Chemical biological studies of small molecules targeting mutant β-catenin tumor

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Hiroaki Ikeda

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Graduate School of Science and Technology

Keio University

Hiroaki Ikeda

Chapter 1:
Introduction

1.1 Cancer chemotherapy

Cancer is the most common cause of death worldwide. Normal human cells maintain their homeostasis by strictly controlling the proliferation or death signals. However, if the signal regulation is disrupted due to mutations on specific genes by chemical substances or radiation, cell growth may be aberrant[1]. Generally, such abnormal cells should be eliminated by the induction of programmed cell death, called apoptosis, but the accumulation of mutations leads to disruption of control and the abnormal proliferation. Moreover, heterogeneity and variety of mutations make it difficult to treat cancer.

In the past, cytotoxic drugs that kill fast-growing cells by targeting DNA or microtubule have been developed as anti-cancer drugs, such as doxorubicin and paclitaxel[2] (Figure 1-1). Doxorubicin (Adriamycin) inhibits DNA replication by inhibiting topoisomerase II[3]. Paclitaxel (Taxol) prevents cell division by inhibiting microtubule depolymerization[4]. However, these drugs simply focus only on the cell growth speed, so that some normal cells may also be damaged, resulting in strong side effects. It is severely problem of classical cytotoxic agents. Owing to remarkable research development for signal transduction related to cancer, molecular targeted drugs targeting proteins on the proliferation signaling pathway have been highlighted. Specific inhibition of gene mutations or related signaling pathways can be expected to inhibit cancerous growth with less toxicity to normal cells, including inhibitors of protein kinase (e.g. imatinib: Bcr-Abl[5], gefitinib: EGFR[6], vemurafenib: BRAF[7]; Figure 1-1). However, they are not completely safe to normal cells, and some have serious side effects. In addition, because these drugs are mainly aimed to inhibit cell proliferation signaling, cancer cells are often

not killed and cause recurrence due to drug resistance of survivor cells[8]. In order to solve these problems, the development of “drugs that selectively kill cancer cells” is required. Based on these background, immune checkpoint inhibitors recently highlighted. PD-L1 expressed on tumor cells contribute to escape from the PD-1 recognition expressed on T cell immunity, thus the blockade of immune checkpoint interaction is believed to provide an effective strategy for tumor immunotherapy[9]. In this study, the author focused on another strategy for targeting cancer cells selectively. That is “synthetic lethality”. Synthetic lethality is a concept of the relationship between two genes; a defect in either one of two genes has no fatal effect, but simultaneous defects in both two genes results in cell death[10]. One notable example is that PARP inhibitors, such as olaparib (Figure 1-1), which exhibit synthetic lethality with BRCA1/2 mutation in breast cancer cells[11,12], are now showing success in the clinic[13].

