GSK-3β activity, resulting in the accumulation of cytoplasmic β-catenin and subsequently translocation of β-catenin into the nucleus. The elevated level of nucleus β-catenin leads to the complex formation with TCF/LEF transcription factor and the complex induces target gene expression. On this line, many evidences suggest that the Wnt signaling pathways are involved in a variety of physiological and pathophysiological processes including embryonic development, tissue homeostasis, degeneration and cancer.[6–8] Therefore, the agonists or antagonists for the Wnt signaling pathways are considered useful tools for studies of adult tissue-homeostasis, regeneration, and embryogenesis.

In the activation of Wnt signaling one of possible mechanism of action is through the inhibition of GSK-3β activity. Indeed, the relatively selective inhibitors against GSK-3β such as pyrrole compounds of SB-216763 and SB-415286 showed the potential induction of transcription of a β-catenin-mediated gene expression and these compounds were elucidated as potential therapeutic effectors for diabetes disease.[9,10]

Recent findings also suggest that an indirubin analog 6-bromoindirubin-3′-oxime (BIO) displayed a remarkable selective inhibition of GSK-3β and thus BIO reduced β-catenin phosphoryaltion on a GSK-3-specific site in cellular models (Fig. 1). These effects are closely mimicked Wnt signaling pathway in in vivo *Xenopus*

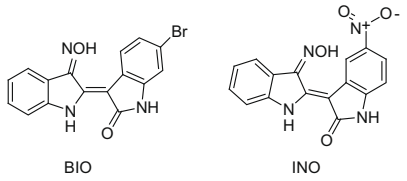

Figure 1. Chemical structures of 6-bromoindirubin-3′-oxime (BIO) and indirubin-5-nitro-3′-oxime (INO).

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doi:10.1016/j.bmcl.2009.02.083

embryo model and in human and mouse embryonic stem cells for the maintenance of its self-renewal stemness.[11] Therefore, BIO is considered a new scaffold for the modulator of Wnt signaling and thus providing practical applications in regenerative medicine or other diseases including Alzheimer's disease and diabetes.

Based on the potential of BIO as a Wnt activator and thus applicable for the development of novel therapeutic agents through the modulation of Wnt signaling, we primarily made an attempt to evaluate indirubin analogs for procurement of novel Wnt activators.

Indirubins are found from various natural sources including indigo-producing plants, bacterial strains and gastropod mollusks. Especially, indirubin is the main active ingredient of Danggui Longhui Wan, a traditional Chinese medicinal recipe used for treatment of various diseases including cancer.[12] Many biological activities of indirubins have been reported by the inhibition of several kinase families.[13–16] Anti-proliferative effects on the human cancer cells were to inhibit CDKs by competing in the ATP binding sites with high selectivity. A series of the indirubin derivatives were also developed for the treatment of Alzheimer's disease (AD) through inhibiting GSK-3β and CDK5, which are related to the control of the abnormal hyperphosphorylation of the microtubule-binding protein tau, one of the diagnostic characteristics of AD.[17] In addition, indirubins have been revealed as potent ligands of the aryl hydrocarbon receptor and inhibitors of c-Jun NH_2-terminal kinase (JNK).[18] More recently, some indirubin derivatives were identified as potent and selective GSK-3 inhibitors such as BIO and were considered as Wnt activators.[11] We here report an identification of some additional novel Wnt activators from a series of indirubin derivatives synthesized in our previous study for the inhibitory activity of CDK.[14]

First, in order to determine the activation of Wnt pathway, we used human embryonic kidney cells (HEK293) which have transiently transfected with a Tcf-luciferase transcription reporter (pTOPFlash). This transcription reporter generates luciferase in response to the activation of β-catenin-mediated Tcf/Lef transcriptional activation.[19] By employing this assay system we primarily evaluated the TOPFlash activity with various concentrations of BIO for an incubation of 24 h. As shown in Figure 2, BIO significantly increased the luciferase activity in a concentration-dependent manner, suggesting BIO is a β-catenin-drived Wnt activator. On this line, for procurement of novel Wnt activator with the similar chemical characteristics in BIO, we evaluated several indirubin derivatives on β-catenin-mediated target gene expression with TOPFlash assay.

