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RESEARCH ARTICLE

NOVEL NAPHTHOQUINONE ANALOGS THAT TARGET Wnt/β-Catenin/TCF4 SIGNALING PATHWAY AND CLONOGENIC ACTIVITY OF COLON CARCINOMA CELLS

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ABSTRACT

The Wnt/β-catenin signaling pathway plays a critical role in cancer development and progression. Our extensive efforts have identified and developed novel small molecule inhibitors that can fight colon cancers caused by aberrant Wnt/β-catenin signaling. A series of naphthoquinone derivatives were designed, synthesized and evaluated using a combination of comprehensive biological and quantitative structure-activity-relationship (QSAR) analysis approaches, which revealed a new class of inhibitors of Wnt/β-catenin/Tcf signaling. In this class, compound 27 (named BC27) was the lead compound. At nanomolar to low micromolar concentrations, BC27 strongly inhibited the colony-forming activity of the β-catenin-overexpressing colon cancer carcinomacells caused by gene mutations of β-catenin and APC and reduced TOP-luciferase activity. This paper presents the chemical synthesis, biological activity and a comparative molecular field analysis (CoMFA) of this new class of compounds to introduce their preliminary structure–activity relationships (SARs).

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INTRODUCTION

Drug resistance and recurrence are two major obstacles that hinder the successful treatment of human cancers. This resistance and recurrence can be caused by escape of cancer stem cells (CSCs) from treatment and by accumulation of genetic mutations. (Reya *et al*., 2001; Li and Neaves, 2006; Sell, 2006; Dawood *et al*., 2014; Wahab *et al*., 2017) Targeted disruption of CSC self-renewal represents a novel therapeutic strategy that is worth developing. The β-catenin dependent Wnt signaling pathway is a promising therapeutic target because β-catenin overexpression caused by the mutations of itself or its upstream proteins in this pathwayplays crucial roles in CSC renewal as well as in tumorigenesis, cancer development, and treatment responses. (Anastas *et al*., 2013; Polakis, 2000; Behrens *et al*., 1998) For example, about 90% of colorectal cancers harbor mutant APC proteins that have lost their ability to down-regulate β-catenin. Additionally, about 50% of colon cancers contain activating β-catenin mutations at putative GSK-3β phosphorylation sites, which render the β-catenin protein resistant to proteasome-mediated destruction. (Lee *et al*., 2006) Targeted over-activation or over-expression of the active form β-catenin can lead to tumorigenesis in transgenic mice and block malignant cell differentiation. (Trowbridge *et al*., 2006) Therefore, targeting of self-renewal, the key 'stemness' property unique to CSCs, may represent a new paradigm in cancer therapy; manipulation of the β-catenin mediated Wnt signaling pathway may be critical for achieving CSC eradication. Accumulating evidence supportsthe validity and feasibility of inhibiting β-catenin function by targeting the region of its surface that binds to the TCF (T-cell factor) family of DNA-binding proteins. (Graham *et al*., 2000; Tian *et al*., 2012) High-throughput screening (HTS) studies performed over the past decade have identified several small-molecule inhibitors of the Wnt/β-catenin signaling pathway. (Anastas *et*

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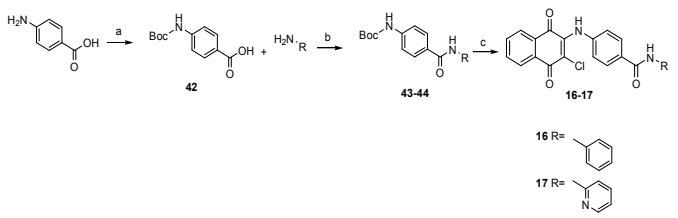
al., 2013; Lepourcelet *et al*., 2004; Wei *et al*., 2010; Handeli *et al*., 2008; Saraswati *et al*., 2010; Watanabe *et al*., 2011; Lepourcelet *et al*., 2004; Barker and Clevers, 2006; Lu *et al*., 2004) Most of these compounds are naturally occurring materials but their complex structures render them unsuitable for use as lead compounds. (Dehnhardt *et al*., 2010; Chen *et al*., 2009) Nevertheless, they point to the possibility that the simplified naphthoquinone mother core might possess inhibitory activity against Wnt signaling mediated by β-catenin/TCF-4. Interestingly, celecoxib, a US FDA approved COX-2 inhibitor NSAID drug for treating colon cancers contains benzenesulfonamide and also inhibits Wnt/β-catenin/TCF signaling. Our recent *in silico* virtual screening study identified a potent β-catenin/TCF inhibitor, BC21 ($C_{21}H_{14}ClN_3O_4S$), which contains both naphthoquinoneand benzenesulfonamidescaffolds/moieties. We demonstrated that this small molecule strongly inhibited β-catenin/TCF4 binding and significantly sensitized non-small cell lung cancer (NSCLC) cells to x-ray radiation. (Zhang *et al*., 2016) This compound has a good drug-likeness and represents an excellent lead compound for further development.

In the present study, we have designed, synthesized, and collected a series of naphthoquinone analogs. Four compounds that share similar naphthoquinoneand/or benzenesulfonamide structures showed promising potency in terms of inhibiting β-catenin/TCF signaling and supressing the clonogenic growth of colon cancer carcinoma cells that overexpress β-catenin and show Wnt/β-catenin signaling pathwayoveractivation. This new class of small molecule inhibitors of β-catenin/TCF4 signaling represent valuable chemical probes and lead candidates for the development of new β-catenin-targeted anticancer therapeutics.

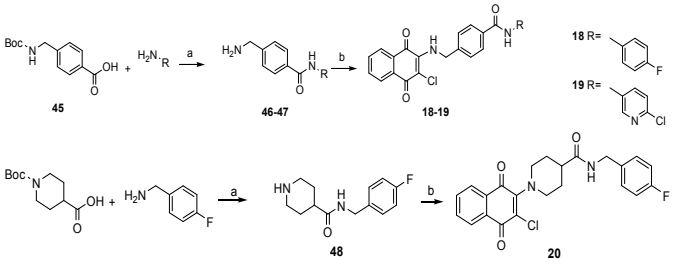
RESULTS AND DISCUSSION

Design and synthesis of new analogs of naphthoquinone

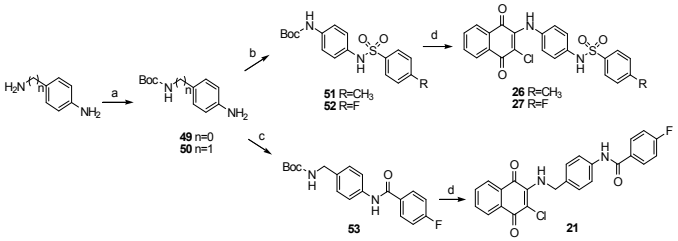
Different naphthoquinone analogs were synthesized and designed by altering substituents on C-2 and C-3 of the naphthoquinone ring (R_1 and R_2, Schemes 1-3, Table 1). Compounds 2-8, 10-15, and 22-25 were synthesized from 2,3-dichloronaphthoquinone (Dichlon, 1) and commercially available amines, and compounds 29 and 30 were prepared by the same method from 3-bromonaphthoquinone (28). (Lawrence *et al*., 2010) The ester 9 was methyl esterified from carboxylicacid8 using sulfuric acid and methanol. The preparation of analogs 16 and 17 is shown in Scheme 1. The intermediates 43-44 were obtained by reacting *N*-Boc-4-aminobenzoic acid 42 with aromatic amines using HBTU/Et_3N. Subsequently, intermediates 43-44 were deprotected and reacted with 1 to give 2-position aminated products 16-17. Compounds 18-20 were prepared by similar methods to those used for preparation of compounds 16 and 17 (Scheme 2). Compounds 21, 26, and 27 were obtained as depicted in Scheme 3, by reacting substituted benzenesulfonyl chloride or benzoyl chloride with intermediates 49 and 50. Compounds 31-41 (Table 1) were obtained from the National Cancer Institute/Development Therapeutic Program (NCI/DTP) open chemical repository using similarity search tools, based on the structure of the lead compound that we identified. (Zhang *et al*., 2016) The NCI codes for these compounds were shown in Experimental Procedures section (4.1.30).



Scheme 1. Reagents and conditions: a. Et_3N, $(Boc)_2O$, dioxane, H_2O, r.t. (room temperature), 24 h; b. HBTU, Et_3N, CH_2Cl_2, r.t., 18 h; c. (i) 50% TFA, CH_2Cl_2, r.t., 4 h; (ii) **1**, Et_3N, 1-butanol, 90 °C, 48 h.



Scheme 2. Reagents and conditions: a. (i) HOBt, DIC, DIEA, CH_2Cl_2, r.t., 14 h; (ii) 50 % TFA, CH_2Cl_2, r.t., 4 h; (iii) 20 % NaOH, CH_2Cl_2, H_2O, 0 °C; b. **1**, Et_3N, 1-butanol, r.t., 48 h.

