Mitochondrial uncoupler exerts a synthetic lethal effect against β-catenin mutant tumor cells

Yuki Shikata,1 Masaki Kiga,1 Yushi Futamura,2 Harumi Aono,2 Hiroyuki Inoue,3 Manabu Kawada,3,4 Hiroyuki Osada2 and Masaya Imoto1

1Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Yokohama; 2Chemical Biology Research Group, RIKEN Center for Sustainable Resource Science (CSRS), Saitama; 3Numazu Branch, Institute of Microbial Chemistry, Shizuoka; 4Laboratory of Oncology, Institute of Microbial Chemistry, Tokyo, Japan

Key words
Antitumor activity, apoptosis, uncoupler, Warburg effect, β-catenin

Correspondence
Masaya Imoto, Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan. Tel/Fax: +81-45-566-1557; E-mail: imoto@bio.keio.ac.jp

Funding Information
JSPS KAKENHI, Grant/Award Number: ‘JP 15H03116’ MEXT KAKENHI; (Grant/Award Number: ‘JP23102006’)

Received November 14, 2016; Revised January 14, 2017; Accepted January 16, 2017

Cancer Sci 108 (2017) 772–784
doi: 10.1111/cas.13172

The wingless/int-1 (Wnt) signal transduction pathway plays a central role in cell proliferation, survival, differentiation and apoptosis. Interaction of a Wnt ligand with its receptors: Frizzled and LRPS/6, allows β-catenin to accumulate in the cytosol and translocate to the nucleus, leading to the activation of Wnt target genes, including cyclin D1 and c-Myc. Typically, this activity is tightly regulated by the “destruction complex” which consists of Axin, an adenomatous polyposis coli (APC), casein kinase 1α (CK1α), glycogen synthase kinase 3β (GSK3β) and β-TrCP. Most human cancer cell lines contain mutations in β-catenin at codons 33, 41, and 45. CK1 initially phosphorylates Ser45 of β-catenin, and phosphorylated Ser45 primes GSK-3-mediated phosphorylation of Thr41, which in turn primes successive phosphorylation of Ser37 and Ser33 by GSK-3. Cancer cells in which these key phosphorylated serines or threonine are mutated have inappropriately high levels of β-catenin that activate Wnt target genes. It has been reported that β-catenin is mutated in a wide variety of human tumors, including colon, liver, ovarian, and prostate cancer. In addition, approximately 10% of all human tumors harbor activating mutations in β-catenin.

As described above, activation of β-catenin plays a critical role in the pathogenesis of human tumors. Therefore, this pathway has been considered a promising target for therapeutic intervention, and several molecularly targeted agents have been developed. Some have already entered Phase I studies for solid tumors, such as a Porcupine inhibitor LGK974, a pan-Frizzled antibody OMP-18R5, a Wnt ligand antibody OMP-54F28, and a CBP inhibitor PRI-724. However, no approved drugs are clinically available for treatments that target the Wnt signaling pathway. In addition, most of these agents induce growth arrest in tumor cells by targeting the Wnt signaling pathway. Thus, the tumor cells do not die and can acquire resistance.

We have previously reported that MEK1/2 inhibitors induce cell growth arrest in β-catenin wild type tumor cell lines, whereas they induce apoptosis in β-catenin mutant tumor cell lines in vitro. Furthermore, we have reported that a MEK inhibitor alone could induce significant tumor regression in β-catenin-mutant xenograft models. These findings support the clinical use of MEK inhibitors as single agents for patients with colorectal carcinoma who carry active β-catenin mutations. To find another effective compound that is effective for patients with tumors who carry active β-catenin mutations, we screened for compounds that exhibited synthetic lethality with β-catenin mutations, other than MEK1/2, from an in-house natural product library. In addition, we also found that V-ATPase inhibitors induced apoptosis preferentially in β-catenin mutant tumor cells. In our continued screening, we have now identified nonactin as a hit compound. Nonactin is a member of a family

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of naturally occurring cyclic ionophores known as macrotetro-
lide antibiotics. In this study, we show that nonactin exhibits
high selectivity against β-catenin mutant tumor cell lines with
apoptosis induction in vitro and in vivo. We also demonstrate
the mechanism by which nonactin selectively induces apoptosis
in cell lines harboring active mutant β-catenin.

