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Identification of 2,3,6-trisubstituted quinoxaline derivatives as a Wnt2/β-catenin pathway inhibitor in non-small-cell lung cancer cell lines

Sang-Bum Lee [a], Young In Park [a], Mi-Sook Dong [a,*], Young-Dae Gong [b,*]

[a] Graduate School of Biotechnology, Korea University, Seoul 136701, Republic of Korea
[b] Department of Chemistry, Dongguk-University-Seoul, Pil-dong 3-ga, Jung-gu, Seoul 100715, Republic of Korea

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ABSTRACT

We screened 1434 small heterocyclic molecules and identified thirteen 2,3,6-trisubstituted quinoxaline derivatives that were able to inhibit the Wnt/β-catenin signal pathway and cell proliferation. In the screen, some of the hit compounds such as the ethylene group-coupled quinoxaline derivatives were shown to hold promise for use as potential small-molecule inhibitors of the Wnt/β-catenin signal pathway in non-small-cell lung cancer cell lines.

Wnt proteins constitute a family of highly conserved secreted glycoproteins that play multiple roles in development and the progression of diseases. Wnt signaling is one of the key signaling pathways that regulate cell proliferation, differentiation, and morphogenesis.[1,2] Aberrant activation of the canonical Wnt signaling pathway is related to many human cancers including colon carcinoma and melanoma.[3,4] Up to now, 19 different Wnt proteins and 10 different Frizzled receptors have been identified in the mouse and human.

Wnt signaling is initiated by the secreted Wnt ligands. Wnt ligands bind to a class of seven-pass transmembrane receptors encoded by the frizzled genes and LRP5/6 coreceptors. Activation of the receptor leads to the phosphorylation of the dishevelled protein and through its association with axin, prevents glycogen synthase kinase 3 beta (GSK3β) from phosphorylating crucial substrates. The GSK3β substrates include the negative regulators axin and APC, as well as β-catenin itself. Unphosphorylated β-catenin avoids recognition by β-TrCP, a component of an E3 ubiquitin ligase, and translocates to the nucleus where it interacts with transcription factors such as TCF, LEF, Pygo and Bcl-9.[5–7]

Lung cancer is the leading cause of cancer-related mortality worldwide. Studies have shown that APC or β-catenin mutations lead to the accumulation of nuclear β-catenin, which is associated with greater than 80% of familial colorectal cancers. However, these mutations are found rarely in lung cancers. Although extensive research on the Wnt pathway and cancer has been performed in colon tumors, more recent work has demonstrated that the Wnt pathway may play a significant role in lung cancer.[8] Several Wnt proteins have been differentially expressed in non-small-cell lung cancers (NSCLC), including Wnt 1, -2 and -7a.[9]

The mRNA of human Wnt2 is highly expressed in fetal lung and weakly expressed in the placenta.[10] Inappropriate activation of the Wnt2/β-catenin pathway has been reported in many human cancers including colorectal, gastric, breast, and cervical cancers.[10–13] You[14] demonstrated that the Wnt2 protein was overexpressed in freshly resected human non-small-cell lung cancer (NSCLC) tissues and inhibition of Wnt2-mediated signaling by siRNA or a monoclonal antibody induced programmed cell death in the NSCLC cell line A549. Moreover, the anti-Wnt2 antibody was shown to inhibit cell growth of primary cultures obtained from patients suffering from NSCLC.[14]

The importance of Wnt2 in the mediation of normal and pathological processes has motivated considerable efforts to identify β-catenin inhibitors. Although a wealth of inhibitory compounds are available, the generation of β-catenin inhibitors with selectivity toward individual Wnt isoforms has proven to be challenging. In particular, drug-like small organic molecule Wnt inhibitors are rare.[15] Here, we report initial evidence showing that 2,3,6-trisubstituted quinoxaline derivatives[16] are highly active β-catenin/Wnt pathway inhibitors and hold promise for treating NSCLC.

To identify small-molecule inhibitors of β-catenin/Wnt pathway, we screened 1434 compounds using a cell proliferation assay to select the first hits and then checked the transcriptional activity

* Corresponding authors. Tel.: +82 2 3290 4146; fax: +82 2 3290 3951 (M.-S.D); tel.: +82 2 2260 3206; fax: +82 2 2268 8204 (Y.-D.G.).
E-mail addresses: msdong@korea.ac.kr (M.-S. Dong), ydgong@dongguk.edu (Y.-D. Gong).