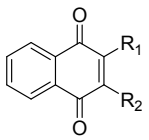


Scheme 3. Reagents and conditions: a. K_2CO_3, $(Boc)_2O$, THF, DMF, r.t., 24 h; b. sulfonyl chloride, Et_3N, CH_2Cl_2, 0 °C,3 h; c. 4-fluorobenzoylchloride, Et_3N, CH_2Cl_2, 0 °C,3 h; d. (i) 50% TFA, CH_2Cl_2, r.t., 4 h; (ii) Et_3N, **1**, EtOH, r.t., 48 h.

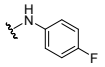
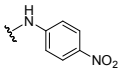
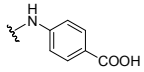
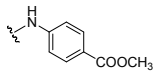
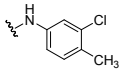
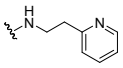
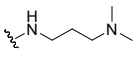
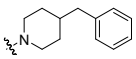
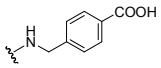
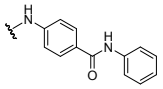
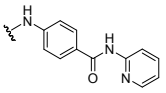
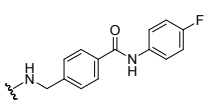
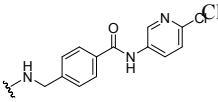
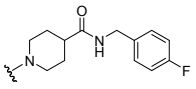
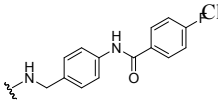
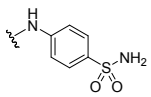
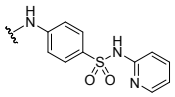
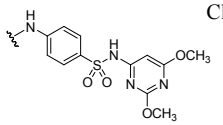
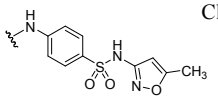
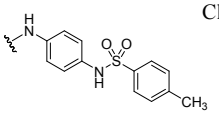
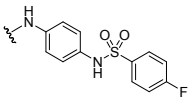
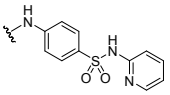
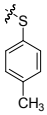
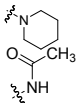
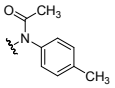
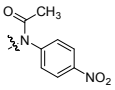
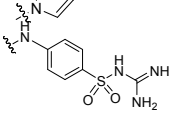
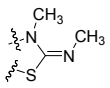
Identification of highly potent compounds that inhibit the growth of colon carcinoma cells

The inhibitory properties of compounds were evaluated using HCT116 (wild-type APC and ΔSer45 β-catenin mutation) and SW480 (APC truncation at position 1338) colon carcinoma cell lines. Table 1 summarizes the structures and percentage of cell growth inhibition of all 41 compounds at 5 μM.

Table 1. Chemical structure of compounds 1-41 and their cell-death inducing activity



No.	R_1	R_2	Cell growth inhibition % [a]	
			SW480 (5 μM)	HCT116 (5 μM)
1	Cl	Cl	NA	14.9
2	NH_2	Cl	49.5	46.9
3	$NHCH_3$	Cl	2.9	14.7
4	$N(CH_3)_2$	Cl	84.5	64.8

5		Cl	96.7	74.0
6		Cl	80.6	17.9
7		Cl	81	45.5
8		Cl	33.6	11.9
9		Cl	98.7	63.8
10		Cl	85	49.2
11		Cl	36.8	4.0
12		Cl	34.4	46.9
13		Cl	NA	28.8
14		Cl	NA	6.4
15		Cl	NA	NA
16		Cl	16.5	30.4
17		Cl	NA	24.4
18		Cl	10.2	27.2
19		Cl	NA	24.7
20		Cl	NA	9.5
21		Cl	NA	29.2
22		Cl	34.8	42.1
23		Cl	99.5	88.8
24		Cl	99.2	32.6
25		Cl	99.6	14.88
26		Cl	99.5	40.4
27		Cl	98.4	63.7
28	Br	H	NA	17.4
29	$N(CH_3)_2$	H	NA	15.6
30		H	NA	49.9
31		Cl	95	37.5
32		Cl	52.3	57.5
33	$N(CH_3)_2$		5.4	45.6
34			0	22.8
35			54.1	75.7
36		H	10.8	16.1
37		Cl	17.9	63.4
38			NA	20.0
39		Cl	NA	ND
40		Cl	NA	ND
41			76.1	76.1

[a] Results are the mean value of at least three independent experimets.NA: no activity detected. ND: not determined.

For the different 2-amino substituents on the naphthoquinone core, the 2-aromatic amines 5-7, 9, 10 exhibited higher inhibition than did 2-aliphatic amines 3, 11-15, and 18-21. However, among these 2-aromatic amines, the modification of *para*-benzoic acid ester 9 to *para*-benzoic acid 8 and *para*-benzoyl amide 16, 17 resulted in a decrease in activity. Compounds 23-27, containing a 3-chloro group and a substituted sulfonamide part at the *para*-position of the 2-aminophenyl, were the most potent analogs. However, the activity of free sulfonamide derivative 22 and sulfonyl guanidine 40 were lower than that of the phenyl substituted sulfonamides. Retaining the sulfonamide part but removing the 3-chloro also led to a decrease in inhibition. The 3-Chloro derivatives 4 and 23 were more potent than 3-hydro compounds 29 and 30, respectively. These results indicated that 3-chloro substituents on the naphthoquinone core were preferred for the activity. In addition, the aliphatic tertiary amine at 2-position (compound 4) was exhibited higher activity than the corresponding secondary (compound 3) and

primary (compound 2) ones. Compounds 31-41 were obtained from the NCI/DTP open chemicals repository in order to enhance the diversity of structures based on fundamental relationships of cell viability and the structures of compounds 1-30. Analogs 31, 32, and 35, possessing an aliphatic tertiary amine at the 2-position, showed higher inhibition. However, substitution of 2-aromatic amines for 2-amide derivatives (36-38) resulted in decreased inhibition. The activity also decreased if the 3-chloro was changed into a 3-*p*-methlyphenylsulfenyl group (*e.g.,*4*vs*33). These features were consistent with above results.

All the compounds with better inhibition (>50 % at 5 μM) were subsequently tested for their IC_{50} values (Table 2). It is worth noting that the most potent inhibitors share three structure types, which are sulfonamides (23-27), aliphatic tertiary amines (4, 31, 32, 34, and 35), and substituted anilines (5-7, 9, and 10). All the sulfonamide derivatives (23-27) exhibited better activity. Compounds 23 and 27 showed lower IC_{50} values (< 2 μM) for both HCT 116 and SW 480 cell growth inhibition and compounds 24 and 25 exhibited selective inhibition for the SW 480 cell line.

Table 2. Cancer cell growth inhibition by selected compounds

Compound	Cell growth inhibition (IC_{50}, μM)[a]	
	SW480	HCT116
4	3.5	3.0
5	1.2	3.7
6	3.0	>10
7	2.4	4.1
9	>10	3.9
10	1.5	3.9
23	1.0	1.9
24	1.7	4.9
25	1.7	5.1
26	1.8	5.2
27	1.5	1.5
31	7.4	2.3
32	9.8	5.9
34	>10	6.3
35	4.2	2.5
37	>10	3.6
41	3.8	1.9

[a] Results are the mean value of at least three independent experiments.

Table 3. Clonogenic cell growth inhibition by representative compounds

Compound	SW480 (IC_{50}, μM)[a]	HCT116 (IC_{50}, μM)[a]
23	0.35	0.45
24	1.37	2.89
25	1.78	3.97
27	0.34	0.35

[a] Results are the mean value of at least three independent experiments.

Clonogenic evaluation of lead representative compounds that inhibit the colony-forming activity of β-Catenin overexpressing colon carcinoma cells

We sought to determine whether these lead inhibitors cause clonogenic cell death for theβ-Catenin overexpressing SW480 and HCT116 cell lines. The results showed that four representative compounds (23-25 and 27) significantly blocked the colony forming activity in a dose-dependent manner (Figure 1, Table 3). Of these compounds, two sulfonamide derives 23 and 27 were the most potent compounds. The IC_{50} values of compounds 23 and 27 were 350 versus 340 nM (SW480 cells), and 450 versus 350 nM (HCT116 cells), respectively (Figure 1, Table 3). The colony forming activity of SW480 (as well as HCT 116) cells was completely inhibited when the concentrations of compounds were ≥1.25 μM.

