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ORIGINAL ARTICLE

A diterpenoid derivative 15-oxospiramilactone inhibits Wnt/β-catenin signaling and colon cancer cell tumorigenesis

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The Wnt/β-catenin signaling pathway is a highly conserved pathway in organism evolution and regulates many biological processes. Aberrant activation of the Wnt/β-catenin signaling pathway is closely related to tumorigenesis. In order to identify potent small molecules to treat the over-activated Wnt signaling-mediated cancer, such as colon cancer, we established a mammalian cell line-based reporter gene screening system. The screen revealed a diterpenoid derivative, 15-oxospiramilactone (NC043) that inhibits Wnt3a or LiCl-stimulated Top-flash reporter activity in HEK293T cells and growth of colon cancer cells, SW480 and Caco-2. Treatment of SW480 cells with NC043 led to decreases in the mRNA and/or protein expression of Wnt target genes *Axin2, Cyclin D1* and *Survivin*, as well as decreases in the protein levels of *Cdc25c* and *Cdc2*. NC043 did not affect the cytosol-nuclear distribution and protein level of soluble β-catenin, but decreased β-catenin/TCF4 association in SW480 cells. Moreover, NC043 inhibited anchorage-independent growth and xenograft tumorigenesis of SW480 cells. Collectively these results demonstrate that NC043 is a novel small molecule that inhibits canonical Wnt signaling downstream of β-catenin stability and may be a potential compound for treating colorectal cancer.

***Keywords*:** Wnt; small molecule; inhibitor; tumorigenesis

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Introduction

The Wnt signaling pathway is involved in multiple developmental events during embryogenesis and is also implicated in adult tissue homeostasis and tumorigenesis [1, 2]. Wnt ligands trigger at least three different intracellular signaling cascades: the canonical Wnt pathway, which results in transcriptional regulation of target genes via β-catenin, the planar cell polarity (PCP) pathway and the Wnt-dependent calcium/protein kinase C (PKC) pathway [1].

In the absence of Wnt ligands, cytoplasmic or soluble β-catenin is phosphorylated by a cytoplasmic 'destruction complex' at key amino-terminal Ser and Thr residues. The phosphorylation of β-catenin targets it for degradation. The 'destruction complex' consists of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), casein kinase 1 (CK1) and protein phosphatase 2A (PP2A) [2]. In the presence of Wnt proteins, transmembrane receptors Frizzled and low density lipoprotein receptor-related protein 5/6 (LRP5/6) recruit the cytoplasmic protein dishevelled (Dvl) and Axin to the receptor complex, which results in the disruption of the 'destrution complex' [3, 4]. β-catenin then accumulates in the cytoplasm and nucleus where nuclear β-catenin interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factors (LEF1/TCFs) to initiate target gene transcription.

In pathological conditions, β-catenin escapes degradation and cells retain canonical Wnt signaling caused by mutations in APC, Axin or β-catenin [2]. More than 90% of all colorectal cancers have an activating mutation of the canonical Wnt signaling pathway [5]. Constant activation of Wnt/β-catenin signaling is thought to be an initiating event in colorectal carcinogenesis. Colorectal carcinomas develop through a series of well-characterized

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histopathological changes: intestinal epithelial crypts, aberrant crypt focus, adenoma and carcinoma [6]. Every change is a result of some specific genetic mutation in a handful of oncogenes and tumor-suppressor genes. The earliest genetic alterations in the genesis of colorectal tumors are the mutations in the APC gene, which cause chromosome instability and β-catenin accumulation. APC mutations occur in the very earliest stages of tumorigenesis, known as aberrant crypt foci, and seem to be required to initiate clonal evolution [6]. Moreover, Wnt/β-catenin signaling is also involved in cancer progression. Some inhibitors of Wnt/β-catenin signaling, such as DKK1 and ICAT, inhibit proliferation of colorectal tumor cells [7]. Conventional treatments for colorectal cancer such as radiation therapy and chemotherapy are mostly dependent on p53-mediated apoptosis [8, 9] and are not satisfactory because of the mutation of *p53* in many cases of colorectal cancer. The concept that the Wnt/β-catenin pathway is a good therapeutic target for colorectal cancer is becoming more accepted as more research unequivocally demonstrates a role of Wnt signaling in tumorigenesis.

Due to mutations in *APC*, *Axin* or even *β-catenin* itself, β-catenin degradation is not properly regulated in these cancer cells. Therefore, a drug targeting the β-catenin/TCF transcription complex is likely to have therapeutic benefit. Screening for new inhibitors of the Wnt/β-catenin signaling pathway, especially that downstream of β-catenin is thus of great importance and significance. Here we report the identification of a novel low molecular weight diterpenoid derivative, NC043, as a Wnt/β-catenin signaling inhibitor that interferes with the β-catenin/TCF4 association.

Results

NC043 inhibits the activity of Wnt/β-catenin signaling reporter Top-flash

In order to identify potential small molecules that regulate β-catenin stability or its downstream signaling, we used a Top-flash (Wnt/β-catenin pathway-responsive firefly luciferase plasmid) reporter gene assay in a three-step screen. In the first step, HEK293T cells were co-transfected with Top-flash and Wnt1 plasmids where over-expressed Wnt1 activates Wnt/β-catenin signaling resulting in expression of the reporter. Chemical compounds were added and the luciferase activity was measured to identify compounds that inhibited Wnt1-induced reporter activity. The compounds whose inhibition ratio was more than 50% were selected for the second step screen, in which Wnt3a conditioned medium (CM) and LiCl (lithium chloride) were added as agonists. LiCl is a GSK-3β inhibitor, which prevents the function of β-catenin degradation complex and leads to the accumulation of β-catenin in cytoplasm and nucleus. Thus, whether inhibiting LiCl-induced Top-flash reporter activity will distinguish compounds functioning upstream or downstream of β-catenin stability. The compounds that inhibit both Wnt3a- and LiCl-stimulated Top-flash activity in the second screen were further tested in the SW480 and Caco-2 colon carcinoma cell lines. β-catenin is stabilized, which leads to constitutive transcription of downstream target genes, due to APC truncation in these cell lines [10, 11]. Compounds whose inhibition ratio was more than 60% were considered positive hits.