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of the first hits using cell-based reporter gene assay that measures the transcriptional activity of β-catenin-TCF/LEF in A549/Wnt2 cells. β-catenin-TCF/LEF transcriptional activity was dependent on Wnt signaling and could be used to monitor the activity of the Wnt/β-catenin signaling pathway. The 1434 compounds that were screened represent 20,000 structurally diverse and druggable, heterocyclic compound libraries, including benzopyrans,[17a] oxazoles,[17b] pyrazoles,[17c] oxadiazoles,[17d] thiadiazoles,[17d] various thiazoles,[17e,17f] and pyrimidinediones.[17g] Calphostin C, a non-specific Wnt/β-catenin pathway inhibitor, was used in the assay as a positive control. Inhibition of cell proliferation of A549/Wnt2 cells by the 1434 compounds were screened at a compound concentration of 5 μM and compounds that reproducibly inhibited growth by over 50% were selected.[18] In the first round of screening, 28 compounds were shown to reproducibly have a IC_{50} below 5 μM.

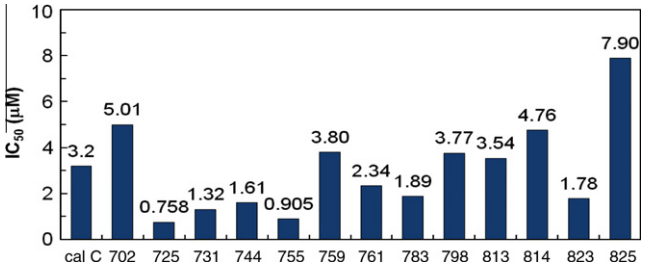

Figure 2. IC_{50} values of the 13 compounds selected from the 1st screen using the cell proliferation assay with A439 cells. Cal C, calphostin C, the positive control.

Among them, 13 compounds that had a 2,3,4-trisubstituted quinoxaline structure were selected (Fig. 1) for further screening. De-