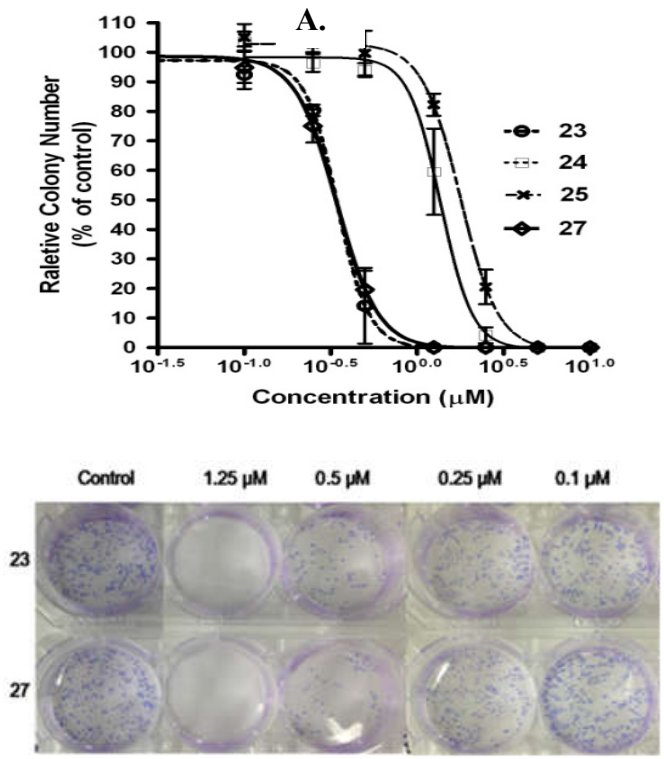


Figure 1. Effects of compounds on SW480 colony forming activity. (A) Dose-response inhibitory curves of four compounds on SW480 colony forming activity. The SW480 cells were treated with a range of concentrations of four potent compounds and incubated for 7 days at 37 °C. Colonies consisting of more than 50 cells were scored. Relative colony number was calculated as [(colony number) $_{treatment}$/(colony number) $_{control}$] × 100%. Results are the average of at least three independent experiments. (B) Effects of the most potent compounds 23 (upper panels) and 27 (lower panels) on SW480 colongenicity. Two compounds reduced colony forming in a dose-dependent manner. The colony forming activity of SW480 cells was inhibited completely at the concentrations ≥ 1.25 μM

Biological and molecular characterization of representative compounds that target the β-Catenin/TCF transcriptional signaling

We saw growth inhibitory activity of the new lead analogs of naphthoquinone in SW480 and HCT116 cells; therefore, we chose both cell lines to investigate the possible modulation of β-catenin-dependent TCF transcriptional activity by these lead inhibitors. The SW480 (APC mutation) and HCT116 (ΔSer45 β-catenin mutation) cell lines harbor upregulated β-catenin expression and function, are good representatives for the majority of human colorectal adenomas. Four potent compounds 23-25 and 27 were assayed for their inhibition of luciferase activities in both SW480 and HCT116 cell lines (see Experimental Procedures). The results are summarized in Figure 2 and Table 4. These results, together with cell survival data, indicated that BC27 could interrupt the β-catenin/TCF-mediated signaling pathway, which led to SW480 and HCT116 cell death, and particularly clonogenic cell death. SW480 and HCT116 cells were transfected with a reporter gene (luciferase) harboring the TCF/LEF binding site (TOP)or a mutant TCF/LEF binding site (FOP). Cell viabilities were

surveyed at the same time point, and combined with luciferase activities in order to rule out nonspecific cytotoxicity. A range of concentrations of four potent compounds were added to transfected cells and then the cells were assayed for luciferase activity. Figure 1 showed that four compounds inhibited TCF activity in SW480 cells in a concentration-dependent manner. The sulfonamide BC27 exhibited the most potent inhibition. The IC_{50} values were 2.1 and 2.5 μM for SW480 and HCT116, respectively (**Table 4**).

Table 4. Anti-β-catenin/TCF transcriptional signaling activity of representative compounds

Compound	SW480 (IC_{50}, μM)[a]	HCT116 (IC_{50}, μM)[a]
23	1.7	2.2
24	2.3	3.3
25	2.7	4.3
27	2.1	2.5

[a] Results are the mean value of at least three independent experiments.

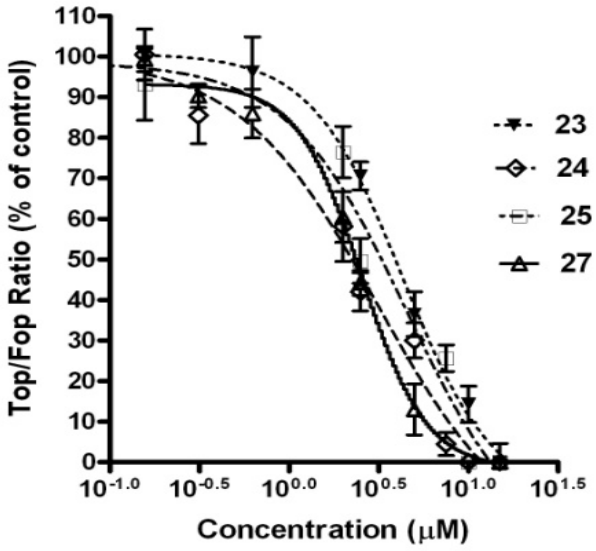


Figure 2. Dose-dependent inhibitory curves of four compounds on the TCF-responsive luciferase activity. HCT116 cells were transfected with a reporter gene (luciferase) harboring the TCF/LEF binding site (TOP) or a mutant TCF/LEF binding site (FOP). Results are the average of three independent experiments performed in duplicate

CoMFA Analysis of compounds

The presentCoMFA model was built by adding 19 compounds, including compounds 4, 6, 7, 9-12, 15, 18, 19, 23, 24, 27, 29-32, 35, and 38. The details of the calculation methods were described in the experimental section. The 19 selected compounds were superimposed with the 1, 4-naphthoquinone core. After the molecule alignment, a cross-validation (Leave-One-Out) analysis was performed to determine the number of optimal components and to evaluate the predictive ability of the model which was measured by r_{cv}^2. In our models, the highest r_{cv}^2 =0.634and the proper component was 5. After that, a non-cross-validated partial least squared (PLS) analysis was performed and an r^2 of 0.974, SD of 0.149 and $F_{5,\ 13}$ of 97.131were obtained, which indicated that the model has a good predictive ability. The CoMFA contour map (A) and superimposition of 19 candidate compounds (B) are shown in Figure 3.

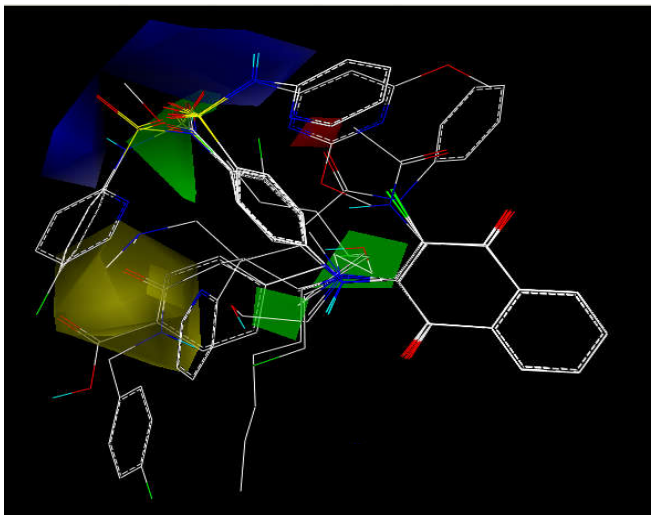


Figure 3. The CoMFA contour map of 19 selected compounds

The biological activity and CoMFA analysis present the preliminary SARs, as shown in Table 1-3 and Figure 3. (1) Steric contours show the main region of favored (green) contribution to the activity that surrounds 2-amines (*e.g.* compounds 23, 24, 25, and 27). (2) The unfavorable (yellow) region of CoMFA map indicated steric bulks result in decreasing activity (*e.g.* compounds 11 and 12). (3) The Blue contour (electropositive substituent favored) is distributed surrounding sulfamines. The activity could be enhanced if sulfonamide were substituted by aromatic rings carrying π electrons (*e.g.* 22*vs.*23-25). (4) The 3-chloro group of naphthoquinone core was preferred for the inhibition. 3-Hydro substituent led to loss of activity (*e.g.* 27*vs.* 30).

Conclusion

A series of naphthoquinone derivatives were designed and evaluated as inhibitors of the β-catenin/TCF4 signaling pathway and of clonogenicity of colon carcinoma cells. Among these compounds, BC27, which has naphthoquinone, sulfonamide, and 2-chloro structure, was the most potent. It specifically reduced the TOP-luciferase reporter gene activity of the Wnt/β-catenin signaling pathway and strongly inhibited the colony-forming activity of β-catenin over expressing HCT116 and SW480 colon carcinoma cell lines (with IC_{50} values of 350 and 340 nM, respectively). These results, together with our QSAR analyses, indicate that the aromatic substituted sulfonamide/aminosulfonyl and chlorine moieties were essential for anti-β-catenin and anticancer activity. The lead BC27has potential for further development of a new class of anticancer therapeutics that target renewable CSCs.