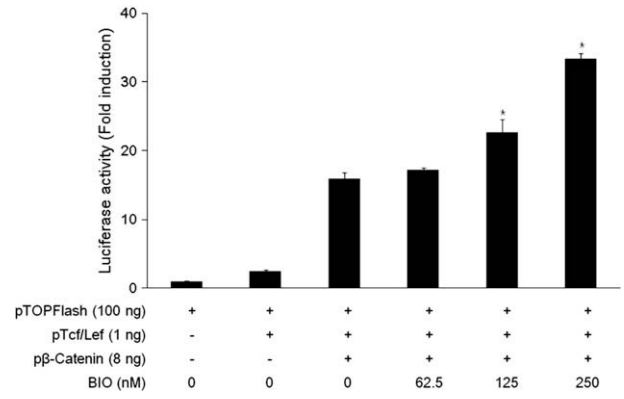


Figure 2. 6-Bromoindirubin-3′-oxime (BIO) activates β-catenin-mediated Tcf/Lef reporter construct ($^{*}p < 0.05$ was considered statistically significant compared to vehicle-treated control group).

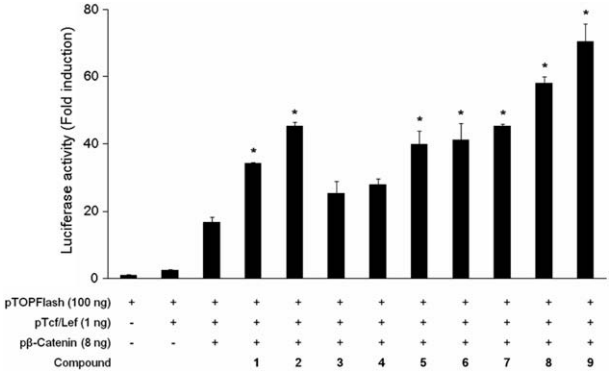
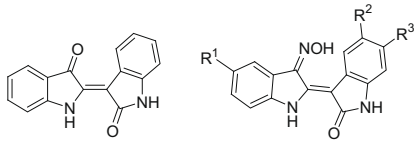


Figure 3. TOPFlash activity of indirubin derivatives ($^{*}p < 0.05$ was considered statistically significant compared to vehicle-treated control group).

As shown in Figure 3, co-treatment of pβ-catenin (8 ng) and pTcf4 (1 ng) induced the TOPFlash activity with approximately 15-fold compared to only pTOPFlash transfected group. Employing the assay system primarily indirubin derivatives (250 nM) were simultaneously treated with pβ-catenin (8 ng) and pTcf 4 (1 ng) and evaluated in the β-catenin/Tcf-mediated luciferase activity (TOPFlash activity). Indirubin exhibited a moderate activating effect on the assay (~2.0-fold induction compared to vehicle-treated control group). Several compounds also showed potential activation in this capacity. Among them, indirubin-5-nitro-3′-oxime (INO) exhibited the most potent activation effect (~4.2-fold induction) (Table 1). In terms of structure–activity relationship of indirubin-3′-oxime derivatives, compounds **3** and **5** which have R^2 with alkyl-substituted amide functional groups (–NCOR) showed rela-

Table 1
Effects of indirubin and its derivatives on β-catenin-mediated Tcf/Lef reporter gene activity (TOPFlash)



1 (Indirubin)

Compound	R^1	R^2	R^3	Fold induction*
1 (Indirubin)	H	H	H	2.04
2	H	H	Br	2.70
3	H		H	1.51
4	Br		H	1.66
5	H		H	1.89
6	H	Cl	H	3.46
7 (INO)	H		H	4.20

*Fold induction was calculated by the comparison of TOPFlash activity between the compound-treated group and vehicle-treated control group.

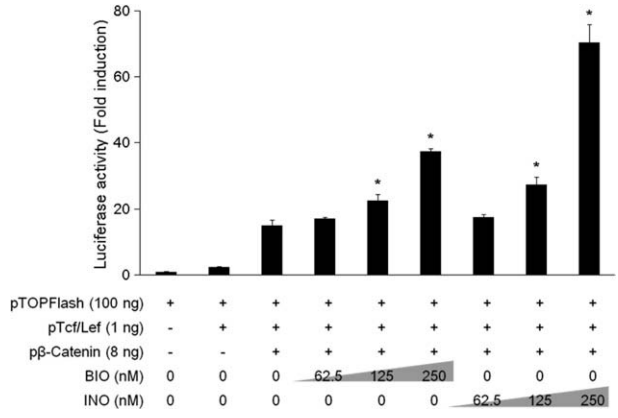


Figure 4. Dose-dependent induction of β-catenin-mediated Tcf/Lef transcriptional activity of INO ($^{*}p < 0.05$ was considered statistically significant compared to vehicle-treated control group).