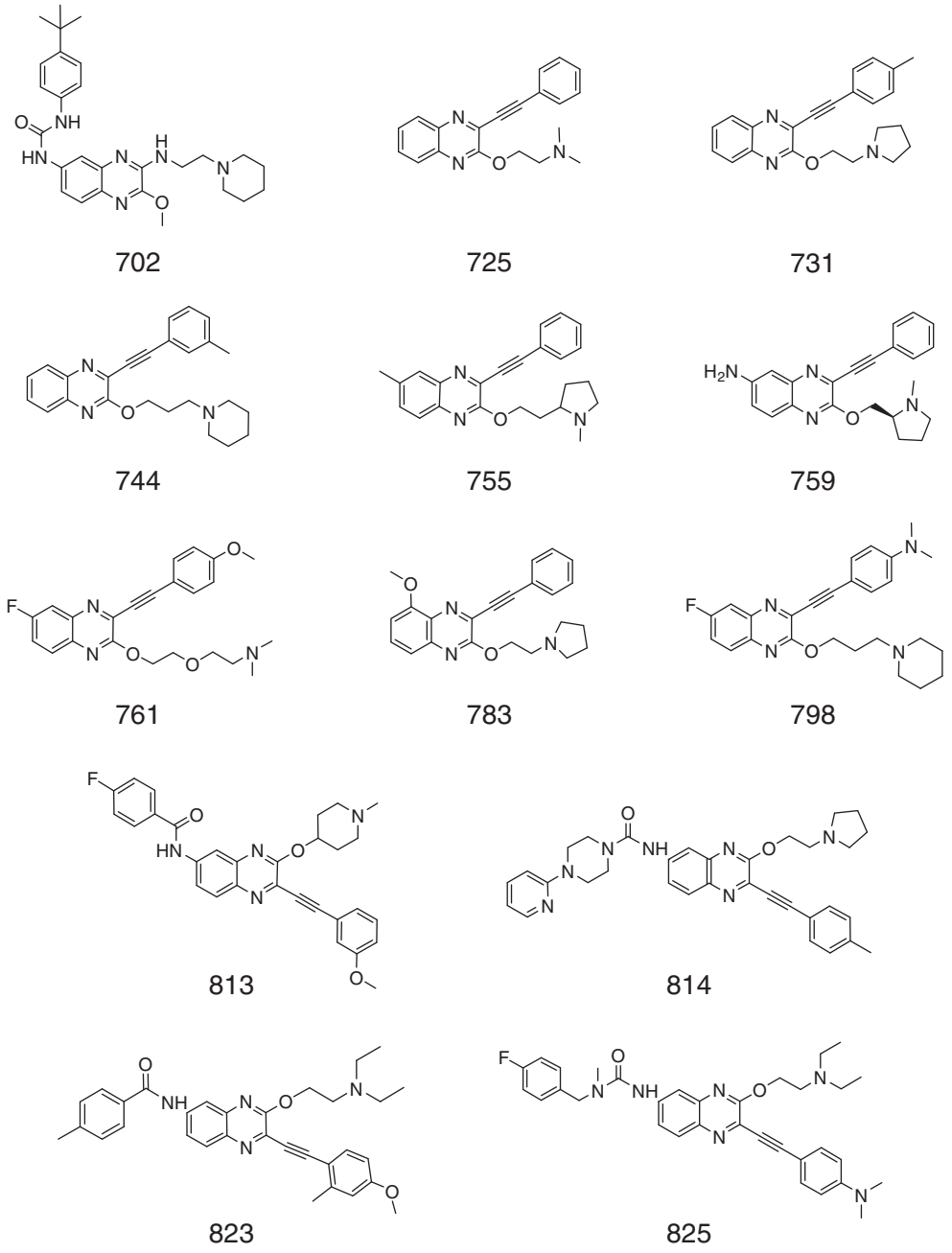

Figure 1. Structure of the Wnt2/β-catenin pathway inhibitors identified in the primary screen.

spite their structural similarity to the quinoxaline core scaffold, the IC_{50} values significantly varied (Fig. 2).

And then we also confirmed that the cell growth inhibition of selected 13 compounds with low concentration, 15% and 30% of IC_{50} (Fig. 3). Cell growth was carried out at 24 and 48 h after treatment by using MTS assay system. These compounds were significantly inhibited the cell proliferation without cytotoxicity at 15% and 30% of IC_{50} (data not shown). Compounds **755**, **761**, **783** and **814** were shown the impediment of cell proliferation in a dose dependent manner.

Next, we examined the inhibitory effect of the 13 derivatives of 2,3,4-trisubstituted quinoxaline, which were chosen from the first