We screened a small molecule library of 4 000 compounds and identified NC043 as the most potent compound to antagonize Wnt3a- and LiCl-stimulated Top-flash reporter activity (Figure 1A). The identity of NC043 is 15-oxospiramilactone, a semi-synthetic diterpenoid derivative (with a molecular weight of 330 Da) from spiramilactone (S2) [12] through an oxidation reaction as described in Materials and Methods. *Spiraea* diterpenoid alkaloids and diterpenoids (including spiramilactone) are a number of atisine-type natural products with a C_{20} skeleton obtained from the complex of *Spiraea japonica* (Rosaceae) [12-16], a Chinese herbal medicine widespread in Yunnan Province and used for anti-inflammation and analgesia in folk and ethnic traditions [17]. We also detected the effect of NC043 on Top-flash activity in colon cancer cell lines, SW480 and Caco-2. NC043 inhibited Top-flash activity in a dose-dependent manner after 24 hours (Figure 1B) or 3 hours (Supplementary information, Figure S1) of treatment.

To characterize the structure-activity relationship of NC043, we synthesized additional NC043 derivatives (Figure 1C**)** and examined their effects on Top-flash activity (Figure 1D). NC043 and its derivatives had different effects on Top-flash activity. S1 and S2 had no effect on both Top-flash and Fop-flash activity (Figure 1D). Although SR-37 exhibited inhibitory effects on both Top-flash and Fop-flash activity, the ratio between Top-flash and Fop-flash luciferase values did not change compared to DMSO (Figure 1D). These results suggest that the 15-carbonyl group of NC043 might be responsible for its inhibitory effect and the spatial configuration formed by its lactone group is responsible for its inhibition specificity.

NC043 selectively inhibits Wnt signaling and its target gene expression

To test the selectivity of NC043, we performed the NF-AT and CRE reporter gene assays using the corresponding agonists ionomycin [18] and forskolin [19] re-

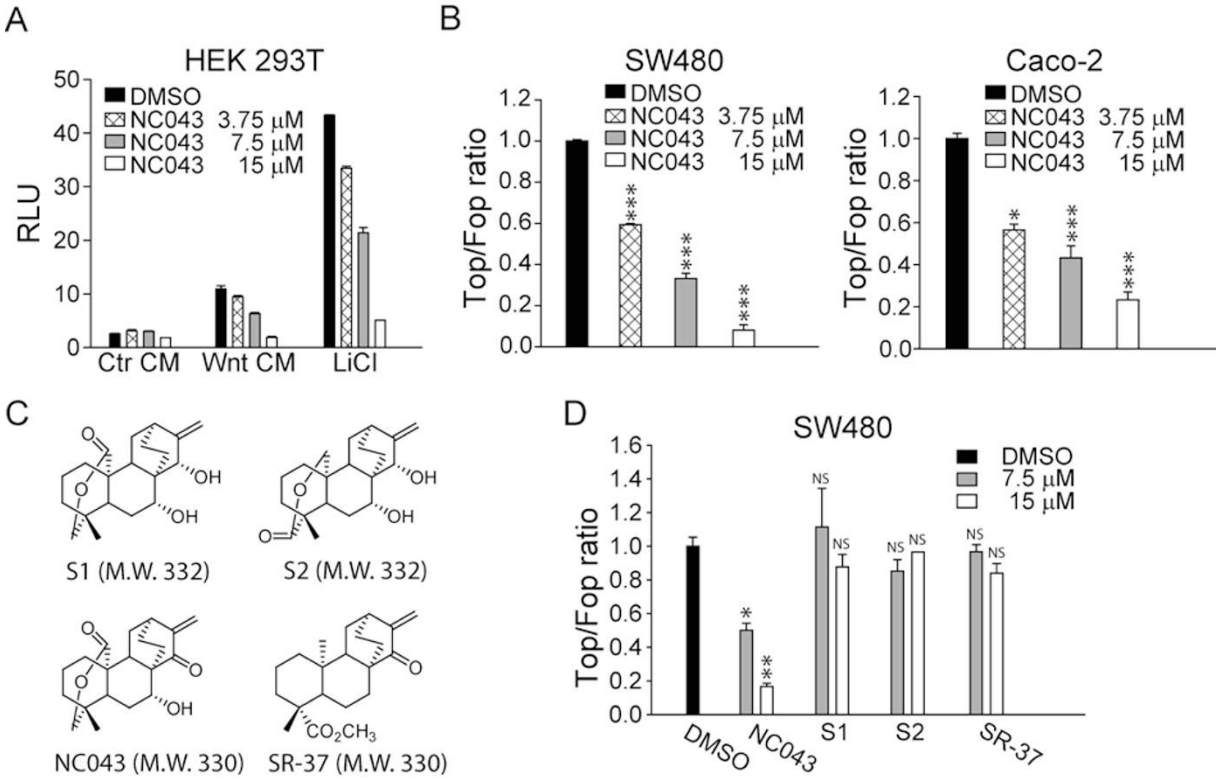

Figure 1 NC043 inhibits the Wnt/β-catenin signaling reporter Top-flash. **(A)** NC043 inhibits Top-flash activity in a dose-dependent manner in HEK293T cells. DMSO or NC043 with indicated dosage was added to cells 17 h after transfection with the Top-flash plasmid. One hour later, the cells were treated with control or Wnt3a conditioned medium (CM) or 20 mM of LiCl containing the same dosage of NC043 for additional 6 h. Luciferase activity was then measured. **(B)** NC043 inhibits Top-flash activity in a dose-dependent manner in colon carcinoma cells, SW480 and Caco-2. Cells were treated with NC043 for 24 h. Data represent the mean±S.D. from three independent experiments. **(C)** The chemical structure of NC043 and its derivatives. **(D)** The effect of NC043 derivatives on Top-flash activity in SW480 cells. Transfected SW480 cells were treated with NC043 and its derivatives and incubated for 24 h. Data represent the mean±S.D. from one experiment. Experiments were repeated three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significant relative to vehicle control. NS, no statistics significance.

spectively. As shown in Figure 2A, NC043 had little effects on NF-AT and CRE reporters at a concentration that significantly inhibited the Top-flash activity. To extend our studies to include the expression of genes known to be regulated by Wnt/β-catenin signaling in human cells, we monitored mRNA expression of *Axin2* [20, 21], *Survivin* [22] and protein expression of *Survivin*, *Cyclin D1* [23, 24]. NC043 showed dose-dependent inhibitory effects on the expression of these target genes, which could be seen as early as 3 hours post-treatment in SW480 cells (Figure 2B and 2C).