Experimental Procedures

Chemistry

All reagents and solvents were purchased from commercially suppliers and used without further purification. ^{1}H NMR spectra were recorded with a Bruker-BioSpin 300 MHz or 600 MHz spectrometer with $CDCl_3$-d_6 or DMSO-d_6 as the solvent. ^{13}C NMR spectra were recorded at 75 MHz or 125 MHz. All chemical shifts (δ_H and δ_C) were reported in parts per million (ppm) and the coupling constants were measured in hertz (Hz). The high-resolution mass spectra (HRMS) were recorded using a LTQ Orbitrap XL^{TM} hybrid Fourier Transform Mass

Spectrometer (Thermo Fisher Scientific). Thin layer chromatography was performed using silica gel 60 F_{254} plates (Sigma-Aldrich) with observation under UV when necessary. Chromatography was performed on 230-400 mesh silica gel.

General procedures for synthesis of naphthoquinone analogs

Method A. The dichlon **1** (2, 3-dichloronaphthoquinone, 284 mg, 1.2 mmol) and commercially available amines (1 mmol) were suspended in 1-butanol (10 mL) at room temperature for 2 days to obtain mixtures of red/orange precipitates. The resultant precipitates were filtered and washed with ethanol. The crude products were purified by column chromatography or recrystallization/precipitation to give final products at yields of 28-99%.

Method B. Triethylamine (10 mL, 73.0 mmol) was added to the solution of 4-aminobenzoic acid or 4-aminomethylbenzoic acid (36.5 mmol) in 75 mL of 1,4-dioxane and 25 mL of water and stirred at room temperature for 5 min. Di-*tert*-butyl dicarbonate (11.9 g, 54.8 mmol) was then added to the solution and stired for 24 h. Following removal of the solvent in *vacuo*, hydrochloric acid (10%) was added to the residue to yield a white precipitate. The slurry was filtered and washed with water (100 mL) and recrystallized from hot methanol to give white crystal *N*-Boc-4-aminobenzoic acid **42** (8.24 g, 95%) or *N*-Boc-4-aminomethylbenzoic acid **45** (8.98 g, 98%). [25]

Method C.HBTU (3.5 equiv.) was added to a suspension of *N*-Boc-4-aminobenzoic acid **42** (1equiv.), amine (1.1 equiv.), and DIEA (3.5 equiv.) in CH_2Cl_2 (30 mL). The mixture was stirred at room temperature for 18 h. The reaction was then diluted with EtOAc (100 mL) before washing with water (100 mL) and brine (100 mL ×3). The EtOAc layer was dried with Na_2SO_4, filtered and concentrated in *vacuo*. The crude products were purified by column chromatography (MeOH/CH_2Cl_2=1/100) to give colorless oil **43** and **44**.

Method D. *N*-Boc-4-aminomethylbenzoic acid **45** (2.5 g, 10 mmol), HOBt (1.4 g, 10 mmol), DIC (1.9 mL, 12 mmol), and DIEA (3.3 mL, 20 mmol) were suspended in CH_2Cl_2 (50 mL) and stirred at room temperature for 1 h; 4-fluoroaniline (0.96 mL, 10 mmol) was added and stirred overnight. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed with brine (100 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. The product was purified by gradient elution column chromatography (Hexane/EtOAc =100/1-10/1). This product was suspended in CH_2Cl_2 (10 mL) and TFA (10 mL), and stirred at room temperature for 4 h. The solvent was removed in *vacuo*. The residue was suspended in CH_2Cl_2 (100 mL) and ice water (50 mL) and the pH adjusted to around 10 with 20% NaOH aqueous solution. The layers were allowed to separate, and the aqueous layer was extracted with CH_2Cl_2 (50 mL×2). The combined organic layers were washed with brine (100 mL×2) and dried over anhydrous Na_2SO_4. Removal of Na_2SO_4 by filtration and evaporation of solvents produced 4-aminomethyl-*N*-(4-fluorophenyl) benzamide **46** (1.83 g, 75%). ^{1}H NMR (DMSO-d_6) δ_H 10.21 (s, 1H), 7.89 (d, *J* = 9.0 Hz, 2H), 7.78 (m, 2H), 7.46 (d, *J* =9.0 Hz, 2H), 7.17 (m, 2H), 3.78 (s, 2H).

Method E. An aqueous solution of potassium carbonate (1.52 g, 11 mmol) in 5 mL of H_2O was added to a solution of *p*-phenylenediamine (3.24 g, 30 mmol) in a mixture of THF (25 mL) and DMF (10 mL). To the mixture, di-*tert*-butyl dicarbonate (2.18 g, 10 mmol) was added dropwise for 0.5 h. The reaction mixture was stirred for an additional 3 h at room temperature. The mixture was then poured into cold water (40 mL) and extracted with CH_2Cl_2 (50 mL×3). The combined organic layers were dried with Na_2SO_4 and concentrated to yield intermediate **49** as a yellow solid (2.018 g, 97%). ^{1}H NMR ($CDCl_3$) δ_H 7.80 (d, *J*= 8.4 Hz, 2H), 7.34 (d, *J*= 9.0 Hz, 1H), 7.32 (d, *J*= 8.4 Hz, 2H), 6.92 (d, *J*= 9.0 Hz, 1H), 6.60 (s, 1H), 1.51 (s, 9H).

Method F. The intermediate **49** (1.2 mmol) obtained from Method E was dissolved in CH_2Cl_2 (10 mL) and cooled in an ice-water bath, and then sulfonyl chloride (1.0 mmol) was added. The reaction mixture was stirred at room temperature for 1 h. Water (5 mL) was added and stirred for 10 min. The suspension was extracted with CH_2Cl_2 (50 mL×3). The combined organic layers were dried with Na_2SO_4 and concentrated. The crude product was purified by column chromatography (MeOH/CH_2Cl_2=1/100) to give a white powder.

2-Amino-3-chloronaphthoquinone (2)

Starting from an ammonia solution (7 N in methanol), a orange solid (151 mg, 73%) was obtained after purification by column chromatography (Hexane/EtOAc=3/1) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.97 (d, *J*= 7.8 Hz, 1H), 7.95 (d, *J*= 7.8 Hz, 1H), 7.80 (dt, *J*= 7.5, 1.5 Hz, 1H), 7.72 (dt, *J*= 7.5, 1.5 Hz, 1H), 7. 47 (br s, 1H); ^{13}C NMR (75MHz, DMSO-d_6) δ_C 179.7, 175.6, 147.5, 135.3, 133.1, 132.8, 130.2, 126.7, 126.3, 109.2; HRMS (ESI) $[M + H]^+$ found m/z 208.0164, calcd. for $C_{10}H_7ClNO_2$ 208.0165.

2-Methylamino-3-chloronaphthoquinone (3)

Starting from a methylamine solution (2.0 M in THF), a red solid (162 mg, 73%) was obtained after purification by column chromatography (Hexane/EtOAc=10/1) following method A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H7.91 (d, *J*=7.8 Hz, 1H), 7.89 (d, *J*=7.8 Hz, 1H), 7.76 (dt, *J* = 7.8, 1.2 Hz, 1H), 7.67 (dt, *J* = 7.8, 1.2 Hz, 1H), 7.49 (br s, 1H), 3.24 (s, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 180.7, 175.7, 146.6, 135.3, 132.9, 132.9, 132.6, 130.4, 126.8, 126.8, 126.2; HRMS (ESI) $[M + H]^+$ found m/z 222.0320, calcd. for $C_{11}H_9ClNO_2$ 222.0322.

2-Dimethylamino-3-chloronaphthoquinone (4)

Starting from a dimethylamine solution (2.0 M in THF), a red solid (218 mg, 65%) was obtained after precipitation from CH_2Cl_2/ MeOH following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.94 (d, *J*= 7.2 Hz, 1H), 7.91 (d, *J*= 7.2 Hz, 1H), 7.78 (m, 2H), 3.17 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 182.1, 177.3, 151.9, 134.6, 133.5, 131.7, 131.5, 127.0, 125.9, 118.1, 44.5, 34.5; HRMS (ESI) $[M + H]^+$ found m/z 236.0477, calcd. for $C_{12}H_{11}ClNO_2$ 236.0478.