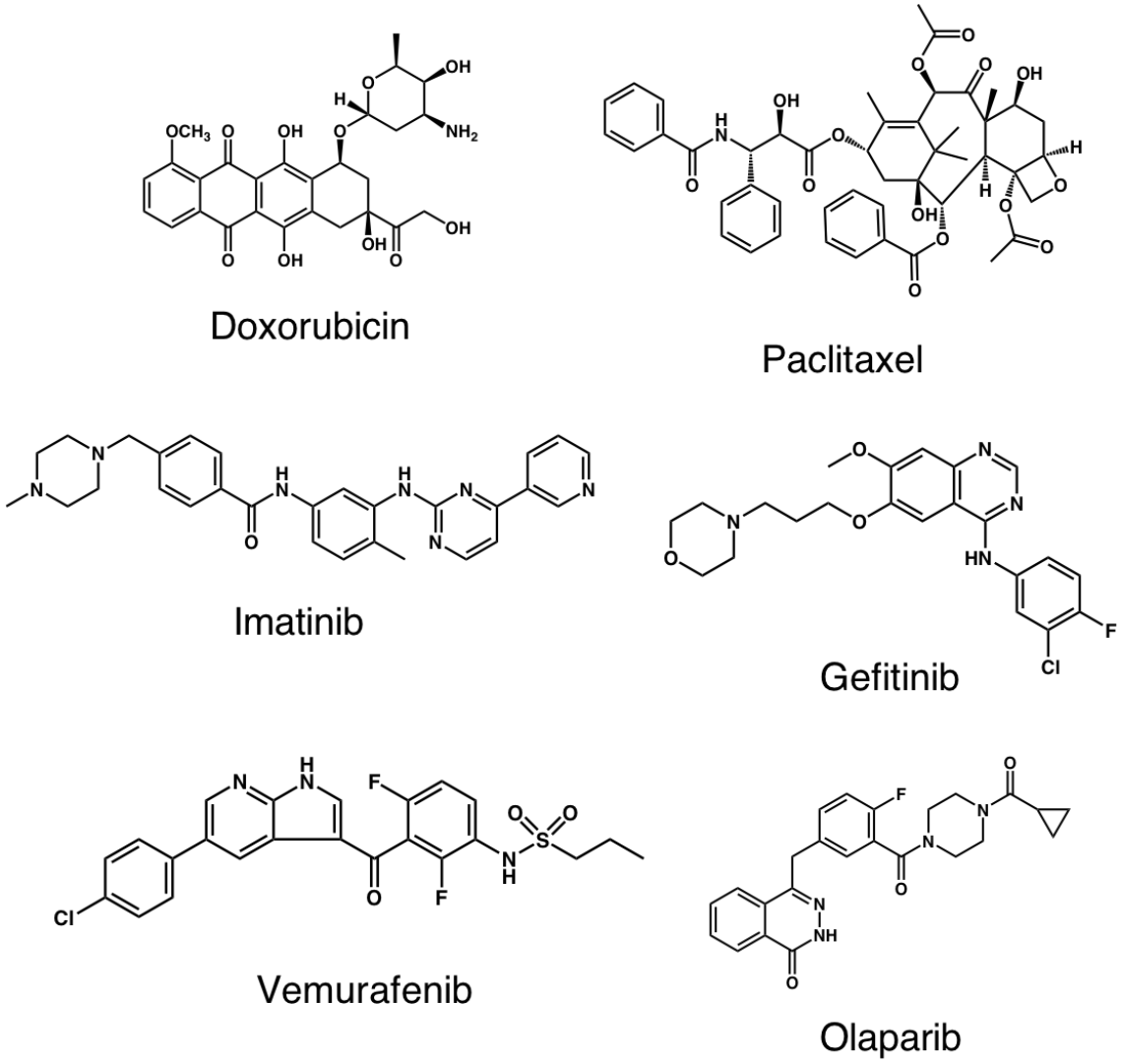

Figure 1-1. Chemical structures of anti-cancer agents.

1.2 Apoptosis

Apoptosis is one of the mechanism of cell death, classified in a programmed cell death, which has been first described by Kerr, Wyllie and Currie[14]. The intrinsic pathway of apoptosis is regulated by a set of cysteine proteases, caspases, in most cases, initiated by caspase-8 and/or -9 activation resulting in caspase-3 activation. Caspase-8 is the key initiator caspase in the death receptor pathway[15]. Upon ligand FasL, TNF-α or TRAIL binding, death receptors Fas, TNFRI/II, DR4/5 form signaling complex including procaspase-8, resulting in the cleavage of caspase-8[16]. The key requirement for caspase-9 cleavage is its association with Apaf-1 and cytochrome *c*, which is called apoptosome[17]. This caspase-9 activation is usually observed in mitochondria-mediated apoptosis with the involvement of Bcl-2 family proteins[18]. On the other hand, mitochondria also contains the caspase-independent apoptosis mediator, apoptosis-inducing factor (AIF). In the presence of specific death stimuli, AIF is released to cytosol, translocates to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation[19]. Although a lot of compounds are reported to induce variety mechanisms of apoptosis, it is unclear that how cells trigger caspase-dependent or -independent apoptosis in response to each death stimuli.

1.3 Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway plays crucial roles in the regulation of diverse cellular processes, including embryogenesis, proliferation, survival, and differentiation[20,21]. Canonical Wnt/β-catenin signaling pathway is overviewed in Figure 1-2. This proliferation signaling is tightly regulated by the destruction complex, which consists of β-catenin, glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), Axin, adenomatous polyposis coli (APC), and β-transducin repeat-containing protein (β-TrCP)[22,23]. In the absence of Wnt ligands stimuli, β-catenin is degraded by the ubiquitin-proteasome system with CK1-dependent phosphorylation of Ser45 and GSK3-dependent phosphorylation of Thr41, Ser37 and Ser33[24,25]. In contrast, Wnt ligands activate Wnt/β-catenin signaling pathway by interacting with the receptor, Frizzled (Fzd) and low-density lipoprotein receptor-related protein-5/6 (LRP5/6), resulting in the disruption of the destruction complex and β-catenin stabilization[26]. Subsequently, β-catenin enters the nucleus and binds to T-cell factor (TCF), with its co-activator Pygopus, CREB-binding protein (CBP)/p300 and Bcl-9[27], activating the transcription of Wnt target genes[28,29], including c-myc and cyclin D1[30,31]. Thus, β-catenin is one of the major components and plays a main role in the Wnt signaling pathway.

Normally, this Wnt/β-catenin signaling pathway is strictly regulated and contributes to the appropriate cell proliferation. However, mutation of the β-catenin gene *CTNNB1* causes stabilization and nuclear accumulation of β-catenin proteins, resulting in aberrant downstream transcriptional activity[32,33]. β-catenin is mutated in a variety of tumors, including 10% and 20% of sporadic colon carcinomas and hepatocellular carcinomas,

respectively[34]. CK1 and GSK3 fail to phosphorylate β-catenin with the mutation in the phosphorylation site (S45/T41/S37/S33) resulting in evasion from proteasomal degradation system and stabilization of β-catenin[35].

In this situation, several small molecules targeting Wnt/β-catenin signaling pathway have been developed and some signal inhibitors have been developed to enter clinical trials[36,37]. LGK974 and ETC-159 inhibit Porcupine, which is important for the secretion of Wnt ligands[38,39]. Anti-Frizzled receptor antibody OMP-18R5 (vantictumab)[40] and anti-Wnt ligand antibody OMP-54F28 (ipafricept)[41] were found as antibodies against Wnt family proteins. PRI-724 inhibits the interaction of β-catenin and CBP[42] resulting in the suppression of transcriptional activity. Tankyrase, which degrades a scaffold protein Axin, has also been reported as a target protein for Wnt signaling inhibition, and Tankyrase inhibitors, XAV939 and JW55[43,44], were developed as Wnt signaling inhibitors, however, there are no ongoing clinical trials with Tankyrase inhibitors. Thus, there are currently no effective therapeutic compounds targeting Wnt/β-catenin signaling pathway.