Material and Methods

Reagents and compounds. Nonactin, valinomycin, carbonyl
cyanide m-chlorophenyl hydrzone (CCCP), and 2-deoxyglu-
cose (2-DG), Z-VAD-FMK were purchased from Sigma-Aldrich
(St. Louis, MO). Valinomycin and monensin were purchased from
Cayman Chemical (Ann Arbor, MI). Wnt-3a ligand was
purchased from Wako Pure Chemical Industries (Osaka, Japan).

Isolation of nonactin from microbial extract. Nonactin was
isolated from a culture broth extract of streptomyces. The broth
was purified using HPLC (MG-II, 4.6 mm, 250 mm; Shi-
ceido, Tokyo, Japan) with 85% aqueous MeOH to obtain pure
nonactin.

Cell lines. A431 cells were provided by M. Kawada (Institute of
Microbial Chemistry, Japan). The other cell lines were obtained
from the ATCC (Rockville, MD, USA). A431 cells were maintained in Dulbecco’s modified eagle medium (DMEM)
supplemented with 5% calf serum (CS), 100 U/mL penicillin
G (Sigma-Aldrich), and 0.1 mg/mL kanamycin (Sigma-Aldrich)
at 37°C in a humidified atmosphere containing 5% CO2. The other cell lines were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin G, and 0.1 mg/mL kanamycin in the same conditions described above.

Western blot analysis. Anti-PARP. Anti-cleaved-PARP and
anti-phospho-ERK1/2 were purchased from Cell Signaling
Technology (Danvers, MA, USA). Anti-β-catenin was pur-
bought from BD Biosciences (San Jose, CA, USA). Anti-β-actin,
HRP-linked anti-mouse IgG, and HRP-linked anti-rabbit IgG
were purchased from Sigma-Aldrich. Cells were treated with
compounds for 24 h. In the co-treatment test using both 2-DG
and an uncoupler, cells were pre-treated with 10 mM 2-DG for
1 h before being treated with the uncoupler. Following treat-
ment, cells were immediately harvested and lysed with RIPA
buffer (25 mM HEPES, 15% TX-100, 1% sodium deoxy-
cholate, 0.1% SDS, 500 mM NaCl, 5 mM EDTA, 50 mM NaF,
0.1 M Na3VO4, and cOmplete Protease Inhibitor Cocktail
Tablets (Roche, Germany); pH 7.8). The lysates were centri-
fuged at 13,000 g for 15 min to remove the insoluble fraction.
Equal amounts of total protein were subsequently separated by
sodium dodecyl sulfate-polyacrylamide gel electrophoresis
(SDS-PAGE) and probed with the indicated antibodies. The
chemiluminescence signal was detected using an Immobilon
Western kit (Merck Millipore, Billerica, MA, USA) and Chemi-
Doc XR5+System (Bio-Rad, Hercules, CA, USA).

Cell cycle and apoptosis analysis by flow cytometry. Percent-
ages of cells in different phases of the cell cycle, were ana-
lyzed by flow cytometry following staining with propidium
iodide (PI; Wako Pure Chemical Industries), modified from a
previously reported method.265 Cells were treated with compo-
unds for 48 h, then were harvested and fixed with 70% EtOH at 4°C, followed by treatment with 10 μg/mL RNase A
(Wako Pure Chemical Industries) for 20 min at 37°C. Subse-
quently, cells were stained with 50 μg/mL PI. PI fluorescence
was measured using an EPICS ALTRA Flow Cytometer
(Beckman Coulter, Brea, CA, USA).

Growth inhibition assay. Cell growth was measured by a
CellTiter-Glo Luminescent Cell Viability Assay (Promega,
Madison, WI, USA) according to the manufacturer’s protocol.
The luminescence was detected by Fluoroskan Ascent FL
(Labsystems, Helsinki, Finland), and this was compared to the
luminescence recorded at 0 h. The growth rate of each cell
type at each concentration of nonactin was calculated using
nonactin-treated luminescence at 48 h (Xi), control lumines-
cence at 48 h (Xctrl), luminescence at 0 h (X0), and the fol-
lowing formula:

\[
\text{Growth rate} = \frac{X_i}{X_{ctrl}}
\]

If \( X_i \geq X_{0, h} \), Growth rate = \( \frac{X_i}{X_{0, h}} - 1 \)

If nonactin induced cell cycle arrest in the cells, the growth
rate would be near zero. On the other hand, if nonactin
induced cell death in the cells, the growth rate would be below
zero.