2-Phenylamino-3-chloronaphthoquinone (5)

Starting from an aniline, a red solid (156 mg, 55%) was obtained after purification by gradient elution column chromatography (Hexane/EtOAc=10/1-1/1) following method A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 9.25 (br s, 1H), 8.01 (d, *J*= 7.8 Hz, 1H), 7.99 (d, *J*= 7.8 Hz, 1H), 7.83 (t, *J*= 7.2 Hz, 1H), 7.77 (t, *J*= 7.2 Hz, 1H), 7. 28 (t, *J*= 7.8 Hz, 2H); 7. 10 (m,

3H); ^{13}C NMR (150MHz, DMSO-d_6) δ_C 180.6, 177.2, 143.6, 139.3, 135.3, 133.7, 132.4, 130.7, 128.4, 128.4, 127.0, 126.6, 124.9, 124.5, 124.5, 114.8.

2-(4-Fluorophenylamino)-3-chloronaphthoquinone (6)

Starting from 4-fluoroaniline, a red solid (256 mg, 85%) was obtained after purification by gradient elution column chromatography (Hexane/EtOAc=10/1-1/1) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H9.24 (br s, 1H), 8.01 (m, 2H), 7.84 (dt, J = 7.5, 1.5 Hz, 1H), 7.77 (dt, J = 7.5, 1.5 Hz, 1H), 7.50 (m, 4H) ; ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 180.4, 177.1, 161.3, 158.1, 143.8, 135.7, 135.2, 133.6, 132.4, 130.6, 126.9, 126.6, 126.5, 115.2, 114.8, 114.2; HRMS (ESI) [M + H]$^+$ found m/z 302.0386, calcd. for $C_{16}H_{10}ClFNO_2$ 302.0384.

2-(4-Nitrophenylamino)-3-chloronaphthoquinone (7)

The 2, 3-dichloronaphthoquinone (200 mg, 0.881 mmol) and 4-nitroaniline (134 mg, 0.969 mmol) were suspended in 1-butanol (8 mL), and heated at 110 °C for 3 days. The reaction mixtures were cooled to room temperature and the precipitates were filtered and washed with ethanol. The resultant precipitates were recrystallized from CH_2Cl_2 and EtOH to give a pure product as a red solid (164 mg, 50%) ; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H9.73 (br s, 1H), 8.14 (d, J=9.0 Hz, 2H), 8.05 (m, 2H), 7.84 (m, 2H), 7.22 (d, J = 9.0 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 180.2, 177.6, 146.6, 142.7, 142.2, 135.1, 134.2, 132.1, 131.1, 127.1, 126.7, 124.5, 124.5, 122.2, 121.6, 121.6; HRMS (ESI) [M + H]$^+$ found m/z 329.0329, calcd. for $C_{16}H_{10}ClN_2O_4$ 329.0329.

4-(2-Chloro-1,4-dihydro-1,4-dioxonaphthalen-3-ylamino) benzoic acid (8)

Starting from 4-aminobenzoic acid, the resultant reaction mixtures were filtered and washed with EtOH and recrystallized from CH_2Cl_2/Hexane. An orange solid (324 mg, 99%) was obtained following method A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H12.68 (br s, 1H), 9.49 (s, 1H), 8.06 (d, J=6.6 Hz, 2H), 7.87 (m, 4H), 7.17 (d, J = 7.2 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 180.4, 177.4, 167.5, 143.8, 143.2, 135.2, 133.9, 132.3, 130.9, 129.9, 129.9, 127.1, 126.7, 125.8, 122.6, 122.6, 118.5; HRMS (ESI) [M + H]$^+$ found m/z 328.0387, calcd. for $C_{17}H_{11}ClNO_4$ 328.0377.

Methyl 4-(2-chloro-1,4-dihydro-1,4-dioxonaphthalen-3-ylamino)benzoate (9)

Concentrated sulfuric acid was slowly added to a mixture of **8** (100 mg, 0.306 mmol) in methanol (5 mL) at 0 °C. After the addition, the mixture was stirred at room temperature for 5 h. The mixture was washed with a saturated solution of sodium bicarbonate and extracted with EtOAc (5 mL × 3). The organic layers were combined and dried over Na_2SO_4 and concentrated. The residue was recrystallized from CH_2Cl_2 /Hexane to give **9** (94 mg, 90%) as a red solid; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H9.49 (br s, 1H), 8.03 (d, J= 7.5 Hz, 2H), 7.87 (m, 4H), 7.16 (d, J= 7.2 Hz, 2H), 3.82 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.4, 177.4, 166.3, 144.2, 143.1, 135.2, 133.9, 132.2, 130.9, 129.7, 129.7, 127.0, 126.6, 124.4, 122.5, 122.5, 118.5, 52.3; HRMS (ESI) [M + H]$^+$ found m/z 342.0540, calcd. for $C_{18}H_{13}ClNO_4$ 342.0533.

2-(3-Chloro-4-methylphenylamino)-3-chloronaphthoquinone (10)

Starting from 3-chloro-4- methylaniline, the resultant reaction mixtures were filtered and washed with EtOH and recrystallized from CH_2Cl_2/EtOH. A dark red solid (315 mg, 95%) was obtained following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 9.24 (br s, 1H); 8.02 (d, J= 7.5 Hz, 1H), 8.00 (d, J= 7.5 Hz, 1H), 7.84 (dt, J= 7.5, 1.5 Hz, 1H), 7.81 (dt, J= 7.5, 1.5 Hz, 1H), 7. 24 (d, J= 7.5 Hz, 1H); 7. 17 (s, 1H), 7. 98 (d, J= 7.5 Hz, 1H), 2.28(s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.4, 177.2, 143.5, 138.5, 135.2, 133.7, 132.8, 132.3, 131.5, 130.8, 130.7, 126.9, 126.5, 124.2, 122.9, 115.5, 19.4; HRMS (ESI) [M + H]$^+$ found m/z 332.0246, calcd. for $C_{17}H_{12}Cl_2NO_2$ 332.0245.

2-(2-(Pyridin-2-yl)ethylamino)-3-chloronaphthoquinone (11)

Starting from 2-(2-pyridyl)ethylamine, a red solid (110 mg, 35%) was obtained after purification by gradient elution column chromatography (Hexane/Acetone =10/1-4/1) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.46 (dd, J= 4.8 Hz, 1H), 7.95 (d, J= 7.5 Hz, 1H), 7.93 (d, J= 7.5 Hz, 1H), 7.80 (dt, J= 7.5, 1.2 Hz, 1H), 7.72 (m, 2H), 7.57 (t, J= 6.6 Hz, 1H), 7. 28 (d, J= 7.8 Hz, 1H), 7. 20 (dd, J= 6.6 Hz, 1H), 4.10 (dd, J= 6.9 Hz, 2H), 2.48 (t, J= 7.2 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.6, 175.8, 159.0, 149.4, 137.1, 135.3, 133.1, 132.4, 130.4, 128.8, 126.9, 126.0, 123.9, 122.1, 111.8, 44.2, 38.7; HRMS (ESI) [M + H]$^+$ found m/z 313.0751, calcd. for $C_{17}H_{14}ClN_2O_2$ 313.0744.

2-(3-(Dimethylamino)propylamino)-3-chloronaphthoquinone (12)

Starting from 3-dimethylamino- 1-propylamine, the resultant reaction mixtures were filtered and washed with EtOH and recrystallized from CH_2Cl_2/EtOH. An orange solid (120 mg, 41%) was obtained following method A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 10.46 (br s, 1H), 7.95 (d, J= 7.2 Hz, 2H), 7.80 (t, J= 7.2 Hz, 1H), 7.72 (t, J= 7.2 Hz, 1H), 3.77 (q, J= 7.2 Hz, 2H), 3.06 (t, J= 7.8 Hz, 2H), 2.70 (s, 6H), 2.00 (t, J= 7.2 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 180.6, 175.9, 147.2, 135.4, 133.2, 132.4, 130.5, 127.0, 126.3, 114.7, 55.4, 54.3, 42.4, 41.4, 26.1; HRMS (ESI) [M + H]$^+$ found m/z 293.1066, calcd. for $C_{15}H_{18}ClN_2O_2$ 293.1057.

2-(Piperidin-1-yl)-3-chloronaphthoquinone (13)

Starting from a piperidine, a red solid (85 mg, 31%) was obtained after purification by column chromatography (Hexane/Acetone=20/1) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.93 (m, 2H), 7.76 (m, 2H), 3.46 (m, 4H), 1.64 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 181.7, 177.5, 151.4, 134.5, 133.7, 131.9, 131.5, 127.1, 126.1, 120.8, 52.8, 52.8, 26.9, 26.9, 23.9; HRMS (ESI) [M + H]$^+$ found m/z 276.0796, calcd. for $C_{15}H_{15}ClNO_2$ 276.0791.