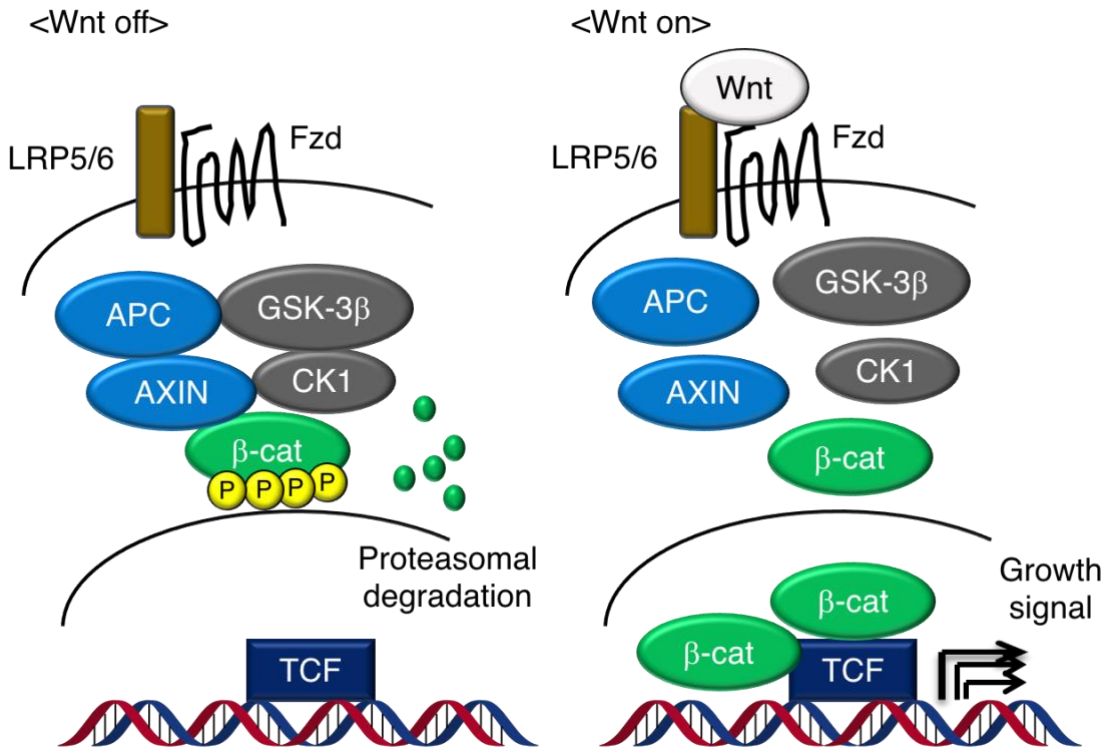

Figure 1-2. Wnt/β-catenin signaling pathway.

1.4 Chemical biology

"Chemical biology" is a relatively new science area which is aimed to understand the biological system using chemical technologies. Gene editing techniques (e.g. gene knockdown/out by siRNA system[45] or CRISPR-Cas9 system[46,47]) are mainly used as classical methods for biological researchers to reveal the relationship between gene and phenotype. On the other hand, chemical biology, also called chemical genetics, is a method to reveal the relationship by using small molecules to regulate gene or protein. Classical gene editing techniques are well modified to regulate objective genes easily, however, irreversible knockdown/out of protein directly affects the expression levels of protein, resulting in the loss of every function of the protein especially in the case of multi-functional protein. Although small molecules have some problems such as off-target effects or difficulties of target identification, it is useful to regulate the specific activity of proteins reversibly by inhibiting functional domain of target proteins. Of note, it is possible to develop small molecules as a chemotherapeutic agents directly.

Chemical genetics is generally classified into two categories; forward and reverse genetics. Phenotype-based and target-based chemical screenings are carried out in forward chemical genetics and reverse chemical genetics, respectively[48]. Target-based screening is now generally performed, starting from target validation. Proteins in proliferation signaling pathway or biomarkers of disease should be established for the target, followed by chemical screening to obtain candidate small molecules. On the other hand, phenotypic screening is now on a declining trend in many companies because of the difficulty of target identification and mode of action studies after finding of interesting

small molecules. However, it sometimes contributes to find unexpected targets for biological events because of the non-biased chemical screening. And now, a lot of useful approaches for target identification are developed, the problems of these studies can be solved.

As described above, we initially need to search for the bioactive small molecules for chemical biological research. Traditionally, novel compounds were obtained from natural sources such as microorganisms or plants. However, the most difficult point for the discovery of small molecules is isolation/purification step. Typically, it takes extremely long period of time for bioassay-guided purification, moreover, often results in obtaining small amount of objective compounds. Currently, major pharmaceutical companies do not continue natural products screening, almost changed to synthetic compounds screening. Although a series of synthesis process was highly complicated formerly, recent technologies for combinatorial chemistry has been well-developed, and it is possible to prepare a large number of compounds simultaneously. Because of a variety of synthesis methods for combinatorial chemistry, it was thought to be a big innovation for researchers. However, the diversity of compounds structures were limited compared with natural products or their derivatives[49].

1.5 Previous studies and this study

In this study, the author searched for the compounds that selectively induced cell death in β-catenin-mutated tumor cells. Previously, our research group reported that SMK-17[50] (MEK inhibitor) and nonactin[51] (mitochondrial uncoupler) induced apoptosis selectively in tumor cell lines harboring β-catenin mutations. However, the mechanism of selective cell death in β-catenin-mutated tumor cells caused by these compounds has not yet been fully elucidated. Our continuous screening resulted in the identification of new compounds, metacycloprodigiosin (mcPG), which was isolated from *streptomyces* strain extracts, and DS37262926 (miclxin), which was provided by Daiichi-Sankyo Pharmaceutical Company. These agents exhibited selective induction of apoptosis in β-catenin-mutated HCT116 cells.

Chapter 2:
Metacycloprodigiosin (mcPG) induced cell death selectively in β-catenin-mutated tumor cells

2.1 Result

2.1.1 Isolation and identification of metacycloprodigiosin (mcPG).

To find the compounds that induced synthetic lethality in β-catenin mutant tumor cells, the author first screened more than 2,000 microbial extracts for the compounds that induced apoptosis in β-catenin mutated HCT116 cells, but not in A375 cells harboring wild type β-catenin. As a result, the author found one *streptomyces* strain produced such active compound, which was isolated from extracts of *streptomyces* cultures following bioassay-guided fractionation; both silica-gel column chromatography and reverse-phase HPLC were utilized. Spectroscopic analysis by NMR and mass spectrometry revealed this active compound to be metacycloprodigiosin (mcPG) (Figure 2-1)[52]. Flow cytometric analysis of PI staining revealed that the subG1 population in β-catenin mutated HCT116 cells was significantly increased upon treatment with 0.1 μM mcPG for 48 h, while the subG1 population in A375 cells expressing wild-type β-catenin was only slightly increased, indicating that mcPG induced apoptosis in HCT116 cells but not in A375 cells (Figure 2-2).