Overexpression of wild type and active mutant β-
catenin. Transfection of wild type and active mutant β-catenin
(S37A, S45A) plasmid vectors and control vector was per-
fomed using PLUS Reagent and Lipofectamine LTX (Invitro-
gen Life Technologies, Carlsbad, CA, USA) according to the
manufacturer’s protocol.

Monitoring of mitochondrial membrane potential. Loss of
mitochondrial membrane potential was assessed using JC-1
(Wako Pure Chemical Industries), modified from a previously
reported method. Cells were treated with compounds for
30 min, then were stained with 2.0 μM JC-1 for 30 min at
37°C. Subsequently, cells were harvested and red and green
fluorescence was observed using a fluorescence microscope
(IX71, Olympus, Japan). The images were captured with a
digital camera (Olympus). Red and green fluorescence was
quantified using ImageJ and the red/green ratio was calcu-
lated.

Assessment of glycolysis ability. The glycolysis ability of
cells were assessed using the XF96 Extracellular Flux Ana-
lyzer (Seahorse Bioscience, Billerica, MA, USA) according to
the manufacturer’s protocol.28 In brief, 20,000 cells were
seeded in 96-well plates designed for XF96 and, following
overnight culture, were resuspended in RPMI medium without
D-glucose, serum and sodium bicarbonate. After incubation
in the absence of CO2, 1 h, the extracellular acidification
rate (ECAR) was measured using 50 mM 2-DG and 11 mM
D-glucose.

Anti-tumor tests in a xenograft model. Specific pathogen-free
female nude mice (BALB/Ca Icl-nu) were purchased from
CLEA Japan (Tokyo, Japan). A tumor cell suspension
(2 × 10^6 cells/mL) was inoculated subcutaneously into the
axillary region of the nude mice on Day 0. Nonactin was dis-
solved in 10% dimethylacetamide (Wako Pure Chemical
Industries), 10% NIKKOL HCO60 (Nikko Chemicals, Tokyo,
Japan), and saline (Otsuka Pharmaceutical, Tokyo, Japan) and
given daily to the animals by intraperitoneal administration
(0.1 mL/10 g body weight). Tumor-bearing nude mice were
randomly grouped (six mice/group), and administration of
nonactin started on Day 9. Tumor volumes were calculated using
a microgauge (Mitsutoyo Corporation, Kawasaki, Japan)
according to the following equations: Tumor volume
(mm^3) = 1/2 × (tumor length) × (tumor width)^2.

TUNEL staining of xenograft tumor tissue was performed using
the FragEL DNA Fragmentation Detection Kit (Merck
Millipore) according to the manufacturer’s protocol. Tissue
sections were viewed at 100 × magnification, and images were
captured with a digital camera.