2-(4-Benzylpiperidin-1-yl)-3-chloronaphthoquinone (14)

Starting from 4-benzylpiperidin, a red oil (108 mg, 30%) was obtained after purification by column chromatography (Hexane/Acetone=40/1) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.91 (d, J= 6.9 Hz, 1H), 7.88 (d, J= 6.9 Hz, 1H), 7.73 (m, 2H), 7.26 (m, 2H), 7.16 (m, 3H), 3.72 (m,

2H), 3.14 (m, 2H), 2.51 (d, *J*= 6.9 Hz, 2H), 1.79 (m, 1H), 1.63 (m, 2H), 1.34 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 181.6, 177.4, 151.3, 140.4, 134.5, 133.6, 131.8, 131.4, 129.5, 129.5, 128.6, 128.6, 127.1, 126.2, 126.1, 120.8, 51.9, 51.9, 42.7, 37.2, 33.1, 33.1; HRMS (ESI) $[M + H]^+$ found m/z 366.1268, calcd. for $C_{22}H_{21}ClNO_2$ 366.1261.

4-[(2-Chloro-1,4-dihydro-1,4-dioxonaphthalen-3-ylamino)methyl]benzoic acid (15)

Starting from 4-aminomethyl benzoic acid, the resultant reaction mixtures were filtered and washed with EtOH and recrystallized from CH_2Cl_2/EtOH. An orange solid (195 mg, 57%) was obtained following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H12.80 (br s, 1H), 8.34 (t, *J*=7.2 Hz, 1H), 7.95 (d, *J*=7.8 Hz, 2H), 7.88 (d, *J* = 8.1 Hz, 2H), 7.81 (dt, *J* = 7.2, 1.2 Hz, 1H), 7.72 (dt, *J* = 7.2, 1.2 Hz, 1H), 7.40 (d, *J* = 8.1 Hz, 2H), 5.00 (d, *J*=7.2 Hz, 2H); HRMS (ESI) $[M + H]^+$ found m/z 342.0534, calcd. for $C_{18}H_{13}ClNO_4$ 342.0533.

4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)-N-phenylbenzamide (16)

The intermediate **43** was prepared from *N*-Boc-4-aminobenzoic acid **42** (100 mg, 0.442 mmol), aniline (58 μL, 0.633 mmol), DIEA (257 μL, 1.477 mmol), and HBTU (560 mg, 1.477 mmol) following method C. A colorless oil was obtained after purification by column chromatography. The product **43** was suspended in CH_2Cl_2 (5 mL) and TFA (5 mL) and stirred at room temperature for 4 h. The solvent was removed in *vacuo*. To the solution of the above residue in 1-butanol (2 mL), Et_3N (0.5 mL) and **1** (48 mg, 0.211 mmol) were added and heated at 90 °C for 2 days. The final product was obtained as a red solid (63 mg, 81 %) following method A. ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 8.03 (m, 2H), 7.88 (d, *J*= 9.0 Hz, 2H), 7.86 (dt, *J*= 8.4, 1.2 Hz, 1H), 7.81 (dt, *J*= 8.4, 1.2 Hz, 1H), 7.75 (d, *J*= 7.8 Hz, 2H), 7.32 (t, *J*= 8.4 Hz, 2H), 7.19 (d, *J*= 8.4 Hz, 2H), 7.06 (t, *J*= 8.4 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 186.8, 180.5, 177.4, 165.4, 143.4, 142.7, 139.8, 135.3, 133.9, 132.4, 131.0, 130.1, 129.3, 129.0, 129.0, 128.2, 127.1, 126.7, 124.0, 122.8, 120.9, 120.9, 117.7; HRMS (ESI) $[M + H]^+$ found m/z 403.0862, calcd. for $C_{23}H_{16}ClN_2O_3$ 403.0849.

4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)-N-(pyridin-2-yl)benzamide (17)

The intermediate **44** was prepared from *N*-Boc-4-aminobenzoic acid **42** (500 mg, 2.11 mmol), 2-aminopyridine (395 mg, 4.22 mmol), DIEA (1.10 mL, 6.33 mmol), and HBTU (1.595 g, 4.22 mmol) following method C. The crude product was purified by column chromatography (CH_2Cl_2/MeOH=100/1-40/1) to give a colorless oil (335 mg, 51%). The product **44** (150 mg, 0.479 mmol) was suspended in CH_2Cl_2 (5 mL) and TFA (5 mL) and stirred at room temperature for 4 h. The solvent was removed in *vacuo*. To the solution of the above residue in 1-butanol (4 mL), Et_3N (1 mL) and **1** (163 mg, 0.719 mmol) were added and heated at 90 °C for 2 days. The final product was obtained as a red solid (170 mg, 88 %) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 8.35 (m, 1H), 8.15 (d, *J*= 8.4 Hz, 1H), 8.05 (t, *J*= 6.9 Hz, 1H), 7.97 (m, 4H), 7.81 (m, 2H), 7.73 (dt, *J*= 7.5, 1.2 Hz, 1H), 7.42 (d, *J*= 8.1 Hz, 2H), 7.13 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 186.8, 180.5, 176.0, 166.2, 152.6, 148.3, 145.8, 144.6, 138.5, 135.3, 133.2, 133.0, 132.3, 130.5, 128.6, 128.6, 127.0, 126.8, 126.8, 126.2, 120.2, 115.1.

4-[(2-Chloro-1,4-dihydro-1,4-dioxonaphthalen-3-ylamino) methyl)-N-(4-fluorophenyl]benzamide (18)

The preparation of 4-aminomethyl-*N*-(4-fluorophenyl) benzamide **46** (1.83 g, 75%) was made from *N*-Boc-4-aminomethylbenzoic acid **45** (2.5 g, 10 mmol), HOBt (1.4 g, 10 mmol), DIC (1.9 mL, 12 mmol), and DIEA (3.3 mL, 20 mmol) following method D. Starting from **46** (150 mg, 0.615 mmol) and **1** (279 mg, 1.23 mmol), the final product **18** (96 mg, 36 %) was obtained as an orange solid following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 10.21 (s, 1H), 8.06 (t, *J*=7.2 Hz, 1H), 7.97 (d, *J*=7.8 Hz, 1H), 7.96 (d, *J*=7.5 Hz, 1H), 7.88 (d, *J*=8.1 Hz, 2H), 7.79 (dt, *J*=7.5, 1.2 Hz, 1H), 7.75 (m, 3H), 7.43 (d, *J*=8.4 Hz, 2H), 7.15 (m, 2H), 5.02 (d, *J*=7.2 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.5, 176.1, 165.7, 160.3, 157.1, 144.2, 135.9, 135.9, 135.3, 133.8, 133.2, 132.3, 130.4, 128.2, 128.2, 127.0, 126.8, 126.8, 126.3, 122.6, 122.5, 115.7, 115.4, 47.2; HRMS (ESI) $[M + H]^+$ found m/z 435.0925, calcd. for $C_{24}H_{17}ClFN_2O_3$ 435.0912.

4-[(2-Chloro-1,4-dihydro-1,4-dioxonaphthalen-3-ylamino)methyl)-N-(6-chloropyridin-3-yl]benzamide (19)

N-(6-chloropyridin-3-yl)-4-[[(2-chloro-6-methylquinazolin-4-yl)- amino]methyl]benzamide (**47**) was prepared from **45** and 6-chloropyridin-3-amine following method D. The intermediate **47** (231 mg, 0.882 mmol) was reacted with **1** (100 mg, 0.441 mmol) to give final product **19** as an orange solid (99 mg, 49 %) following method A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 10.53 (s, 1H), 8.78 (d, *J*=2.4 Hz, 1H), 8.24 (dd, *J*=8.4, 2.4 Hz, 1H), 8.09 (t, *J*=7.2 Hz, 1H), 7.98 (m, 2H), 7.93 (d, *J*=8.4 Hz, 2H), 7.83 (dt, *J*=7.8, 1.8 Hz, 1H), 7.75 (dt, *J*=7.8, 1.8 Hz, 1H), 7.49(m, 3H), 5.05(d, *J*=7.2 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.5, 176.1, 166.3, 145.8, 144.8, 144.4, 141.9, 136.0, 135.4, 133.2, 133.1, 132.4, 131.2, 130.5, 128.4, 128.4, 127.1, 127.0, 126.9, 126.3, 124.5, 124.5, 47.3; HRMS (ESI) $[M + H]^+$ found m/z 452.0584, calcd. for $C_{23}H_{16}Cl_2N_3O_3$ 452.0569.