The author further examined whether mcPG induced apoptosis selectively in β-catenin mutant tumor cells by using several types of human tumor cells in detail. For this, the author selected twelve tumor cells including five β-catenin mutant tumor cells (HCT116 cells: S45 deletion, LS-174T cells: S45F, SW48 cells: S33Y, HepG2 cells: W25-I140 deletion and Colo-205 cells: N287S), and these tumor cells were treated with 0.1, 0.3, 1.0, 3.0, 10 μM mcPG for 48 h and the subG1 populations were measured by flow cytometer (Table 2-1). The extent of apoptosis induction was calculated using the area under the

curve (AUC), which represents the area under the line in plots of concentrations of each chemical against subG1 population, and *Z*-score. As shown in Figure 2-3, mcPG induced apoptosis not only in HCT116 cells but also in LS-174T, SW48 cells and HepG2 cells, which are all β-catenin mutant tumor cells. Individual value plots of mcPG confirmed that β-catenin mutated cell lines are significantly sensitive to mcPG, compared with cell lines harboring wild-type β-catenin (Figure 2-4). On the other hand, significant differences were not observed in cell lines harboring KRAS and p53 mutations, and BRAF mutation seemed to be a negative factor of mcPG-induced apoptosis.

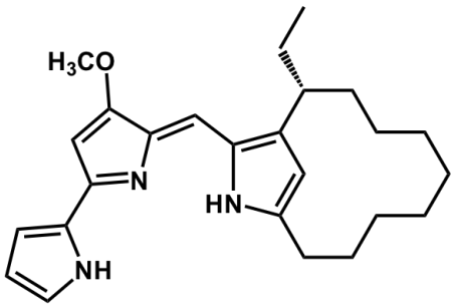

Figure 2-1. Structure of metacycloprodigiosin (mcPG).

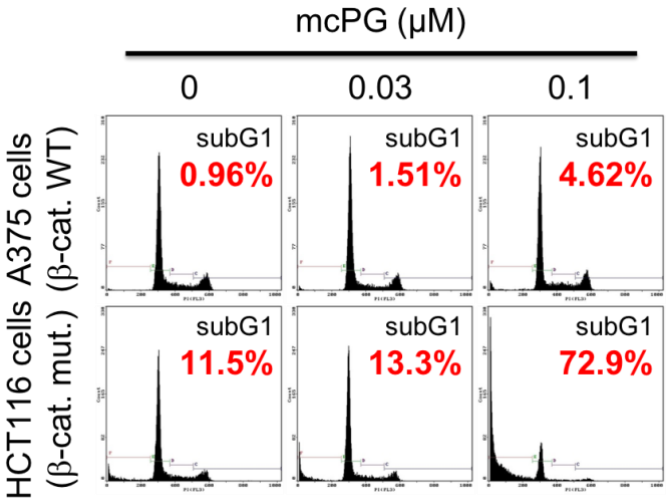

Figure 2-2. Apoptosis-inducing activity of mcPG.

A375 and HCT116 cells were treated with the indicated concentrations of mcPG for 48 h. SubG1 populations were measured by flow cytometer.

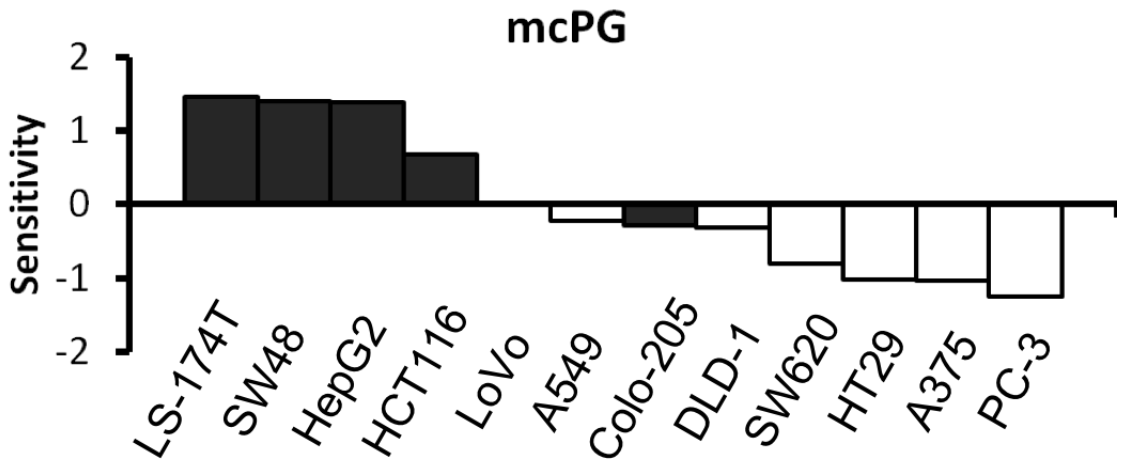

Figure 2-3. Sensitivity against mcPG.

Twelve cell lines were treated with mcPG for 48 h, and subG1 populations were measured by flow cytometer. Sensitivity represents *Z*-score of compounds against twelve cell lines. *Z*-score was calculated based on the values from area under the curve (AUC). Black Bar; β-catenin-mutated tumor cell line.