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Results

Nonactin was identified as inducing apoptosis in β-catenin mutant HCT 116 cells. We screened more than 3000 microbial extracts for compounds that induced apoptosis in β-catenin mutated HCT 116 cells, but not in A375 cells harboring wild type β-catenin. One culture broth extract of *streptomyces* gave a positive result in the screening, and subsequently the active compound produced by this strain was isolated and identified as nonactin (Fig. 1a). Nonactin is well-known as a macrotetrolide antibiotic ionophore. Western blot analysis using anti-cleaved-PARP antibody revealed that the expression levels of cleaved-PARP in β-catenin mutant HCT 116 cells significantly increased upon treatment with concentrations above 0.1 μM nonactin for 24 h. The apoptosis-inducing ability of nonactin in HCT 116 cells was further confirmed by measuring sub-G1 populations of tumor cells via flow cytometry, and nonactin-induced apoptosis was significantly suppressed by Z-VAD-FMK, a pan-caspase inhibitor (Fig. S1). On the other hand, cleaved-PARP was not detected at nonactin concentrations of up to 10 μM in A375 cells expressing wild type β-catenin. This outcome indicates that nonactin induced apoptosis in HCT 116 cells at least 100 times more effectively than in A375 cells. We have previously reported that MEK1/2 inhibition in HCT 116 cells at least 100 times more effectively than nonactin in HCT 116 cells was further confirmed by measuring sub-G1 populations of tumor cells via flow cytometry, and nonactin-induced apoptosis was significantly suppressed by Z-VAD-FMK, a pan-caspase inhibitor (Fig. S1). On the other hand, cleaved-PARP was not detected at nonactin concentrations of up to 10 μM in A375 cells expressing wild type β-catenin. This outcome indicates that nonactin induced apoptosis in HCT 116 cells at least 100 times more effectively than in A375 cells. We have previously reported that MEK1/2 inhibitors induced apoptosis selectively in β-catenin mutant tumor cell lines. However, nonactin did not inhibit ERK1/2 phosphorylation in either cell line (Fig. 1b), indicating that nonactin induced apoptosis in HCT 116 cells but not in A375 cells with a mechanism other than MEK inhibition.

Nonactin induced apoptosis preferentially in β-catenin mutant tumor cells. To further confirm the selectivity of nonactin-induced apoptosis against the β-catenin mutant tumor cell lines, we examined the effects of nonactin on cell viability in various types of human tumor cell lines. For this, we selected 11 tumor cells including four β-catenin mutant tumor cells harboring mutations in key β-catenin N-terminal phosphorylation sites: A427 cells (T41A); HCT 116 cells (S45 deletion); LS-174T cells (S45F); and SW48 cells (S33Y). These tumor cells were treated with 0.1, 0.3, 1.0, 3.0, or 10 μM nonactin for 48 h and the number of cells was recorded. As shown in Fig. 2a, nonactin induced cell death at 0.1 μM in tumor cells harboring mutant β-catenin (growth ratio < 0). By contrast, nonactin induced cell death in concentrations of up to 10 μM in tumor cells harboring wild type β-catenin, including APC mutant tumor cells (growth ratio > 0). This indicates that nonactin induced cell death in β-catenin mutant tumor cells at least 100 times more effectively than in β-catenin wild type cells.

Furthermore, nonactin-induced cell death was detected by western blot using anti-cleaved-PARP antibody. As shown in Fig. 2b, the expression levels of cleaved-PARP increased upon treatment with nonactin concentrations above 0.1 μM in four β-catenin mutant tumor cell lines, but nonactin did not induce PARP-cleavage in tumor cells expressing wild type β-catenin (including APC mutant tumor cell lines). However, nonactin did not significantly affect the sub-G1 population in any other β-catenin wild type tumor cell lines. Taken together, nonactin induced apoptosis selectively in tumor cell lines harboring active mutations of β-catenin.

Nonactin induced apoptosis in β-catenin mutant tumor cells via mitochondrial uncoupling activity. Nonactin is known for its ability to form complexes with alkali cations, most notably potassium and sodium. Therefore, we next investigated whether such ionophore activities are involved in nonactin-induced apoptosis against β-catenin mutated tumor cells by

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Fig. 1. Nonactin induces selective apoptosis in β-catenin mutant HCT 116 cells without phospho-ERK1/2 inhibition. (a) Structure of nonactin. (b) A375 and HCT 116 cells were treated with nonactin, and the PARP-cleavage and ERK1/2-phosphorylation were detected by western blot.
Fig. 2. The antitumor activity of nonactin against various types of human tumor cell lines. (a) Cells were treated with nonactin, and cell growth was measured by a CellTiter-Glo Luminescent Cell Viability Assay. (b) Cells were treated with nonactin, and the PARP-cleavage was detected by western blot.
using salinomycin (a K⁺ ionophore), valinomycin (a K⁺ ionophore), and monensin (a Na⁺ ionophore). As shown in Fig. 3a, salinomycin induced a dose-dependent increase in the expression levels of cleaved-PARP in β-catenin mutant HCT 116 cells, but not in β-catenin wild type A375 cells. On the other hand, neither salinomycin: another K⁺ ionophore, nor monensin exhibited significant selectivity against β-catenin mutated tumor cells. As nonactin and valinomycin are also able to uncouple the oxidative phosphorylation of mitochondria,(31,32) we next examined whether another uncoupler: carbonyl cyanide m-chlorophenyl hydrazone (CCCP) could also induce apoptosis selectively in β-catenin mutant tumor cells. As shown in Fig. 3a, CCCP increased cleaved-PARP levels in β-catenin mutant HCT 116 and SW-48 cells in a dose-dependent manner, but not in β-catenin wild type A375 and HT-29 cells. These outcomes indicate that mitochondrial uncoupling activity is, at least in part, responsible for selective apoptosis induction in β-catenin mutant tumor cells.