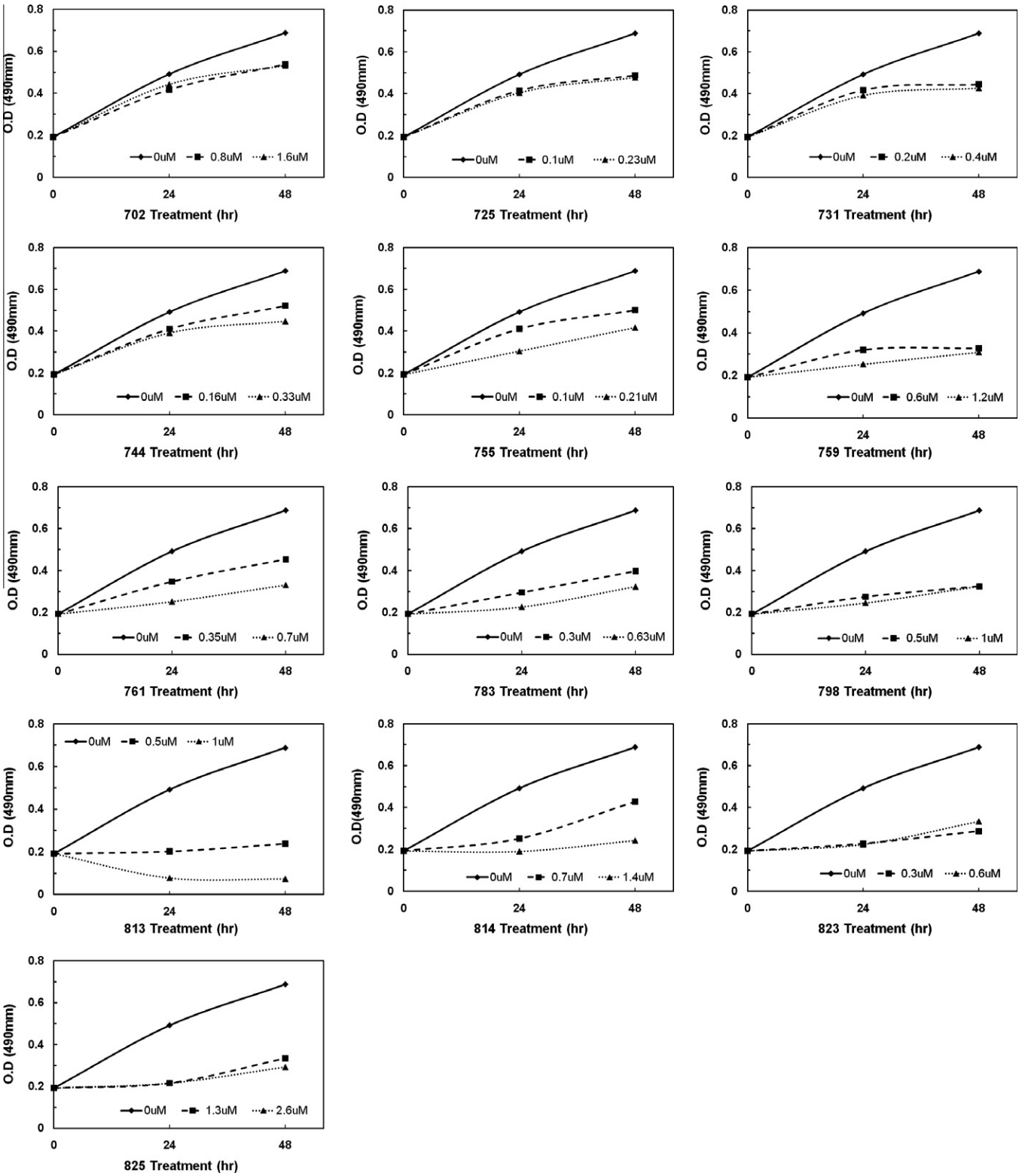

Figure 3. The effect of compounds chosen from the first screen on the cell proliferation at the 15% and 30% of IC_{50} in A549/Wnt2 cells.

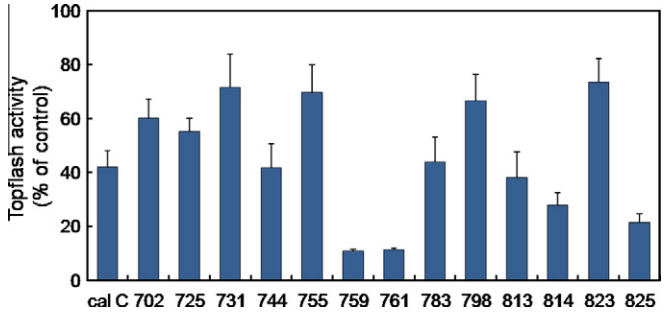

Figure 4. The effect of compounds chosen from the first screen on the Tcf/β-catenin transcriptional activity at the IC_{50} of cell proliferation in A549 cells. Cal C, calphostin C, the positive control.

screen, on the Wnt/β-catenin signal pathway at the IC_{50} determined above. For the Topflash assay, A549/Wnt2 cells were transiently transfected with pSuperTopflash and treated with the 13 compounds at IC_{50} (Fig. 4).[19]

pFopflash, which has eight mutated TCF/LEF-binding sites and has no response to the Wnt/β-catenin signal pathway, was used as a negative control. As shown in Figure 3, the 13 compounds inhibited the reporter gene activity with a reduction of approximately 30–90% compared to the non-treated cells without significantly affecting the mutant reporter Fopflash. Although three compounds (**759**, **761** and **825**) among them dramatically decreased Topflash reporter activity about below 20%, we could not figure the relationship between the inhibition of Wnt/β-catenin signal pathway and the structure of side chain.