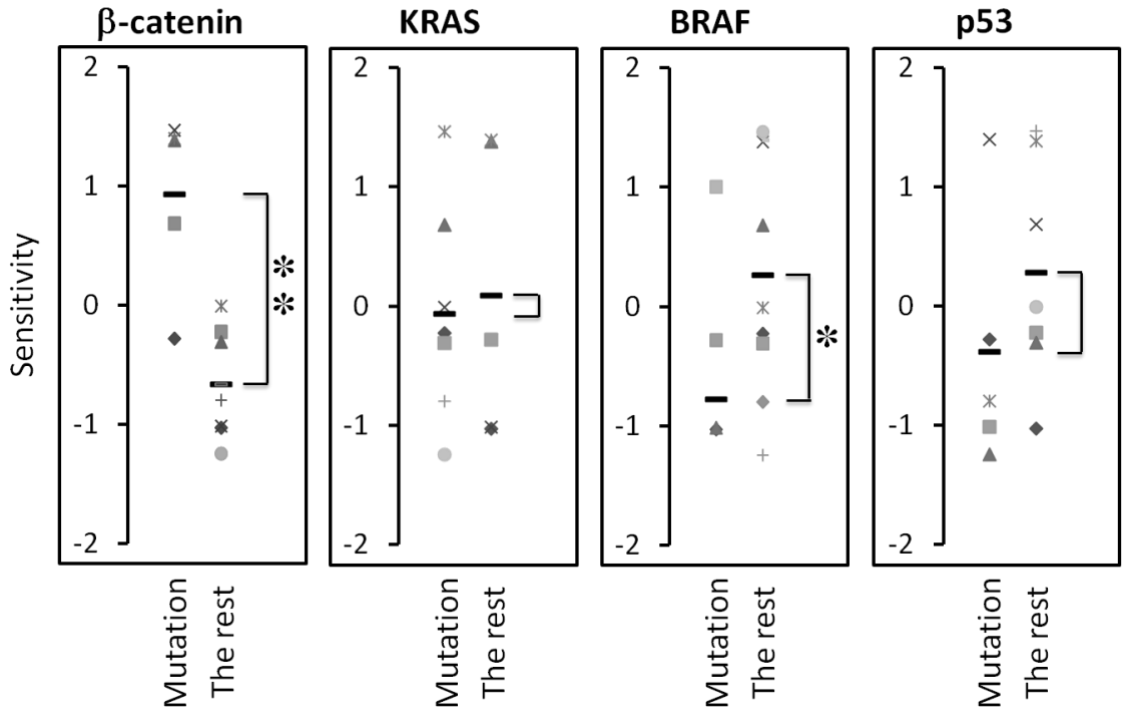

Figure 2-4. Individual value plots for the indicated mutations.

Individual value plots show the *Z*-score of mutated type and the rest cell lines in tumor-related proteins including β-catenin, KRAS, BRAF and p53. *P*-values were obtained by performing a Student's *t*-test for group comparisons. **P*<0.05 and ***P*<0.01.

Table 2-1. The list of subG1 populations after treatment of compounds.

		subG1 population (%)											
chemical	conc.	LS-174T	SW48	HepG2	HCT116	LoVo	A549	Colo-205	DLD-1	SW620	HT29	A375	PC-3
mcPG (μM)	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.1	40.09	42.08	7.58	29.27	15.22	9.09	5.92	8.64	11.47	4.72	3.65	2.38
	0.3	54.29	49.38	12.81	32.57	16.92	11.96	8.94	17.54	14.87	4.89	3.67	2.39
	1	75.99	61.28	91.11	51.27	21.02	17.06	20.34	25.64	13.47	8.15	4	1.88
	3	67.09	68.18	97.41	49.17	35.42	28.86	42.84	30.04	8.18	9.83	4.99	1.65
	10	58.19	77.58	97.81	58.87	73.32	75.56	43.14	30.64	6.27	5.29	24.28	0.7
BMA (nM)	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	2.86	9.39	0.49	0.38	0.34	0.21	0.23	2.71	0	1.9	0	3.33
	3	49.46	17.69	11.7	15.28	12.73	2.7	2.52	13.67	7.15	5.1	3.29	6.64
	10	58.76	22.49	25.6	29.28	18.63	16.74	31.4	19.37	14.61	6.65	9.99	3.58
	30	55.06	18.19	25.9	29.58	14.63	16.14	34.1	20.07	14.61	7.39	11.89	3.51
	100	57.16	28.69	27.9	27.18	16.43	19.54	37.7	19.47	13.21	7.07	10.59	4.3
CMA (nM)	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.1	39.3	16.53	9.63	33.3	12.79	10.58	23.75	13.8	7.03	5.26	7.72	3.5
	0.3	53.8	27.83	25.13	35.5	16.99	17.58	45.75	17.5	15.54	6.59	9.43	1.97
	1	54.3	26.23	23.33	34.9	16.29	16.78	44.75	19.7	16.54	7.04	9.22	1.88
	3	52.4	24.73	24.93	31.8	19.39	15.18	45.25	15.1	16.64	7.09	8.93	2.07
	10	55	30.13	25.53	35.5	17.99	16.18	52.45	17.7	19.64	6.61	8.98	2.35