NC043 does not affect β-catenin stability and distribution but impairs β-catenin/TCF association

Based on its effect on the Top-flash activity induced by LiCl treatment (Figure 1A) or in APC-deficient colon cancer cells (Figure 1B), we deduced that NC043 may not affect β-catenin stability but rather affect its downstream transcription activity. To test this hypothesis, the cytosolic and nuclear fractions of cells with or without NC043 treatment were separated and β-catenin protein expression was determined. As shown in Figure 3A and Supplementary information, Figure S2, NC043 did not affect protein expression and distribution of β-catenin in HEK293T or SW480 cells.

Next we wanted to determine whether NC043 impairs β-catenin/TCF interaction. Nuclear lysates of HEK293T cells treated with NC043 or its derivatives were immunoprecipitated by a β-catenin antibody or mouse IgG as a control, and TCF4 was detected by western blot. NC043 but not its derivatives inhibited the β-catenin/TCF4 interaction (Figure 3B). NC043 also inhibited the binding of TCF4 to β-catenin in a dose-dependent manner in SW480 cells (Figure 3C).

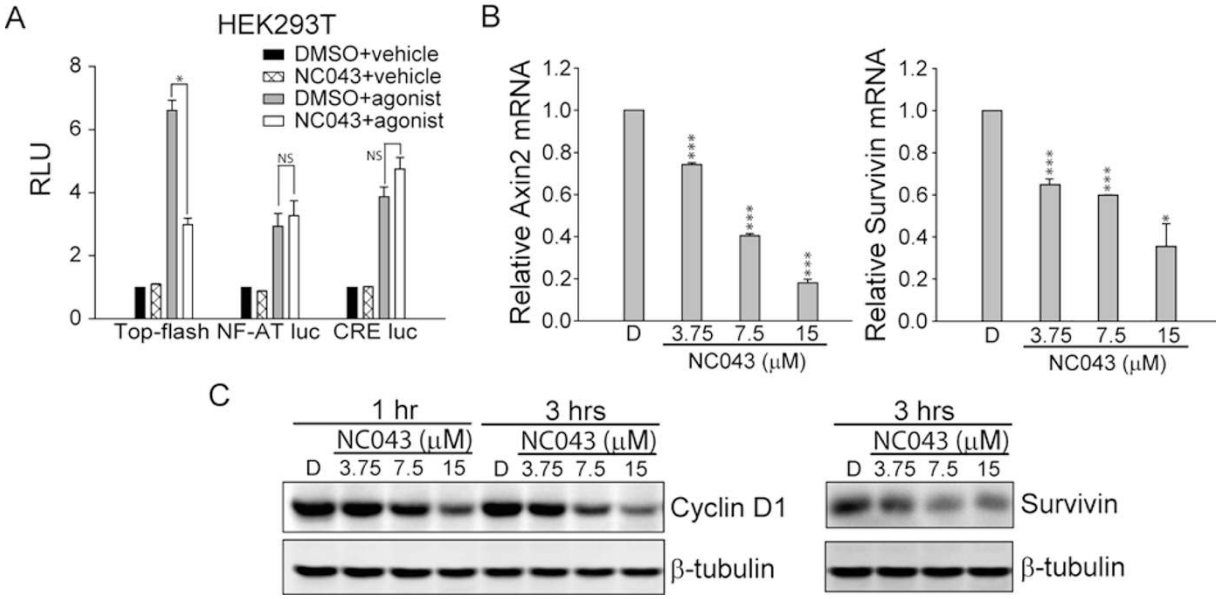

Figure 2 NC043 selectively inhibits Wnt signaling and its target genes expression. **(A)** NC043 specifically inhibits Top-flash activity. DMSO or NC043 was added to the cells 17 hrs after transfection. One hour after the incubation of NC043 (7.5 μM), Wnt3a CM for Top-flash, Ionomycin (1 μg/ml) for NF-AT luc and Forskolin (10 μM) for CRE luc (all of them containing the same dosage of NC043) were added for additional 6 h. **(B**, **C)** NC043 inhibits mRNA and protein expressions of *Axin2*, *Survivin* and *Cyclin D1*. SW480 cells was incubated with DMSO (shorten for **D**) or NC043 with indicated dosage for 3 h. Samples were then prepared for qPCR **(B)** or western blot **(C)**. Data represent the mean±S.D. from one experiment. Experiments were repeated three times. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant relative to vehicle control. NS, no statistics significance.

Component changes of the β-catenin/TCF transcription complex can also affect the stability and transcription activity of the complex. For example, we have previously reported that Dvl and c-Jun are components of the complex and loss of either one of them results in a reduction of β-catenin recruitment to its target promoter *in vivo* [25]. To validate whether the inhibition of β-catenin/TCF4 association caused by NC043 is a direct effect, biosensor binding studies were performed, and the result indicated that NC043 does not affect the direct binding of β-catenin and LEF1 or TCF4 *in vitro* (Figure 3D and Supplementary information, Figure S4). These data suggest that NC043 may inhibit β-catenin/TCF association indirectly, probably through additional components involved in the β-catenin/TCF complex.

β-catenin is involved in many cellular processes including an association with membrane-bound E-cadherin to form adherent junctions through domains that overlap with the TCF-binding armadillo repeats [26, 27]. This association mediates homotypic cell adhesion regulation. This overlap presents a potential risk that an active compound that inhibits β-catenin/TCF association may be toxic to normal cells. To exclude this possibility, we investigated the effect of NC043 on E-cadherin/β-catenin association by immunoprecipitating endogenous E-cadherin from SW480 colon cancer cells. NC043 had little effect on the interaction between β-catenin and E-cadherin (Figure 3E).

In order to investigate whether NC043 could also affect the DNA binding affinity of LEF1/TCFs, we performed Top-flash reporter gene assays with overexpression of ΔN-β-catenin or ΔN-LEF-VP16. ΔN-β-catenin is a constitutively stabilized form due to the lack of N terminal region which contains the phosphorylation sites responsible for mediating degradation. ΔN-LEF-VP16 consists of ΔN-LEF (lacking N-terminal β-catenin binding region) fused to the trans-activation domain from the herpes virus VP16 protein. As shown in Figure 3F, NC043 could inhibit Top-flash activity activated by ΔN-β-catenin but not by ΔN-LEF-VP16. Consistently, ChIP assays using β-catenin and TCF4 antibodies showed that treatment of NC043 led to a decrease in β-catenin but not TCF4 binding to the promoter region of the Wnt target gene *Cyclin D1* (Figure 3G). These results demonstrate that NC043 impairs β-catenin/TCF4 association without affecting TCF4 binding to TBE.