The apoptosis-inducing ability of uncoupler was further confirmed by measuring sub-G₁ populations of tumor cells via flow cytometry. As shown in Fig. 3b, correlating with the western blot analysis, 48 h treatment of tumor cells with uncouplers increased the sub-G₁ populations in β-catenin mutant HCT 116 and SW-48 cells, but not in β-catenin wild type A375 and HT-29 cells. Therefore, these results suggest that the mitochondrial uncoupling activity is closely related to the selective apoptosis induction in β-catenin mutated tumor cells.

Expression of the active form of mutated β-catenin was required for uncoupler-induced apoptosis. To examine whether the expression of mutant β-catenin was a cause for apoptosis induction following treatment with uncouplers, a mutant active form of β-catenin (S37A, S45A) were transfected into A375 cells and A549 cells, which usually harbor only wild type β-catenin, and tested for the apoptosis-inducing activity of uncouplers. As shown in Fig. 4a,b, nonactin or CCCP did not induce apoptosis in vector control-transfected A375 cells or wild type of β-catenin-transfected A375 cells; whereas they induced apoptosis in mutant β-catenin-transfected A375 cells, as judged from the sub-G₁ population. Similar results were obtained when A549 cells were used. These results suggest that active mutant β-catenin is the key factor in uncoupler-induced apoptosis.

Dysfunction of the Warburg effect is involved in the apoptosis induced by uncouplers. As mitochondrial uncouplers induced apoptosis selectively in tumor cells harboring active mutant β-catenin, we hypothesized that these tumor cells may have a higher sensitivity to mitochondrial uncouplers than tumor cells harboring wild type β-catenin. To examine this possibility, we investigated the effect of nonactin on the mitochondrial membrane potential in both β-catenin wild type and mutant tumor cells. As shown in Fig. 5a,b, nonactin, at the concentration which induced selective apoptosis in β-catenin mutant tumor cells, significantly reduced the red fluorescence (aggregate form) of JC-1 in both β-catenin wild type and mutant tumor cell lines to a similar extent, as judged from quantifying red and green fluorescence and calculating the red/green ratio. Similar results were obtained when CCCP was used instead of nonactin. Thus, contrary to expectations, mitochondrial uncouplers induced loss of mitochondrial membrane potential not only in β-catenin mutant tumor cells, but also in β-catenin wild type tumor cells that did not undergo apoptosis after treatment with mitochondrial uncouplers.