2.1.2 V-ATPase inhibitors induced apoptosis preferably in β-catenin mutant tumor cells.

Because prodigiosin analogs are reported to inhibit vacuolar-type H^+-ATPase (V-ATPase)[53,54], the author next examined whether other V-ATPase inhibitors, bafilomycin A1 (BMA)[55] and concanamycin A (CMA)[56], also could induce apoptosis selectively in β-catenin mutant tumor cells. The author asked the possibility that this selective apoptosis in β-catenin mutant tumor cells caused by V-ATPase inhibitors is related to the difference of the sensitivity to V-ATPase inhibitors between β-catenin mutant tumor cells and β-catenin wild-type tumor cells. The low pH of intracellular acidic organelles including lysosomes is maintained by V-ATPase, and this acidification is detected as an orange fluorescence when the cells are stained with acridine orange, a weak base fluorescent reagent[57]. When HCT116 cells and A375 cells were incubated with acridine orange, intracellular organelles of these cells were stained with orange fluorescence, whereas the orange fluorescence in these organelles almost completely disappeared upon treatment with mcPG (0.1 μM) for 4 h, respectively, indicating that this concentration of mcPG inhibited V-ATPase not only in HCT116 cells but also in A375 cells (Figure 2-5). Moreover, this concentration of mcPG is comparable to that of the induction of apoptosis in HCT116 cells, but they failed to induce apoptosis in A375 cells. Similar results were obtained when BMA (3.0 nM) or CMA (0.1 nM) were used instead of mcPG (Figure 2-6). The author further examined whether BMA and CMA also induced apoptosis selectively in β-catenin mutant tumor cells by calculating *Z*-score. As shown in Figure 2-7, BMA and CMA also showed the similar sensitivity pattern with mcPG. Thus, although inhibition of V-ATPase activity is not sufficient for the induction of apoptosis, apoptosis

induction by V-ATPase inhibitors in β-catenin-mutated tumor cells was closely correlated to its V-ATPase inhibitory activities.

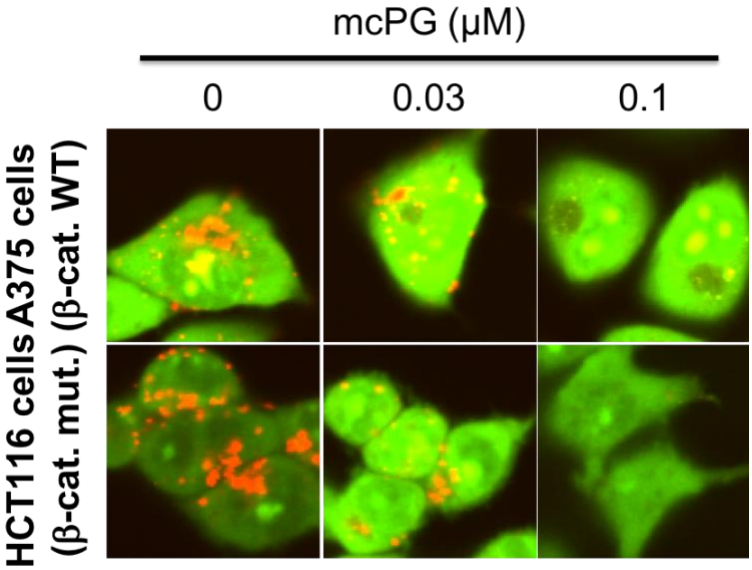

Figure 2-5. V-ATPase inhibitory activity of mcPG.

A375 and HCT116 cells were treated with the indicated concentrations of mcPG for 4 h and then stained with 3 μM acridine orange for 30 min. After being washed, cells were observed under confocal laser scanning microscope.

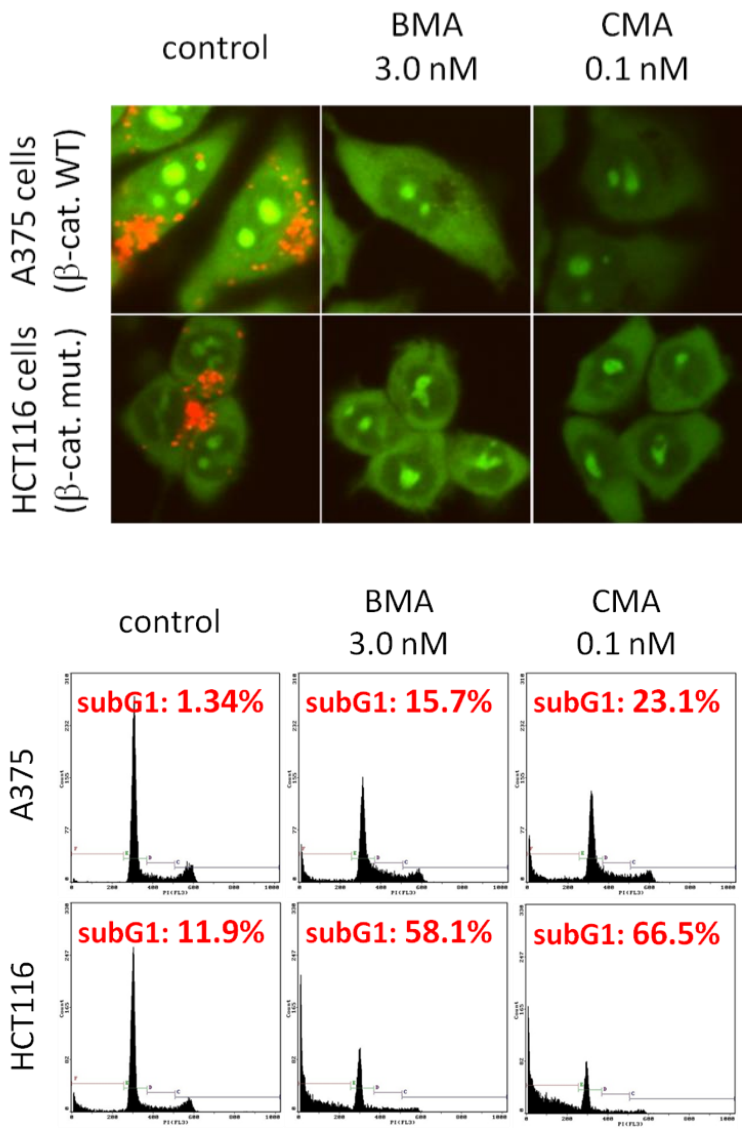

Figure 2-6. The relationship between V-ATPase inhibitory activity and apoptosis-inducing activity.