N-(4-fluorobenzyl)-1-(2-chloro-1,4-dihydro-1,4-dioxonaphthalen-3-yl)piperidine-4-carboxamide (20)

N-(4-fluorobenzyl)piperidine-4-carboxamide (**48**) was prepared starting from 1-Boc-piperidine-4-carboxylic acid and 4-fluorobenzylamine following procedure D.The final product **20** was synthesized starting fromintermediate **48** (236 mg, 1 mmol) and **1** (227 mg, 1 mmol) as a red solid (120 mg, 28%) following method A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 8.35 (t, *J*= 6.0 Hz, 1H), 7.96 (d, *J*= 7.2 Hz, 1H), 7.93 (d, *J*= 7.2 Hz, 1H), 7.78 (dt, *J*= 7.2, 1.2 Hz, 1H), 7.76 (dt, *J*= 7.2, 1.2 Hz, 1H), 7.25 (d, *J*= 8.4 Hz, 1H), 7.23 (d, *J*= 9.0 Hz, 1H), 7.12 (d, *J*= 9.0 Hz, 1H), 7.10 (d, *J*= 9.0 Hz, 1H), 4.23 (d, *J*= 6.0 Hz, 2H), 3.80 (m, 2H), 3.22 (m, 2H), 1.79 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 181.7, 177.6, 174.4, 151.4, 136.4, 134.6, 133.8, 131.9, 131.5, 129.5, 129.4, 127.2, 126.2, 121.3, 115.5, 115.4, 115.4, 78.9, 51.4, 51.4, 41.7, 29.9, 29.9; HRMS (ESI) $[M + H]^+$ found m/z 427.1268, calcd. for $C_{23}H_{21}ClFN_2O_3$ 427.1225.

N-(4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)methylbenzyl)-4-fluorobenzamide (21)

N-Boc-[4-[[(2- chloroquinazolin-4-yl)amino]methyl]phenyl]-4-fluorobenzamide (**53**) was prepared starting from *N*-Boc-4-aminobenzylamine (**50**), 4-fluorobenzoyl chloride following

method F. The intermediate **53** (323 mg, 1.323 mmol) was stirred in TFA (5mL) and CH_2Cl_2 (5mL) at room temperature for 4 h. To the solution of this residue in 1-butanol (10 mL), added **1** (100 mg, 0.441 mmol) and Et_3N (0.5 mL) were added, and stirred at room temperature for 2 days. The final product **21** was obtained as a red solid (190 mg, 99 %) following methods A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 10.23 (s, 1H), 8.01 (d, *J*=8.4 Hz, 1H), 7.99 (d, *J*=9.0 Hz, 1H), 7.95(m, 2H), 7.91 (t, *J*=7.2 Hz, 1H), 7.80 (dt, *J*=7.8, 1.2 Hz, 1H), 7.72 (dt, *J*=7.8, 1.2 Hz, 1H), 7.68 (d, *J*=8.4 Hz, 2H), 7.33 (d, *J*=9.0 Hz, 1H), 7.31 (d, *J*=8.4 Hz, 1H), 7.26 (d, *J*=8.4 Hz, 2H), 4.92 (d, *J*=7.2 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.6, 176.1, 165.3, 164.8, 163.7, 138.3, 135.6, 135.4, 133.2, 132.5, 131.8, 131.8, 130.9, 130.8, 127.4, 127.4, 127.0, 126.3, 121.0, 121.0, 115.8, 115.7, 110.0, 47.1; HRMS (ESI) $[M + H]^+$ found m/z 435.0926, calcd. for $C_{24}H_{17}ClFN_2O_3$ 435.0912.

4-(2-Chloro-1,4-dihydro-1,4-dioxonaphthalen-3-ylamino)benzenesulfonamide (22)

The sulfanilamide (258 mg, 1.5 mmol) and **1** (237 mg, 1 mmol) were suspended in 1-butanol (10 mL), and heated at 110 °C for 3 days. The resultant precipitates were recrystallized from CH_2Cl_2 and EtOH to give pure product as a red solid (224 mg, 62 %) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H9.47 (br s, 1H), 8.03 (d, *J*=7.2 Hz, 2H), 7.82 (m, 2H), 7.71 (d, *J* = 8.4 Hz, 2H), 7.25 (br s, 2H), 7.21 (d, *J* = 8.7 Hz, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C 180.4, 177.4, 143.2, 142.7, 139.0, 135.2, 133.9, 132.2, 130.9, 127.0, 126.6, 126.1, 126.1, 122.8, 122.8, 118.4; HRMS (ESI) $[M + H]^+$ found m/z 363.0218, calcd. for $C_{16}H_{12}ClN_2O_4S$ 363.0206.

4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)-N-(pyridin-2-yl)benzenesulfonamide (23)

The sulfapyridine (249 mg, 2.6 mmol) and **1** (590 mg, 2.6 mmol) were suspended in 1-butanol (10 mL), and heated at 110 °C for 3 days. The resultant precipitates were purified by gradient elution column chromatography (CH_2Cl_2/ MeOH=100/1-20/1) to give pure product as an orange solid (806 mg, 76 %) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 9.44 (s, 1H), 8.07 (m, 3H), 8.07 (m, 3H), 7.61-7.88 (m, 6H), 7.19 (m, 3H), 7.86 (t, *J*= 6.0 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.3, 177.4, 153.4, 144.2, 143.2, 140.5, 136.3, 135.2, 133.9, 132.2, 130.9, 129.3, 127.5, 127.2, 127.0, 126.6, 122.6, 121.8, 118.8, 116.3, 114.1; HRMS (ESI) $[M + H]^+$ found m/z 440.0476, calcd. for $C_{21}H_{15}ClN_3O_4S$ 440.0394.

4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)-N-(2,6-dimethoxypyrimidin-4-yl)-benzenesulfonamide (24)

Starting from a sulfadimethoxine, an orange solid (396 mg, 79%) was obtained after purification by column chromatography (CH_2Cl_2/MeOH=50/1) following method A; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 9.51 (br s, 1H), 8.02 (d, *J* = 7.8 Hz, 1H), 8.01 (d, *J* = 7.8 Hz, 1H), 7.84 (dt, *J*= 7.2, 1.2 Hz, 1H), 7.80 (dt, *J*= 7.2, 1.2 Hz, 1H), 7.79 (d, *J*= 9.0 Hz, 2H), 7.19 (d, *J*= 9.0 Hz, 2H), 5.91 (s, 1H), 3.77 (s, 3H), 3.72 (s, 3H); ^{13}C NMR (150MHz, DMSO-d_6) δ_C 180.3,177.5, 172.2, 161.2, 144.1, 143.1, 135.2, 134.1, 132.2, 131.0, 127.9, 127.9, 127.1, 126.7, 126.3, 122.7, 122.5, 122.5, 119.0, 85.1, 54.9, 54.3; HRMS (ESI) $[M + H]^+$ found m/z 501.0639, calcd. for $C_{22}H_{18}ClN_4O_6S$ 501.0636.

4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)-N-(5-methylisoxazol-3-yl)benzenesulfonamide (25)

Starting from a sulfamethoxazole, the resultant reaction mixtures were filtered and washed with EtOH and recrystallized from CH_2Cl_2/EtOH. A red solid (142 mg, 32%) was obtained following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 9.50 (br s, 1H); 8.03 (m, 2H), 7.85 (m, 2H), 7.71 (d, *J*= 8.7 Hz, 2H), 7.20 (d, *J*= 8.7 Hz, 2H), 6.12 (s, 1H), 2.28 (s, 3H); ^{13}C NMR (75MHz, DMSO-d_6) δ_C 180.2, 177.5, 170.7, 158.0, 144.3, 143.0, 135.2, 134.1, 133.5, 132.1, 130.9, 127.5, 127.5, 127.1, 126.7, 122.5, 122.5, 120.0, 95.9, 12.45; HRMS (ESI) $[M + H]^+$ found m/z 444.0427, calcd. for $C_{20}H_{15}ClN_3O_5S$ 444.0421.

N-(4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)phenyl)-4-methylbenzenesulfonamide (26)

The intermediate **51** was prepared from **49** and *p*-toluenesulfonyl chloride following method F. A white solid **51** was obtained (293 mg, 81%);^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7.61 (d, *J*= 8.4 Hz, 2H), 7.25 (d, *J*= 8.7 Hz, 2H), 7.22 (d, *J*= 6.9 Hz, 2H), 6.98 (d, *J*= 6.9 Hz, 2H), 6.54 (s, 1H), 6.47 (s, 1H), 2.39 (s, 3H), 1.53 (s, 9H). The intermediate **51** (100 mg, 0.276 mmol) was suspended in CH_2Cl_2 (2 mL) and TFA (2 mL) at room temperature for 4 h, and then the solvent was removed in *vacuo*. The residue was dissolved in 1-butanol (3 mL), and Et_3N (0.5 mL) and **1** (125 mg, 0.552 mmol) were added. The final product **26** was obtained as a red solid (72 mg, 58 %) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 10.07 (s, 1H), 9.16 (s, 1H), 8.01 (d, *J*= 7.8 Hz, 1H), 7.99 (d, *J*= 7.5 Hz, 1H), 7.83 (dt, *J*= 7.5, 1.5 Hz, 1H), 7.79 (dt, *J*= 7.5, 1.5 Hz, 1H), 7.60 (d, *J*= 8.1 Hz, 2H), 7.31 (d, *J*= 8.1 Hz, 2H), 6.98 (m, 4H), 3.32 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.4, 177.0, 143.6, 137.0, 135.6, 135.2, 134.7, 133.5, 132.4, 130.6, 129.9, 129.9, 128.3, 127.2, 127.2, 126.9, 126.5, 125.5, 125.5, 120.7, 120.7, 113.9, 21.4; HRMS (ESI) $[M + H]^+$ found m/z 453.0689, calcd. for $C_{23}H_{18}ClN_2O_4S$ 453.0676.