The mitochondrial membrane potential plays a crucial role in the function of the respiratory chain to produce ATP;(33) however, the mitochondrial uncouplers did not induce apoptosis in β-catenin wild type tumor cell lines (Figs 2,4). Therefore, we next examined the possibility that β-catenin wild type tumor cells, but not β-catenin mutant tumor cells, produced ATP by relying on aerobic glycolysis rather than mitochondrial oxidative phosphorylation for their proliferation,(34) thereby surviving in the condition where mitochondrial function was damaged by uncouplers. For this, β-catenin wild type A375, A549, and HT-29 cells were treated with a glycolysis inhibitor: 2-deoxyglucose (2-DG), in the presence or absence of 0.1 μM nonactin or 10 μM CCCP for 24 h, and apoptosis status was analyzed by western blot. As shown in (Fig. 5c), 2-DG alone did not affect the expression levels of cleaved-PARP in these cells, but co-treatment of cells with 2-DG and the uncouplers induced an increase in the expression levels of cleaved-PARP in a dose-dependent manner. These results indicated that glycolysis-dependent production of ATP is required for the survival of β-catenin wild type tumor cell lines when cells were treated with uncouplers. Furthermore, these results indicated that the glycolysis-dependent production of ATP was suppressed in β-catenin mutant tumor cell lines. Therefore, we next examined the possibility that active mutant β-catenin could suppress glycolysis. The active mutant form of β-catenin (S37A, S45A) was transiently transfected into β-catenin wild type A375 and A549 cells. The extracellular acidification rate (ECAR) in response to glucose was subsequently measured by an Extracellular Flux Analyzer. As shown in Fig. 5d, glucose-induced ECARs were significantly suppressed in active mutant β-catenin expressing cells, compared to control cells or wild type β-catenin expressing cells. These results suggested that the expression of active mutant β-catenin suppressed the ability to perform glycolysis and limited tumor cells to relying on mitochondrial oxidative phosphorylation to survive.

Antitumor effect of nonactin in xenograft models. As nonactin induced apoptosis selectively in β-catenin mutant cell lines in vitro, we examined the antitumor activity of nonactin in vivo. β-catenin wild type A375 cells and β-catenin mutant HCT 116 cells were injected subcutaneously into nude mice.

Table 1. Apoptosis-inducing ability of nonactin detected by flow cytometer. Cells were treated with nonactin, and the sub-G₁ populations were detected by flow cytometry.

| Cell line | sub-G₁ population (%) | None | Nonactin 0.1 μM |
|-----------|------------------------|------|----------------|
| APC & β-catenin wild type | A2058 | 0.8 | 3.5 |
| A375 | 0.8 | 1.7 |
| A431 | 2.1 | 5.1 |
| A549 | 1.6 | 4.3 |
| JIMT-1 | 1.4 | 2.2 |
| APC mutant | DLD-1 | 2.7 | 9.0 |
| HT-29 | 1.2 | 2.6 |
| LoVo | 0.6 | 6.6 |
| SW480 | 3.3 | 5.2 |
| SW620 | 3.5 | 6.7 |
| β-catenin mutant | A427 | 12.4 | 42.0 |
| HCT 116 | 3.0 | 48.1 |
| LS-174T | 14.8 | 65.7 |
| SW48 | 1.5 | 43.4 |
The mice were then intraperitoneally administered nonactin once-daily. As shown in Fig. 6a, the maximum effect of nonactin at MTD dosing (100 mg/kg) was growth inhibition of A375 tumors expressing wild type β-catenin. However, tumor regression in response to nonactin at 100 mg/kg was observed in a β-catenin mutant xenograft model without significant body weight loss. Furthermore, TUNEL staining revealed significant apoptosis induction in HCT 116 tumor tissues from nonactin-treated mice, but not in HCT 116 tumor tissues from control mice (Fig. 6b). These results suggested that nonactin induced apoptosis selectively in β-catenin mutant tumor cell lines also in vivo.

**Discussion**

β-Catenin is one of the main components of the Wnt signaling pathway, playing a central role in cell proliferation, survival, differentiation and apoptosis. β-Catenin is mutated in a wide variety of tumors, and mutations can be detected in up to 10% of all sporadic colon carcinomas and 20% of hepatocellular carcinomas. The mutation in the N-terminus of β-catenin stabilizes β-catenin and allows it to escape from the destruction complex, after which the Wnt signaling pathway is hyperactive and drives oncogenesis. Several small molecules targeting the Wnt signaling pathway have been identified;

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**Fig. 3.** Mitochondrial uncoupling activity is required for nonactin-induced apoptosis. (a) A375, HT-29, HCT 116, and SW48 cells were treated with indicated ionophores, after which the PARP cleavage was detected by western blot. (b) The apoptosis-inducing ability of uncouplers was investigated by flow cytometry.
however, no approved drugs are clinically available for treatment via targeting of the Wnt signaling pathway. As the Wnt signaling pathway plays a crucial role in normal development and adult tissue homeostasis, interference with this pathway will not only target β-catenin mutant tumor cells, but also normal cells, resulting in significant toxicity. Therefore, to obtain compounds that act on targets other than the Wnt signaling pathway itself, we conducted a synthetic lethal chemical screen with a β-catenin mutation from our in-house natural product library. As a result, we found that nonactin induced apoptosis in β-catenin mutant tumor cells that harbor mutations in the N-terminus resulting from point mutations or in-frame