NC043 causes G2/M arrest and inhibits colon cancer cell growth

We also investigated the effect of the compound on

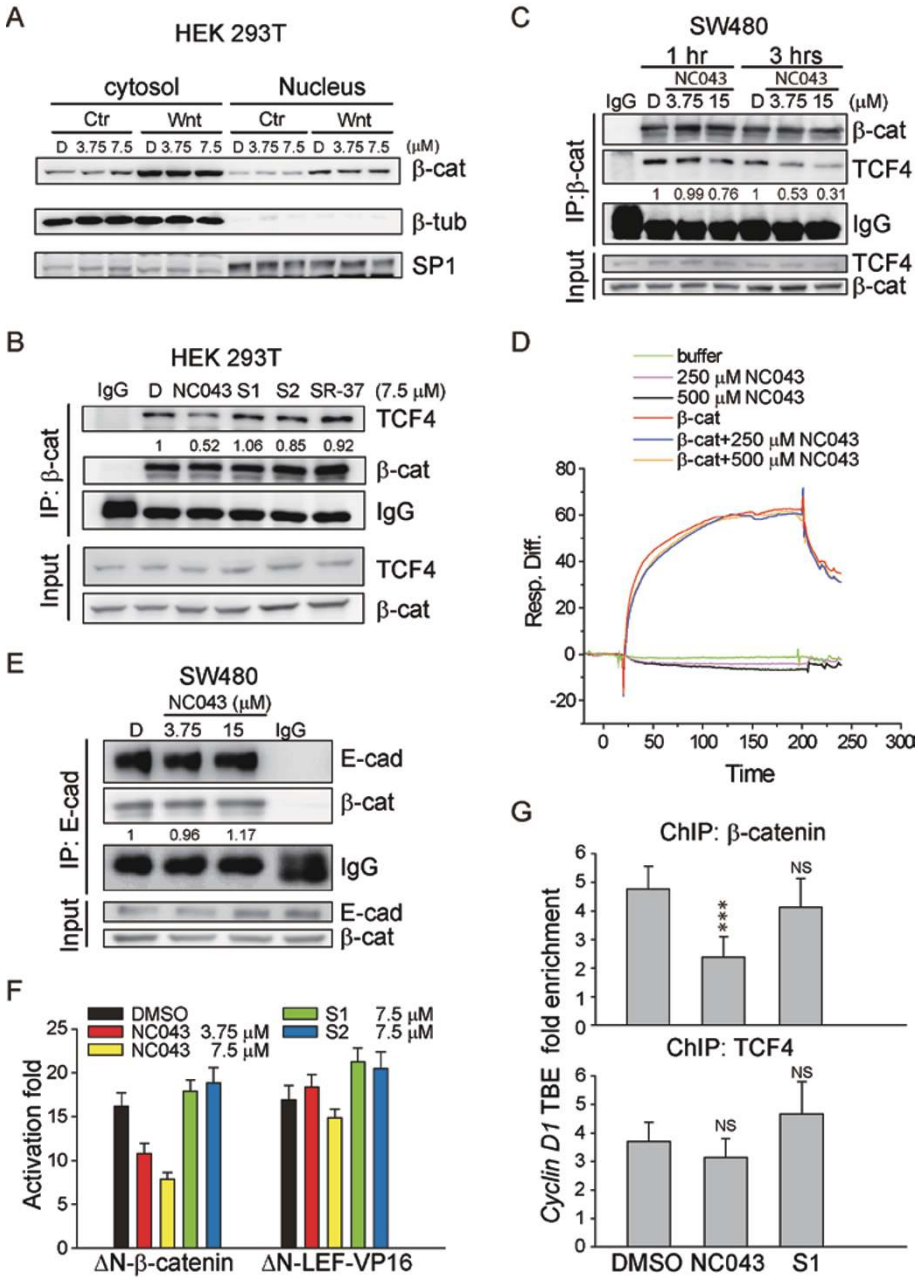

Figure 3 NC043 does not affect β-catenin stability and distribution but does impair β-catenin/TCF4 association. **(A)** NC043 has no effect on β-catenin protein expression and distribution in HEK293T cells. Cells were incubated with NC043 or DMSO. After 1 h cells were treated with control or Wnt3a CM containing the same dosage of NC043 for additional 3 h. The cells were then fractioned as described in Materials and Methods, and the samples were analyzed by western blot. β-tubulin was used as a cytosolic marker and loading control. SP1 was used as a nuclear marker and loading control. **(B**, **C)** NC043 but not its derivatives impair β-catenin/TCF4 association. HEK293T cells **(B)** were incubated with NC043 and its derivatives. One hour later, cells were treated with Wnt3a conditioned medium (CM) containing the same dosage of small molecules for additional 3 h. SW480 cells **(C)** were incubated with NC043 or DMSO. Nuclear fractions were immunoprecipitated by the β-catenin antibody and analyzed by western blot. For quantitative western blot analysis, the integrated intensity of each signal was measured. The TCF4/β-catenin signal ratios were shown. **(D)** NC043 does not affect β-catenin and LEF1 interaction *in vitro*. Biosensor Binding Studies were performed as described in Material and Methods. The equilibrium response data for 6His-β-catenin was fitted to 1:1 binding site model to obtain the K_D of ~1.84 μM. NC043 was then added with 6His-β-catenin. The equilibrium response data for NC043 was the same compared to the data without NC043. Data are representative of one experiment. Experiments were repeated at least three times. **(E)** NC043 does not affect β-catenin/E-cadherin association. SW480 cells were incubated with NC043 for 1 h. Whole cell lysates were then immunoprecipiataed by an E-cadherin antibody and analyzed by western blot. For quantitative western blot analysis, the integrated intensity of each signal was measured. The β-catenin/E-cadherin signal ratios were shown. **(F)** NC043 inhibits ΔN-β-catenin but not ΔN-LEF-VP16 induced Top-flash activity. DMSO or NC043 and its derivatives with indicated dosage was added to HEK293T cells for 6 h after transfection with the Top-flash, LacZ, ΔN-β-catenin and ΔN-LEF-VP16 plasmids. Luciferase activity was then measured. Data were calculated as the fold activation induced by ΔN-β-catenin and ΔN-LEF-VP16 compared with control plasmid (LacZ) and presented the mean±S.D. from three independent experiments. **(G)** NC043 depresses the recruitment of β-catenin but not TCF to the *Cyclin D1* promotor. SW480 cells were incubated with 30 μM of NC043 for 2 h and a ChIP assay was performed using the β-catenin and TCF4 antibodies. The product of ChIP was then analyzed by qPCR. Data represent the mean±S.E. from at least three independent experiments. ***P < 0.001, significant relative to vehicle control. NS, no statistics significance.