N-(4-(3-Chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)phenyl)-4-fluorobenzenesulfonamide (27)

The intermediate **52** was made from **49** and 4-fluorobenzenesulfonyl chloride following method F. A white solid **52** was obtained (275 mg, 75%);^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7.73 (m, 2H), 7.27 (d, *J*= 8.7 Hz, 2H), 7.14 (m, 2H), 7.00 (d, *J*= 9.0 Hz, 2H), 6.47 (s, 1H), 6.45 (s, 1H), 1.53 (s, 9H). The intermediate **52** (120 mg, 0.328 mmol) was suspended in CH_2Cl_2 (2 mL) and TFA (2 mL) at room temperature for 4 h, and then the solvent was removed in *vacuo*. To the solution of this residue in 1-butanol (3 mL), Et_3N (0.5 mL) and **1** (93 mg, 0.393 mmol) were added. The final product **27** was obtained as a yellow solid (94 mg, 63 %) following method A; ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 10.17 (s, 1H), 9.18 (s, 1H), 8.01 (d, *J*= 7.5 Hz, 1H), 7.99 (d, *J*= 7.5 Hz, 1H), 7.83 (dt, *J*= 7.5, 1.2 Hz, 1H), 7.77 (m, 3H), 7.37 (m, 2H), 6.99 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ_C 180.4, 177.0, 166.4, 163.0, 143.6, 136.0, 135.2, 134.3, 133.6, 132.4, 130.6, 130.3, 130.2, 126.9, 126.5, 125.5, 125.5, 121.1, 121.1, 116.9, 116.6, 114.2; HRMS (ESI) $[M + H]^+$ found m/z 457.0451, calcd. for $C_{22}H_{15}ClFN_2O_4S$ 457.0425.

2-Dimethylamino-naphthoquinone (29)

The 2-bromo-1,4-naphthoquinone (237 mg, 1 mmol) and dimethylamine solution (1.5 mL, 2.0 M, 3 mmol) were

suspended in 1-butanol (10 mL), and stirred at room temperature for 2 days. After evaporation of solvent, the crude product was purified by column chromatography (Hexane/Acetone=10/1) to give a red solid (110 mg, 55%); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 7.90 (d, J= 7.8 Hz, 1H), 7.86 (d, J= 7.8 Hz, 1H), 7.76 (dt, J= 7.2, 1.2 Hz, 1H), 7.69 (dt, J= 7.2, 1.2 Hz, 1H), 5.73 (s, 1H), 3.14 (s, 6H); ^{13}C NMR (75MHz, DMSO-d_6) δ_C 183.5, 181.5, 153.2, 134.4, 134.4, 132.8, 132.7, 126.6, 125.0, 105.8, 42.6, 42.6; HRMS (ESI) $[M + H]^+$ found m/z 202.0870, calcd. for $C_{12}H_{12}NO_2$ 202.0868.

4-(1,4-Dioxo-1,4-dihydronaphthalen-2-ylamino)-N-(pyridin-2-yl)benzenesulfonamide (30)

The 2-bromo-1,4-naphthoquinone (284 mg, 1.2 mmol) and sulfapyridine (249 mg, 1 mmol) were suspended in 1-butanol (10 mL), and heated at 100 °C for 2 days. After evaporation of solvent, the crude product was purified by gradient elution column chromatography (MeOH/CH_2Cl_2=1/100-1/1) to give a dark red solid (186 mg, 46%); ^{1}H NMR (300 MHz, DMSO-d_6) δ_H 11.94 (s, 1H), 9.35 (s, 1H), 8.05 (d, J= 7.5 Hz, 1H), 8.00 (m, 1H), 7.95 (d, J= 7.5 Hz, 1H), 7.88 (d, J= 8.7 Hz, 2H), 7.75-7.85(m, 2H), 7.71 (dt, J= 7.5, 1.5 Hz, 1H), 7.54 (d, J=8.7 Hz, 2H), 7.16 (d, J=8.4 Hz, 1H), 6.86 (t, J=6.3 Hz, 1H), 6.30(s, 1H); HRMS (ESI) $[M + H]^+$ found m/z 406.0874, calcd. for $C_{21}H_{16}N_3O_4S$ 406.0862.

Compounds obtained from the NCI database

Compounds **31-41** were collected from the NCI chemical repository [http://dtp.cancer.gov.]. The NCI codes for these compounds are described as follows: **31** (NSC No. 35851); **32** (NSC No. 106714); **33** (NSC No. 121713); 34 (NSC No. 116929); 35 (NSC No. 35854); 36 (NSC No. 129141); 37 (NSC No. 136219); 38 (NSC No. 141316); 39 (NSC No. 40340); and 40 (NSC No. 45380).

CoMFA Calculation

The CoMFA studies were carried out by means of the SYBYL-X suite software (Tripos Inc. St. Louis, MO). The conformation of all compounds with lowest energy was obtained by a conformation search and used as a template to build the other analogs. All the compounds were energy minimized and superimposed by the 1, 4-naphthoquinone core. The method of partial least-squares (PLS) implemented in the Quantitative Structure−Activity Relationship (QSAR) module of SYBYL was used to construct and validate the models. The van der Waals potentials and coulombic terms were calculated using the Tripos force field. A sp^3 hybridized carbon atom with +1 charge served as probe atom to calculate steric and electrostatic fields. The steric and electrostatic contributions were truncated to +30.0 kcal/mol. The CoMFA descriptors were used as independent variables, and pIC_{50} values as dependent variables in partial least square regression analysis. The minimum sigma (column filtering) was set to 2.0 kcal/mol to improve the signal-to-noise ratio. Cross-validation was performed with the leave-one-out procedure. The optimum number of the components, N, retained for final PLS analyses was defined as the one that yielded the highest cross validated r_{cv}^2. The robustness of the models was internally evaluated by calculating the r^2, SD, and F test values from the training set.

Biological evaluation

The colorectal carcinoma cell lines HCT116 and SW480 were obtained from American Type Culture Collection (ATCC). The cell lines were cultured in RPMI 1640 (Hyclone, Thermal Scientific), containing 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

Cell Growth Inhibition Assay

Human colon cancer HCT116 and SW480 cells were plated in 96-well plates at a density of 5×10^3 cells per well. Cells were treated with different concentrations of compounds and incubated at 37 °C for 72 h. Cell viability was measured by a Cell Titer-Blue reagent-based assay (Promega). The resulting luminescence was read on a Synergy 2 reader.

Clonogenic Assays

SW480 and HCT116 cells were mixed in RPMI 1640, 10% FBS, and penicillin/streptomycin and plated at 250 cells/well onto 6-well plates. Cells were incubated at 37 °C for 7 days with different concentrations of compounds (final concentration was 0.1-20 μM), and colonies were counted after fixation with acetic acid/methanol (1:9, v/v) and staining with 0.5% Crystal Violet solution.

Luciferase Reporter Gene Assay

The SW480 and HCT116 cells at a density of 1×10^4 cells/well (in 96-well plates) were transfected with 100 ng of TOP-FLASH (containing TCF binding sites), or FOP-FLASH harboring mutant TCF binding sites. Various concentrations of candidate compounds in medium were added to the cells at 4 h post-transfection. Luciferase activity was measured 24 h after adding the compounds using the Firefly Luciferase Assay Kit (Biotium, Inc. Hayward, CA). Cell viabilities were surveyed at the same time point, and combined with luciferase activities to drive normalizations for ruling out nonspecific cytotoxicity. The relative luciferase activity was calculated as (TOP-luciferase activity/FOP-luciferase activity) $_{\text{treatment}}$ /(TOP-luciferase activity/FOP-luciferase activity) $_{\text{control}} \times 100$.

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Abbreviations Used

Wnt, wingless int-1; TCF, T-cell transcription factor; CoMFA, comparative molecular field analysis; SAR, structure-activity relationship; CSC, cancer stem cell; APC, adenomatous polyposis coli; LEF, lymphoid enhancer-binding factor; FDA, Food and Drug Administration; HBTU, N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluoro phosphate; TFA, Trifluoroacetic acid; HOBt, hydroxybenzotrizole; DIC, N, N'-diisopropylcarbodiimide; DIEA, N,N-diisopropylethylamine; THF, tetrahydrofuran; DMF, N,N-Dimethylformamide; DMSO, dimethyl sulfoxide; PLS, partial least-squares; NMR, nuclear magnetic resonance; HRMS, high resolution mass spectra; UV, Ultraviolet.

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