Fig. 3. Continued.
Fig. 4. The expression of active mutant β-catenin is required for uncoupler-induced apoptosis. (a) A375 and A549 cells were transiently transfected with wild type and mutated β-catenin (S37A, S45A), and treated with indicated uncouplers, after which the sub-G₁ populations were detected by flow cytometry. (b) The expressions of transfected β-catenin were confirmed by western blot.
Fig. 5. Dysfunction of glycolysis may be involved in uncoupler-induced apoptosis. (a,b) A375, HT-29, HCT 116, and SW48 cells were treated with indicated uncouplers. Following treatment, cells were stained with 2.0 μM JC-1, after which cells were harvested and the red and green fluorescence was observed by fluorescence microscopy. Each level of fluorescence was quantified using ImageJ and the red/green ratios were calculated. (c) A375, A549, and HT-29 cells were treated with the indicated concentrations of 2-DG in the presence or absence of indicated uncouplers, and the PARP-cleavage was detected by western blot. (d) A375 and A549 cells were transiently transfected with wild type β-catenin or mutated β-catenin, and the extracellular acidification rate (ECAR) in response to 11 mM D-glucose was measured in the presence or absence of 50 mM 2-DG.
deletions of the serine or threonine residues phosphorylated by CK1α or GSK3β (A427 cells: T41A, HCT 116 cells: S45 deletion, LS-174T cells: S45F, and SW48 cells: S33Y). The selective apoptosis induction by nonactin was not found to simply correlate to nuclear accumulation of β-catenin.

A requirement for an active mutation in β-catenin for nonactin-induced apoptosis was confirmed by the finding that nonactin did not induce apoptosis in A375 or A549 cells harboring wild-type β-catenin, but it induced apoptosis in A375 or A549 cells by the expression of the active form of β-catenin (S37A, S45A) (Fig. 4a,b). These results demonstrate that nonactin exhibits synthetic lethality with an active mutant form of β-catenin. Interestingly, nonactin did not induce apoptosis in Colo-201 and Colo-205 cells harboring β-catenin N287S mutation(39) (Fig. S3). Furthermore, at the concentration that induced apoptosis in β-catenin mutant tumor cells, nonactin did not induce apoptosis in adenomatous polyposis coli (APC) mutant tumor cell lines. APC is a negative regulator of the Wnt signaling pathway, and the truncated mutations of APC fail to form a scaffold for the destruction complex, leading to stabilized β-catenin and an aberrantly activated Wnt signaling pathway.(40) Moreover, stimulation of β-catenin wild type A375 cells with Wnt-3a enhanced the ability of SMK-17 to induce apoptosis in β-catenin wild type A375 cells, as previously reported.(24) However, nonactin failed to induce apoptosis in A375 cells even after stimulation with Wnt-3a (Fig. S4). These results suggest that activation of the Wnt signaling pathway is not sufficient for nonactin-induced selective apoptosis in β-catenin mutant tumor cells.

Nonactin has been suggested to act as a K⁺ ionophore, and another K⁺ ionophore: valinomycin, showed similar selective apoptosis-inducing ability. Although salinomycin, which is also a K⁺ ionophore, has been recently reported to induce programmed cell death in cancer stem cells (CSCs),(41) as well as in various other cancer cells,(42–44) it did not show a significant selective induction of apoptosis in β-catenin mutant tumor cells. Thus, nonactin action is comparable to that of valinomycin while being distinct from the effects of salinomycin. This difference can be explained by the observations reported by the Szabó group, who found that salinomycin is also known...
to mediate $K^+/H^+$ exchange and induces rapid hyperpolarization of mitochondria. In contrast, the common mechanism of nonactin and valinomycin is one of mitochondrial uncoupling, causing depolarization. As proton influx occurs in exchange for $K^+$ and is electroneutral, it does not by itself have any effect on potential. Hyperpolarization derives from the collapse of $\Delta \varphi$, which the mitochondrion compensates for by increasing mitochondrial membrane potential so as to keep the overall electrochemical potential nearly constant. Indeed, the concentration of salinomycin that induced apoptosis in tumor cells did not induce a loss of mitochondrial membrane potential in both $\beta$-catenin wild type and mutant tumor cell lines (Fig. S5). Further studies will be necessary to delineate the exact factors that contribute to the selective cell death in $\beta$-catenin mutant tumor cells by mitochondrial uncouplers.