A375 and HCT116 cells were treated with the indicated concentrations of mcPG for 4 h and then stained with 3 μM acridine orange for 30 min. After being washed, cells were observed under confocal laser scanning microscope. A375 and HCT116 cells were treated with V-ATPase inhibitors, the indicated concentrations of bafilomycin A1 (BMA) and concanamycin A (CMA), for 48 h. SubG1 populations were measured by flow cytometer.

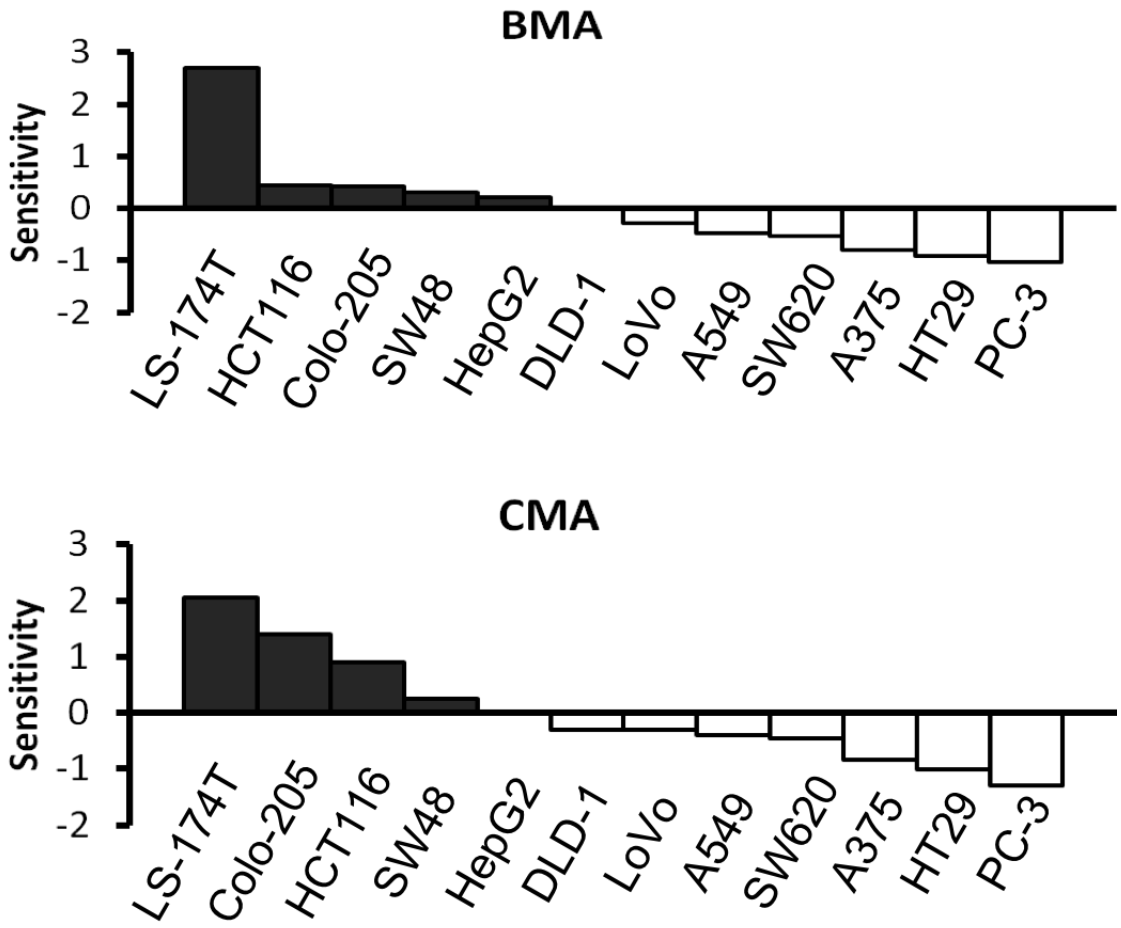

Figure 2-7. Sensitivity against V-ATPase inhibitors.

Twelve cell lines were treated with V-ATPase inhibitors, bafilomycin A1 (BMA) and concanamycin A (CMA) for 48 h, and subG1 populations were measured by flow cytometer. Sensitivity represents *Z*-score of compounds against twelve cell lines. *Z*-score was calculated based on the values from area under the curve (AUC). Black Bar; β-catenin-mutated tumor cell line.

2.1.3 Mutation of β-catenin is required for V-ATPase inhibitors-induced apoptosis.

V-ATPase inhibitors induced apoptosis selectively in β-catenin mutant tumor cells, therefore, the author next examined whether expression of the active form of β-catenin in tumor cells harboring wild-type β-catenin could induce apoptosis following V-ATPase inhibitor treatment. Because most of human cancer cell lines contained mutations in β-catenin at codons 33, 37, 41 and 45 that altered potential CK1 or GSK-3β phosphorylation sites[25], the mutant β-catenin (S37A, S45A)[50,51] were transfected into HEK-293T cells harboring wild-type β-catenin, and tested for apoptosis inducing activity of V-ATPase inhibitors. As shown in Figure 2-8, mcPG did not induce apoptosis in HEK-293T cells but it induced apoptosis in mutant β-catenin-expressing HEK-293T cells, as judged from PARP cleavage. These results suggested that actively mutated β-catenin is exactly the key factor of V-ATPase inhibitors-induced apoptosis.