cellular activities. The MTT cell viability assay was carried out to assess the effects of NC043 and its derivatives on growth of colon cancer cells, SW480 and Caco-2. Normal colonic epithelial cells, CCD-841-CoN, were used as a control cell line. NC043 showed 4- to 6-fold higher IC_{50} in CCD-841-CoN cells than in carcinoma cells (Figure 4A). SR-37 had a 13-fold higher IC_{50} in Caco-2 cells and 3.3-fold higher IC_{50} in SW480 cells compared to NC043. S1 had little inhibitory effect on both carcinoma cell lines (Figure 4A). Considering the different inhibitory effects of SR-37, S1 and NC043 on Top-flash activity (Figure 1D) and β-catenin/TCF4 association (Figure 3B), these results demonstrate the inhibitory effect of NC043 on Wnt signaling and ultimately on colon cancer cell growth.

We have also observed a change in SW480 cell morphology after compound treatment. After 24-hours of NC043 treatment SW480 cells appeared flat and the nuclear size was increased (data not shown). This phenomenon suggests that NC043 may affect cell cycle progression. The distribution of SW480 cells in different phases of the cell cycle after the incubation of NC043 was investigated. As shown in Figure 4B, NC043 arrested SW480 cells at the G2/M phase of the cell cycle and increased apoptosis in a dose-dependent manner. It was previously shown that over-expression of ICAT or the central region of APC, which causes inhibitory effects on Wnt/β-catenin signaling, resulted in an arrest of colorectal cancer cells at the G2 phase of the cell cycle [28]. In light of this finding, the protein expression of Cdc25c and Cdc2, which are involved in regulating the G2/M checkpoint, was analyzed by western blot. Both Cdc25c and Cdc2 protein levels were greatly decreased in response to NC043 treatment (Figure 4C). These data together suggest that NC043 treatment leads to cell cycle arrest and suppression of SW480 cell growth through inhibitory the Wnt signaling pathway.

NC043 inhibits SW480 cell tumorigenesis in vitro and in vivo

In order to test for the potential application of NC043 in the treatment of human colon cancers, the inhibitory

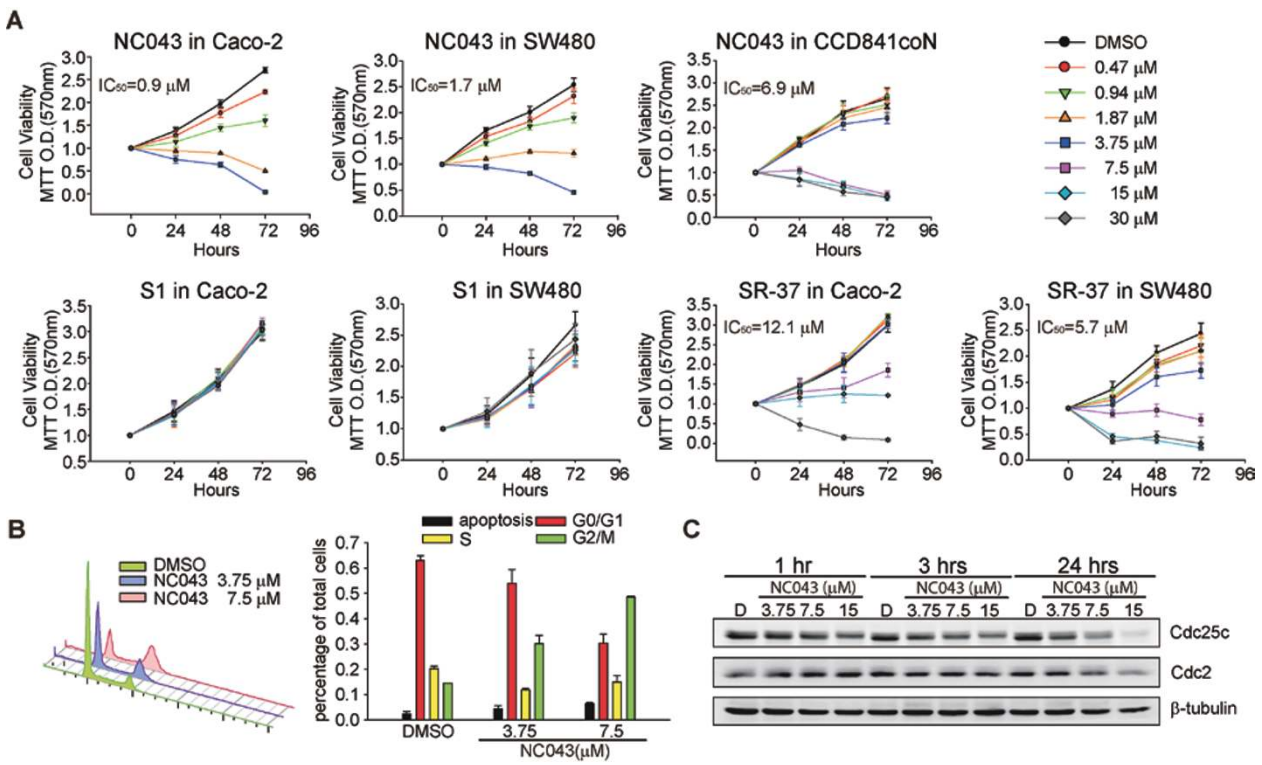

Figure 4 NC043 causes G2/M arrest and inhibition of colon cancer cells growth. **(A)** The effect of NC043 and its derivatives on cell growth of colon carcinoma cells (SW480 and Caco-2) or normal colonic epithelial cells (CCD-841-CoN). Cells were incubated with chemicals and then analyzed by MTT assay. IC_{50} of 72 h is shown. Data represent the mean±S.E. from three independent experiments. **(B)** NC043 arrests SW480 cells at G2/M phase of the cell cycle. SW480 cells were incubated with NC043 for 36 h, and were stained with PI and analyzed by FACS. Data represent the mean±S.D. from two independent experiments. **(C)** NC043 decreases Cdc25c and Cdc2 protein expression in a dose-dependent manner.