The regulation of target genes in Wnt/β-catenin pathway is dependent on the level of β-catenin and nuclear β-catenin is the hallmark of activated Wnt signaling. To compare the inhibition of Wnt/β-catenin pathway with the change of β-catenin levels by these 13 compounds, the effect of the 13 compounds on the β-catenin protein level in whole cells, cytosol and nuclear fractions was evaluated in A549/Wnt2 cells using an immunoblot assay (Fig. 4).[20] Although the 13 compounds had structural similarity to the quinoxaline core scaffold, the levels of β-catenin in whole cells, cytosol, and nuclear fractions were diversely changed. The activity of Wnt/β-catenin pathway was proportional to the level of nuclear β-catenin. Compounds **759**, **761** and **825** inhibited the

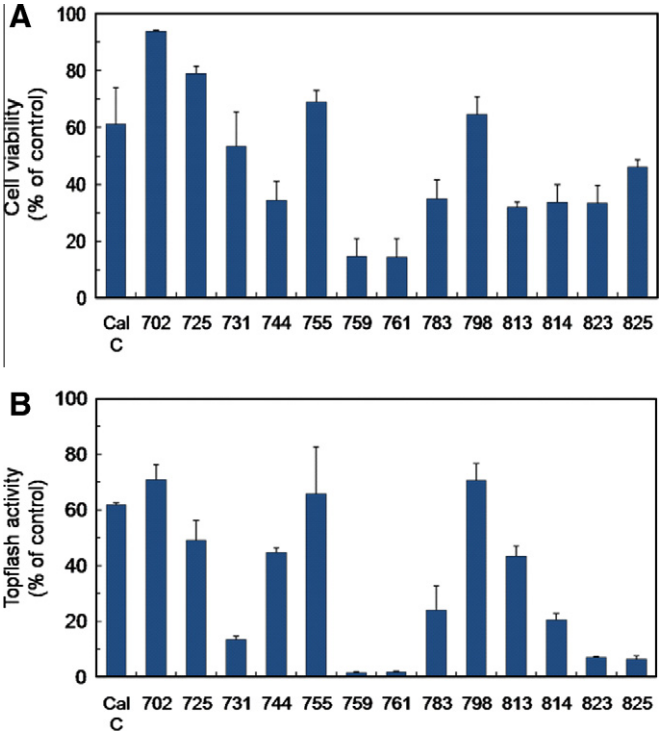

Figure 6. Effect of the 13 compounds on inhibiting cell proliferation and Tcf/β-catenin transcriptional activity at the IC_{50} of A549 cell proliferation. (A) Inhibition of cell proliferation in H460 cells treated with the 13 compounds using the MTS assay. (B) Topflash assay of the Tcf/β-catenin dependent transcriptional activity in H460/Wnt2 cells.

activity of Topflash blow 20% (Fig. 5) and reduced significantly the nuclear β-catenin level compared with control.

Finally, the inhibitory effect of the 13 compounds at IC_{50} in H460, a non-small-lung cancer cell line, was confirmed. The results, shown in Figure 6A, were confirmed using the MTS assay: treatment of the cells with the 13 compounds for 24 h significantly decreased cell proliferation, as was observed in the A549/Wnt2 cells. To test whether the 13 compounds at the IC_{50} concentration reduced the β-catenin/Tcf dependent transcriptional activity in