Mitochondrial membrane potential plays a crucial role in the function of the respiratory chain to drive the synthesis of ATP by $F_0F_1$ ATPase. A significant loss of mitochondrial membrane potential induces cytotoxicity resulting from ATP depletion. Nonactin and CCCP induced loss of mitochondria membrane potential in both $\beta$-catenin wild type and mutant tumor cells at the same concentration of apoptosis induction. Nevertheless, nonactin, and CCCP did not induce apoptosis in $\beta$-catenin wild type tumor cells despite the loss of mitochondrial membrane potential. One possible explanation is that “the Warburg effect” may be responsible for the cell survival of $\beta$-catenin wild type tumor cells. This explanation can be confirmed by our findings that inhibition of glycolysis using 2-DG: a hexokinase 2 inhibitor, enables $\beta$-catenin wild type cells to undergo apoptosis following the treatment with uncouplers (Fig. 5c). On the other hand, because $\beta$-catenin mutant tumor cells underwent apoptosis following the treatment with the uncoupler alone, we hypothesized that the Warburg effect might be unable to function in $\beta$-catenin mutant tumor cell lines. Indeed, the expression of active mutant $\beta$-catenin (S37A, S45A), but not of wild type $\beta$-catenin, induced a decrease in glycolysis rate. This indicates that mutant $\beta$-catenin (S37A, S45A) might inhibit the Warburg effect. At present, we do not know how mutant $\beta$-catenin (S37A, S45A) inhibited the Warburg effect. $\beta$-Catenin reportedly binds several proteins including cadherins, $\alpha$-catenin, axin, the EGF receptor, APC, the actin-bundling protein fascin, the LEF/TCF transcription factors, the MUC-1 breast cancer antigen, the Alzheimer’s-associated protein presenilin, protein phosphatases, and the regulator of small GTPases: IQ GAP. In addition, it
has been reported that c-Src-phosphorylated β-catenin at Tyr333 directly binds to nuclear Pyruvate kinase M2 (PKM2), leading to a regulation of the Warburg effect.56) Therefore, this raises the possibility that mutant β-catenin could bind to and modulate binding proteins that regulate glycolysis, thereby inducing a dysfunction of the Warburg effect. The mechanism by which the mutation of N-terminus phosphorylation site in β-catenin inhibits glycolysis is currently under investigation.

To link these findings to a clinically relevant model, we conducted in vivo studies using active β-catenin mutant xenograft models. Significant tumor regression without any severe toxicity was observed in β-catenin mutant HCT 116 cells in response to daily administration of nonactin (Fig. 6a,b).

In conclusion, our findings suggest that tumor cells harboring active mutant β-catenin showed a dysfunction of the Warburg effect, and produced ATP by relying on mitochondrial oxidative phosphorylation for survival. Therefore, mitochondrial uncouplers such as nonactin, valinomycin, and CCCP induced apoptosis selectively in β-catenin mutant tumor cells. Our results provide new insights into the development of potential chemotherapeutic strategies for tumor cells harboring β-catenin mutations.

Disclosure Statement

The authors have no conflicts of interest to declare.
Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Z-VAD-FMK significantly suppressed nonactin-induced cell death.

Fig. S2 The selectivity of nonactin against β-catenin mutant tumor cells investigated by flow cytometry.

Fig. S3 Colo-201 and colo-205 cells exhibited resistance to nonactin.

Fig. S4 The effect of the Wnt pathway activation on the apoptosis-inducing ability of nonactin.

Fig. S5 Salinomycin did not induce loss of mitochondrial membrane potential.