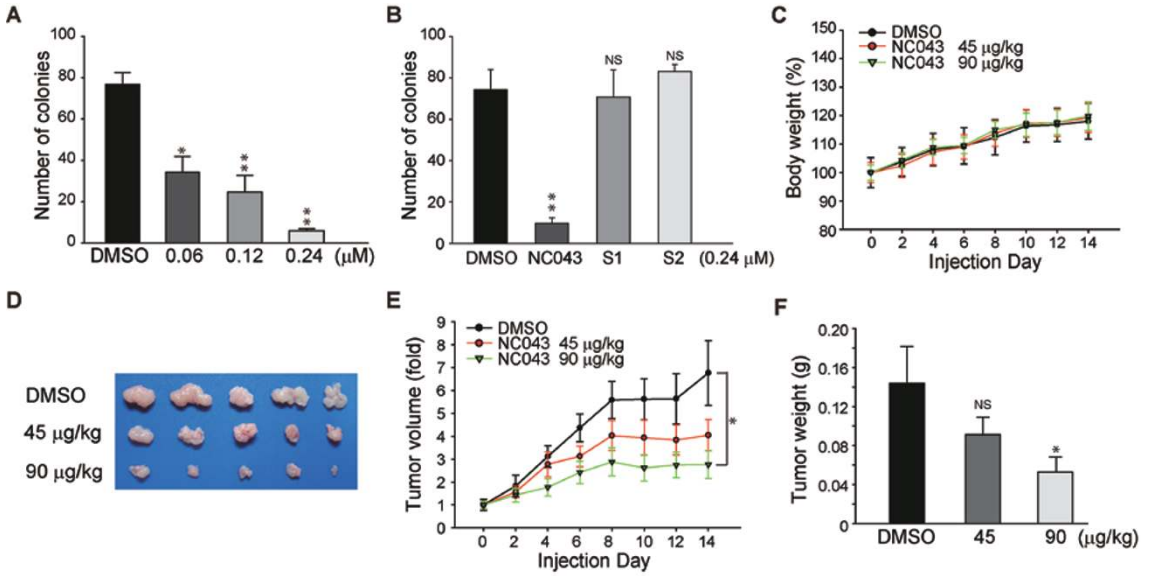

Figure 5 NC043 inhibits SW480 cell tumorigenesis *in vitro and in vivo*. **(A**, **B)** NC043 but not its derivatives inhibit anchorage-independent growth of SW480 cells. SW480 cells were seeded in soft agar with NC043 **(A)** and its derivatives **(B)** for 18 days and stained with crystal violet. Data represent the mean±S.E. from three independent experiments. **(C-F)** NC043 inhibits SW480 cell tumorigenesis in a xenograft model. Tumor bearing mice were treated intraperitoneally with either vehicle or NC043 (45 and 90 μg/kg) daily for 17 days. Body weight **(C)**, tumor size **(E)** and tumor weight **(F)** were measured as described in *Materials and Methods*. The external appearance of tumors is shown **(D)**. Data represent the mean±S.E., $*P < 0.05$, $**P < 0.01$, significant relative to vehicle control.

effect of NC043 on anchorage-independent growth of human colon cancer SW480 cells was determined by crystal violet staining. The result showed that incubation of SW480 cells with NC043 but not S1 or S2 in soft agar inhibited anchorage- independent growth of SW480 cells in a dose-dependent manner (Figure 5A, 5B and Supplementary information, Figure S3).

We further investigated its inhibitory effect on SW480 cell tumorigenesis *in vivo* using a mouse xenograft model. SW480 cells were injected subcutaneously into the flanks of nude mice to initiate tumor formation. Seven days after SW480 cell injection, tumor size reached around 80 mm^3. Tumor-bearing mice were randomly separated into three groups and intraperitoneally injected with vehicle or two dosages of NC043 (45 and 90 μg/kg) daily for 17 days. NC043 treatment did not have any effect on body weight as no difference in weight was observed between control and NC043-treated animals (Figure 5C). However, treatment with 90 μg/kg of NC043 resulted in a significant reduction in both tumor volume (Figure 5D and 5E) and tumor weight (Figure 5F) compared to the vehicle-treated group. These data demonstrate that NC043 inhibits tumor growth *in vivo*. Collectively our *in vitro* and *in vivo* results indicate that NC043 has the potential to inhibit tumorigenesis.

Discussion

There is compelling evidence that aberrant activation of the Wnt/β-catenin pathway is involved in the development and progression of many cancers [29-31]. Therefore, inhibition of Wnt/β-catenin signaling may have potentials in cancer treatment. This inhibition may be achieved by small-molecule compounds that block the interaction between β-catenin and TCF [32] or CREB-binding protein (CBP) [33] or stabilize Axin2 [34], siRNAs that target Dvl, and antibodies against Wnt proteins. Other potential therapeutics include inhibitors that indirectly inhibit Wnt/β-catenin signaling such as extracellular calcium, non-steroid anti-inflammatory drugs (exisulind, sulindac, aspirin), and the tyrosine kinase inhibitor STI571/Gleevac [35]. In previous research, several compounds have been found to be able to interrupt β-catenin/TCF association directly, such as PKF115-854 and CGP049090, etc. Although these compounds inhibit β-catenin/TCF4 binding, their effects on *in vivo* tumor formation were not investigated [32, 33].

Our screening has resulted in the identification of a novel small molecule NC043 that inhibits β-catenin/TCF transcription activity and colon cancer cell tumorigenesis *in vitro* and *in vivo*. NC043 may affect β-catenin/TCF4

association indirectly due to the finding that NC043 can impair β-catenin/TCF4 association *in vivo* but not *in vitro* (Figure 3B-3D and Supplementary information, Figure S4). It is reasonable to hypothesize that NC043 may target or function through a known or unknown component in the β-catenin/TCF4 transcription complex.