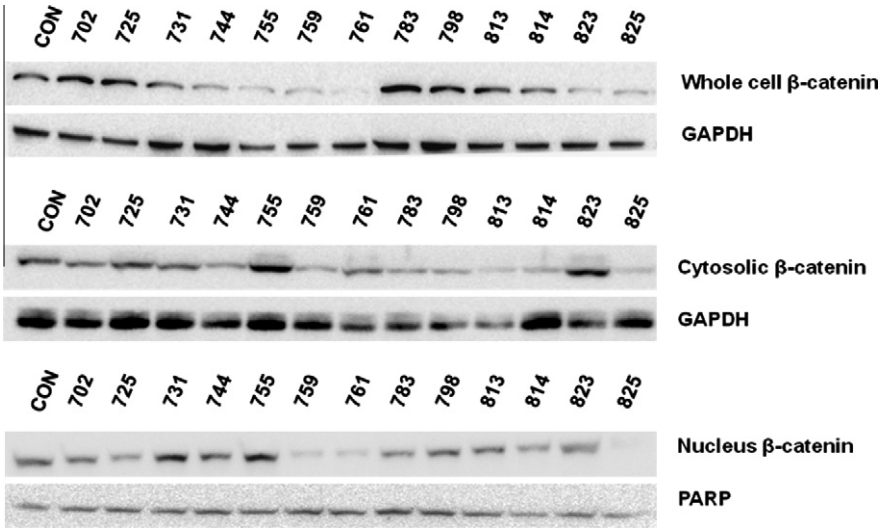

Figure 5. β-Catenine protein levels in whole cells, cytosolic and nuclear fraction prepared from the A549/Wnt2 cells treated with the 13 first 'hit' compounds at the IC_{50} of cell proliferation.

H460/Wnt2, the cells were transiently transfected with SuperTopflash and treated with the 13 compounds at the IC_{50}, as described in the materials and methods. As illustrated in Figure 6B, these compounds decreased the β-catenin/Tcf dependent transcriptional activity in H460/Wnt2. Compounds **759** and **761** significantly decreased the Tcf/β-catenin dependent transcriptional activity by more than 80% in both cell lines, A549/Wnt2 and H460/Wnt2.

In summary, we screened 1434 small heterocyclic molecules and identified 13 number of the 2,3,6-trisubstituted quinoxaline derivatives that were able to inhibit the Wnt/β-catenin signal pathway and cell proliferation.

Among the 13 number of quinoxaline derivatives, some compounds, including compounds **759**, **761** and **825**, hold promise for use as potential small-molecule inhibitors of the Wnt/β-catenin signal pathway. Further studies are currently underway to optimize the potency and selectivity of the 2,3,6-trisubstituted quinoxaline derivatives and address their in vivo efficacy and therapeutic potential including the selectivity to cancer cells. These molecules may serve as useful mechanistic probes of the cellular function of the Wnt/β-catenin signal pathway and anticancer mechanism.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.088.

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18. Cell proliferation assay: Cells were seeded in 96-well plates (5×10^3 cells/well) and allowed to attach for about 24 h. The MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) was used to measure the inhibition effect of the chemical library on cell proliferation. Cells were seeded onto 96-well microplates and treated for 24 h with test the compounds at various concentrations. Cell proliferation was determined using a CellTiter 96 non-radioactive cell proliferation assay kit according to the manufacturer's protocol (Promega, USA). The plate was incubated at 37 °C in a CO_2 incubator for 30 min and the absorbance was measured on a molecular dynamic plate reader (Bio-Rad, Germany) at 490 nm.
19. Luciferase reporter gene assay: Cells were transfected with the luciferase reporter constructs pSuperTopflash containing eight Tcf consensus binding sites upstream of the firefly luciferase cDNA, or pSuperFopflash, a plasmid containing mutated Tcf binding sites (kindly provided by professor Ja-Hyun Baek at the School of Life Sciences & Biotechnology at Korea University, Seoul, Korea). Cells (4×10^4) were transfected using the Transfast transfection reagent (Promega, USA) with 1 μg reporter construct and 15 ng pRL-TK plasmid and incubated with various concentrations of selected compounds at 37 °C. After 24 h, the cells were lysed in 50 μl passive lysis buffer (Promega, USA). Firefly luciferase and Renilla luciferase activity were determined using the Dual-Glo Luciferase Assay System (promega, USA). Results are expressed as the mean ± SEM of normalized ratios of firefly luciferase activity and renilla luciferase activity measurements for each triplicate set.
20. Western blot analysis of β-catenin expression: Whole cell lysates were generated by scraping cells into an ice-cold RIPA buffer 150 mM NaCl, 1% NP_40, 0.25% sodium deoxycholate, 50 mM Tris–HCl (pH 7.4), 1 mM NaF, 1 mM Na_3OV_4 supplemented with a complete protease inhibitor cocktail (Roche, USA). The protein content was determined using the Bradford assay (Bio-Rad, Germany). Protein samples (20–30 μg of protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrile cellulose membranes (Whatman, Germany) for western blotting. The primary antibodies used were β-catenin and GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA). Immunoreactive proteins were visualized with ECL (Amersham Biosciences, USA) according to the manufacturer's protocol. The densities of the bands were quantitatively analyzed using the TINA 2.0 software (Raytest, Straubenhardt, Germany).