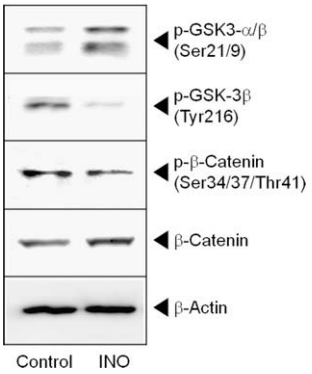


Figure 5. Effects of INO (250 nM) on the phosphorylation and expression of GSK-3β and β-catenin. β-Actin was used as an internal standard.

tively low activities compared to INO. On the other hand, compounds **6** which substituted with Cl at R^2 position exhibited a potential induction (~3.5-fold). Therefore, the introduction of an electronegativity at R^2 position seems to affect the increase of TOPFlash activity. However, in comparison with INO, the introduction of Br at R^1 position caused dramatic decrease of the activity. It suggests that the introduction of an electrowithdrawing group in Ring A might decrease the TOPFlash activity.

In addition, INO was comparable to the known Wnt activator BIO (~2.7-fold induction), and was dose-dependent induction of reporter gene activity (Fig. 4).

Further study was designed to determine whether INO affects to the modulation of β-catenin and its upstream molecule GSK-3β expression in Wnt activating pathway. As shown in Figure 5, Western blot analysis[20] showed that INO treatment increased the phosphorylation at Ser9 of GSK-3β and oppositely decreased the phosphorylation at Tyr216 of GSK-3β in HEK293 cells, leading to the inactivation of GSK-3β. GSK-3β is known to destabilize β-catenin by phosphorylating at Ser33, Ser37 and Thr41.[17] Therefore, the inactivation of GSK-3β resulted in the decreased phosphorylation of β-catenin at Ser34/37/Thr41 residues, leading to the accumulation of β-catenin. Consequently, the accumulated β-catenin in cytosol was translocated into nucleus and then promoted the Tcf/Lef transcriptional activity (TOPFlash).

In summary, the present study demonstrates that a novel indirubin derivative, indirubin-5-nitro-3′-monoxime (INO), might be served as a novel Wnt activator and thus has a potential candidate for the development of therapeutic agents in the modulation of Wnt signaling pathway.

Acknowledgements

This work was supported by Grant No. R15-2006-020 from the National Core Research Center (NCRC) program of the Ministry of Education, Science and Technology (MEST) and the Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signalling & Drug Discovery Research at Ewha Womans University.

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19. *Reporter gene assay:* HEK293 cells (1.5×10^4 cells in a 48 well plate) were seeded and incubated for 24 h. The cells in each well were transiently transfected with pTOPFlash (100 ng, pβ-catenin (8 ng), pTcf4 (1 ng), and pRL-SV40 (5 ng) with 4.0 μl of Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. *Renilla* luciferase (pRL-SV40) was transfected as an internal control to normalize the transfection efficiency of each well. After 24 h incubation, the cells were treated with test samples (250 nM) for 24 h. The cells were lysed and performed dual luciferase assay by using the dual luciferase assay kit (Promega) according to the manufacturer's protocol. Results are presented as a relative value calculated by fold increase over control after normalizing ratios of firefly luciferase/renilla luciferase and averaging triplicate samples.
20. *Western blot analysis for GSK-3β and β-catenin expression:* HEK293 cells (1.2×10^5 cells in a 60 mm dish) were seeded and incubated for 24 h, and then replaced with fresh media containing test compounds or vehicle (0.1% DMSO). After incubation for an additional 24 h, cells were harvested and denatured with boiling 2× sample loading buffer. Proteins (40 μg) were electrophoresesed on SDS-polyacrylamide gel and electrotransfered to PVDF membrane. The membranes were blocked with blocking buffer [5% skimmed milk in phosphate buffered saline with 0.1% Tween-20 (PBST)] for 1 h at room temperature. Then membranes were incubated with specific primary antibodies for 3 h and washed three times with PBST. After washing, membranes were incubated with the corresponding secondary HRP-conjugated antibodies. The amount of protein is determined with West Save® HRP-chemiluminescent detection kit (Lab Frontier, Seoul, Korea) by LAS-3000 Imager. β-Actin was used as an internal standard.