Our data as well as published data demonstrate that Wnt signaling regulates the cell cycle. Although the mechanisms by which inhibition of Wnt/β-catenin signaling down-regulates the expression of Cdc25c, Cdc2, and cyclin B1, and finally causes G2 arrest are not clear, the fact that the Wnt target gene *Axin2* has a function in mitosis may answer the question partially. Axin2 (also known as conductin) is highly up-regulated in the majority of colorectal tumors [21] and localizes to the mitotic spindles and centrosomes where it forms protein complexes with the mitotic and spindle checkpoint regulator polo-like kinase 1 (PLK1). Knock-down of Axin2 by siRNA in colon carcinoma cells or gene ablation in mouse embryo fibroblasts allows over-activation of the spindle checkpoint in response to spindle stress [36]. By contrast, activated Wnt signaling stimulates the APC or β-catenin mutant cells to escape from mitotic arrest and apoptosis [37]. Perhaps NC043 regulates the activity of Axin2 leading to cell cycle arrest in the G2/M phase (Figure 4B). In a recent report, LRP6 phosphorylation and Wnt/β-catenin signaling have been shown to be under cell cycle control and peak at the G2/M phase [38]. This finding suggests that Wnt signaling and the cell cycle may have some unexplored and intimate relationship.

The differences in the observed effects on Top-flash activity, β-catenin/TCF4 association and SW480 cell growth between NC043 and its derivatives provide information regarding the relationship between NC043's biological functions and its structure. S1 and S2, which lack the 15-acetonyl group, did not inhibit Top-flash activity, β-catenin/TCF4 association and carcinoma cell growth (Figures 1D, 3B and 4A), indicating that the 15-acetonyl group is responsible for the inhibitory effects of NC043. SR-37, a derivative which loses the spatial configuration formed by the lactone bond, did not specifically inhibit Wnt signaling activity as measured by Top/Fop ratio and β-catenin/TCF4 association but did slightly inhibit cancer cell growth (Figures 1D, 3B and 4A), suggesting that the spatial configuration formed by the lactone bond may be essential for NC043 to bind its target molecule, resulting in specific inhibition of Wnt signaling. Collectively, these results demonstrate that NC043 is a potent and selective Wnt/β-catenin signaling inhibitor.

NC043 comes from spiramines through chemical reactions *in vitro*. Spiramines, separated from the extract of the roots of a Chinese herbal medicine *Spiraea japonica* (Rosaceae), inhibit platelet aggregation, increase antioxidant enzymatic activity and inhibit nitric oxide production [39, 40]. The anti-tumor activity shown in this report ascribes new anti-cancer properties to diterpene alkaloids including NC043.

Materials and Methods

Synthesis of 15-oxospiramilactione

Active MnO_2 (900 mg, 6.34 mM) was added to a CH_2Cl_2 solution (25 ml) of spiramilactone (S1 in Figure 1C, 200 mg, 0.60 mM) [11]. The reaction solution was mixed at room temperature for 3 days. After filtration and solvent removal, the reaction residure was chromatographed over column of silica gel and eluted with $CHCl_3$:Me_2CO (12:1) to give 120 mg of NC043 (15-oxospiramilactione) with a yield of approximately 60%.

NC043 (15-oxospiramilactione): mp 160-165 ºC; $[\alpha]^{10}_D$-63.7 (c 0.10, $CHCl_3$); UV ($CHCl_3$) λmax (log ε) 240 (3.71) nm; EIMS *m/z* (%): 330 $[M]^+$ (56), 312 $[M\text{-}H_2O]^+$ (100), 284 (55), 238 (15), 210 (17), 197 (22), 171 (10), 145 (14), 95 (5), 69 (3), 55 (3); 1H NMR ($CDCl_3$, 400 MHz) δ: 0.92 (3H, s, H-18), 4.07 (1H, d, J = 11.6 Hz, H-19a), 4.10 (1H, dd, J = 4.2, 11.9 Hz, H-7), 4.23 (1H, dd, J = 2.3, 11.6 Hz, H-19b), 5.23 (1H, d, J = 1.5 Hz, H-17a), 5.93 (1H, d, J = 1.5 Hz, H-17b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 40.7 (C-1, t), 20.3 (C-2, t), 37.5 (C-3, t), 32.8 (C-4, s), 47.1 (C-5, d), 16.7 (C-6, t), 69.8 (C-7, d), 50.8 (C-8, s), 43.7 (C-9, d), 46.0 (C-10, s), 27.3 (C-11, t), 36.0 (C-12, d), 23.5 (C-13, t), 27.1 (C-14, t), 205.1 (C-15, s), 147.9 (C-16, s), 117.2 (C-17, t), 23.6 (C-18, q), 76.5 (C-19, t), 174.2 (C-20, s).

Reagents and antibodies

The antibodies used were: Cdc25c, Cdc2 and Cyclin D1 (Santa Cruz Biotechnology, Inc.), E-cadherin and β-catenin (BD Biosciences), TCF-4 (Millipore), SP1 (Sigma, Inc.), Survivin (Cell Signaling, Inc.). Wnt-3a conditioned medium (CM) and control medium were described previously [4].

Cell culture

SW480 cells were maintained at 37 °C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Caco-2, CCD-841-CoN and HEK293T were maintained according to recommendations of ATCC.

Cell transfection and luciferase assay

HEK293T, SW480 and Caco-2 cells were transfected using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. The LacZ plasmid was also added to make the total amount of transfected DNA equal. For reporter gene assays, HEK293T, SW480 and Caco-2 cells were seeded in 24-well plates. Each well of HEK293T cells was transfected with 250 ng of plasmids in total, including 20 ng of Top-flash or Fop-flash or TAL-CRE luc or 40 ng of CRE luc, 25 ng of EGFP-C1 and other plasmids as indicated. Each well of SW480 or Caco-2 cells was transfected with 500 ng of plasmids in total, including 100 ng of Top-flash or Fop-flash, 100 ng of EGFP-C1 and other plasmids as indicated. 3 or 24 h after transfection, cells were lysed and luciferase activity was measured and normalized to GFP expression levels as described previously [41].

Cytosol and nucleus fractionation

HEK293T or SW480 cells were grown in 6-well plates or SW480 cells were grown in 60 mm dishes and harvested with a cell scraper into 1.5 ml PBS and spun at 700× *g* for 10 min. The pelleted cells were resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 10 mM NaF, 2 mM $NaVO_4$, 1 mM pyrophosphoric acid and Complete™ protease inhibitors) and incubated on ice for 10 min. Cells were then passed through the 0.4 mm needlepoint until the cell membrane was broken up and centrifuged at 700× *g* at 4 °C for 10 min. The supernatant was collected and centrifuged at 100 000× *g* at 4 °C for 1 hr. The supernatant from the ultracentrifugation was collected as the cytosolic fraction. For nuclear protein extraction, the pellet from the 700× *g* centrifugation was washed by buffer A once and then resuspended in buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 10 mM NaF, 2 mM $NaVO_4$, 1 mM pyrophosphoric acid and Complete™ protease inhibitors) and incubated on ice for 30 min. Nuclear extracts (supernatants) were recovered after centrifugation at 100 000× *g* at 4 °C for 1 h.

Endogenous co-immunoprecipitation

The nuclear fraction of 3 ~ 5 × 10^7 SW480 or HEK293T cells was isolated as described above. Then the fraction was lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% [vol/vol] Triton X-100, 5 mM EDTA, and proteinase inhibitors) and centrifuged at 16 000× *g* for 15 min at 4 °C. The supernatant was incubated with a specific primary antibody overnight at 4 °C in addition to A/G plus agarose (Santa Cruz Biotechnology, Inc.) for the last 2 h of incubation. The beads were washed five times and resuspended in 50 μl SDS loading buffer.

RT-PCR and quantitative real-time PCR

Total RNAs were extracted from cultured cells with TRIzol and reverse transcription of purified RNA was performed using oligo (dT) priming and superscript III reverse transcription according to the manufacturer's instructions (Invitrogen). Quantification of all gene transcripts was done by quantitative PCR (qPCR) using the TaKaRa SYBR *Premix Ex Taq* kit and a Rotor-Gene RG-3000A apparatus (Corbett Research). The values of *Axin2* and *Survivin* were shown against the value of *GAPDH* which was used as a control. The primer pairs used for the human Axin2 gene were 5′-CTGGCTTTGGTGAACTGTTG-3′, 5′-AGTTGCTCACAGCCAAGACA-3′. The primer pairs used for the human GAPDH gene were 5′-GCACCACCAACTGCTTA-3′ and 5′-AGTAGAGGCAGGGATGAT-3′. The primer pairs used for the human Survivin gene were 5′-CCGACGTTGCCCCCTGC-3′ and 5′-TCGATGGCACGGCGCAC-3′. The primer pairs used in ChIP assay for the human *Cyclin D1* TBE (TCF Binding Element) were 5′-CCTCCCGCTCCCATTCTCTGCCG-3′, 5′- CCTCGCCGGAGCGTGCGGACTCTG-3′.

ChIP assays

1 × 10^7 SW480 cells were prepared for the ChIP assay. The ChIP protocol was performed as described previously [42]. Quantification of all ChIP sample was done by quantitative PCR (qPCR) using the TaKaRa SYBR *Premix Ex Taq* kit and a ABI 7500 fast. Data are presented as fold changes calculated by each antibody ChIP value (IP/Input, the percentage of input) relative to IgG control ChIP value.

Biosensor Binding Studies

The 6His-β-catenin, GST-LEF1 and GST-TCF4-N (residues 8-53) [32] were expressed in *E. coli* BL21 DE3 grown in a standard Luria-Bertani (LB) broth medium, and were purified by affinity chromatography. The experiments were carried out at 10 °C using an optical binding sensor (Biacore T100, Core Facility of Molecular Biology) equipped with CM5 sensor chips. Purified GST-LEF1 or GST-TCF4-N was coupled to the chip through amine coupling while purified GST was coupled as a control. 6His-β-catenin was added by injection at 10 μl/min for 3 min with increasing concentrations.

FACS analysis of cell cycle

SW480 cells were grown in 60 mm dish, harvested and spun down (800 rpm, 5 min, 4°C). The pellet was washed twice with PBS and resuspended with 300 μl of PBS. Cells were fixed with ethanol (final concentration of 70%) overnight at –20 °C, spun down, and washed with PBS. Cells were then dyed with 500 μl of PBS containing 50 μg/ml Propidium Iodide, 200 μg/ml RNase A and 1% Triton X-100 for 1 h at 37 °C and analyzed on a BD FACS Calibur.

SW480 cell anchorage-independent growth assay

2.5 ml of 0.6% agar in DMEM supplemented with 10% FBS was layered onto each well of 6-well tissue culture plates. SW480 cells (4 × 10^3) were added to 0.3% agar-basal DMEM supplemented with 10% FBS and the mixture was added to the top of the 0.6% agar layer. To analyze the inhibitory effect of NC043 and its derivatives on SW480 cell anchorage-independent growth, both layers of agar were supplemented with small molecules. Cells were incubated at 37 °C in 5% CO_2 for 18 days, and the number of colonies was scored by crystal violet staining.

MTT cell viability assay

SW480 (1 × 10^4), Caco-2 (5 × 10^3) and CCD-841-CoN (5 × 10^3) cells were seeded into each well of 96-well plates. After being cultured for 24 h in a CO_2 incubator, the cells were treated with the dose rang from 0.47 to 10 μM of NC043 and its derivatives or DMSO control for 72 h. The medium was then changed and replaced with 200 μl of fresh growth medium with 10% FBS and 20 μl of MTT solution (Sigma). Cells were incubated for another 4 hrs and the medium replaced by with 200 μl of DMSO. After 10 min of incubation at 37 °C the optical absorbance was measured using a micro-plate reader at a 570 nm. The results are presented as percentage of cell viability. Data of 0 hour was set to 1 in each panel.

Xenograft studies

Fifteen male athymic nude mice (4 weeks old) were purchased from the Chinese Academy of Science Shanghai SLAC Laboratory Animal Co. (SLACCAS, Shanghai, China) and acclimated for 4 days. 3.3 × 10^6 SW480 cells in 80 μl of growth medium with 10% FBS were implanted into the flanks of each nude mouse. Tumor size was measured every three days in two dimensions with calipers and calculated using the formula $(L \times W^2)/2$, where L is length and W is width. After the tumor size reached around 80 mm^3, which occurred 7 days after cell injection, the mice were randomly assigned into two NC043 treatment groups with dose of 45 and 90 μg/kg and a vehicle control group. NC043 in 60 μl PBS

(with 3% DMSO) was injected intraperitoneally into each mouse daily for 17 days. Control mice were intraperitoneally injected with 60 μl PBS (with 3% DMSO) as a vehicle control daily for 17 days. Tumors were removed from mice 24 days after SW480 cell injection and flash-frozen in liquid Nitrogen.

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