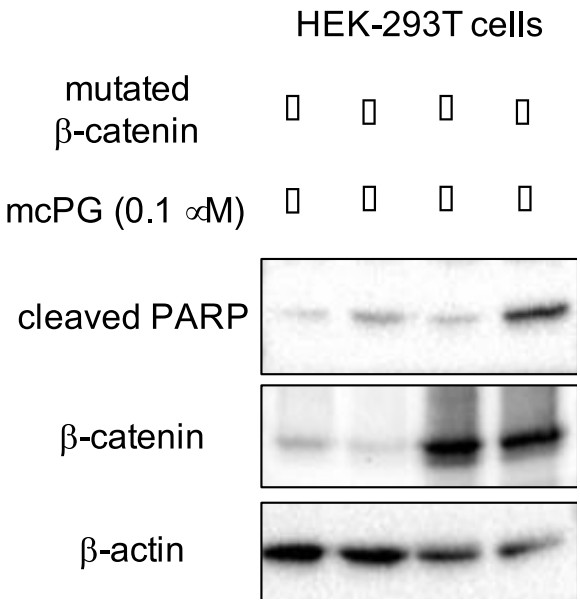

Figure 2-8. The effect of mutant β-catenin on mcPG-induced apoptosis.

HEK-293T cells were transiently transfected with control vectors or mutant β-catenin (S37A and S45A) plasmids. Then, cells were treated with 0.1 μM of metacycloprodigiosin (mcPG) for 24 h, and cell lysates were prepared for western blot analysis to detect the expression of β-catenin and cleaved PARP.

2.1.4 V-ATPase inhibitors induced caspase-8-dependent apoptosis.

Cleavage of poly(ADP-ribose) polymerase (PARP) and caspase activation are known to contribute to apoptosis[58,59], therefore, the effect of V-ATPase inhibitors on caspase activation was studied. A375 (β-catenin wild-type) and HCT116 (β-catenin mutant type) cells were treated with 0.1 μM mcPG for the indicated periods, and caspases were detected by western blot analysis. The results showed that significant increase in cleaved caspase-8 expression after 12 h treatment with mcPG, and subsequently, cleaved caspase-9, cleaved caspase-3 and cleaved PARP were observed upon 18 h treatment only in HCT116 cells (Figure 2-9). Furthermore, 1 h pre-incubation of HCT116 cells with the pan-caspase inhibitor z-VAD-FMK, caspase-8 inhibitor z-IETD-FMK, caspase-9 inhibitor z-LEHD-FMK and caspase-3 inhibitor Ac-DEVD-CHO suppressed the mcPG-induced cleavage of PARP (Figure 2-10), respectively. Similar results were obtained when BMA and CMA were used instead of mcPG (Figure 2-11). Therefore, these results suggested that V-ATPase inhibitors induced caspase-dependent apoptosis initiated from the activation of caspase-8 in HCT116 cells.

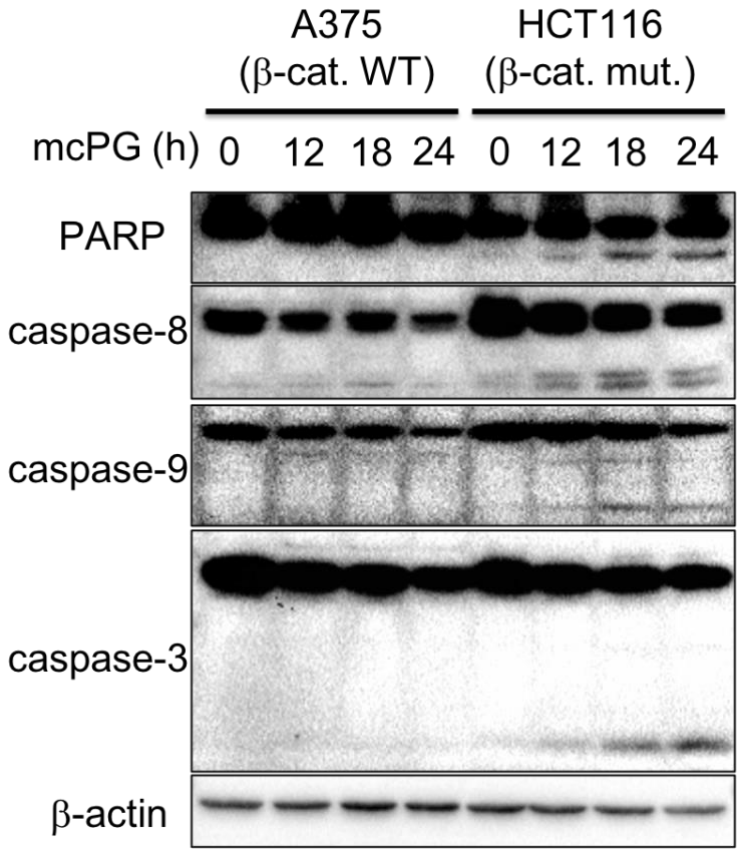

Figure 2-9. Caspase activation by mcPG treatment.

A375 and HCT116 cells were treated with 0.1 μM mcPG for the indicated periods. Cell lysates were prepared for western blot analysis to detect PARP, caspase-8, -9, -3.

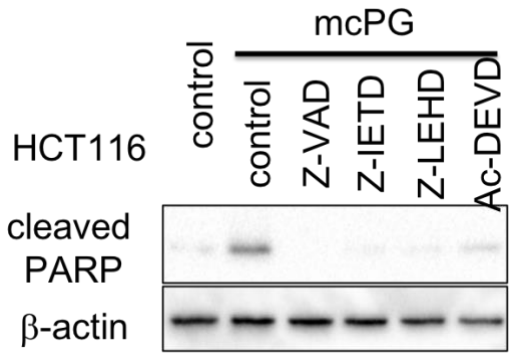

Figure 2-10. The effect of caspase inhibitors on mcPG-induced apoptosis.

HCT116 cells were treated with 0.1 μM mcPG after 1 h pre-treatment of 100 μM z-VAD-FMK, z-IETD-FMK, z-LEHD-FMK, Ac-DEVD-CHO. After 24 h treatment, cell lysates were prepared for western blot analysis to detect cleaved PARP.

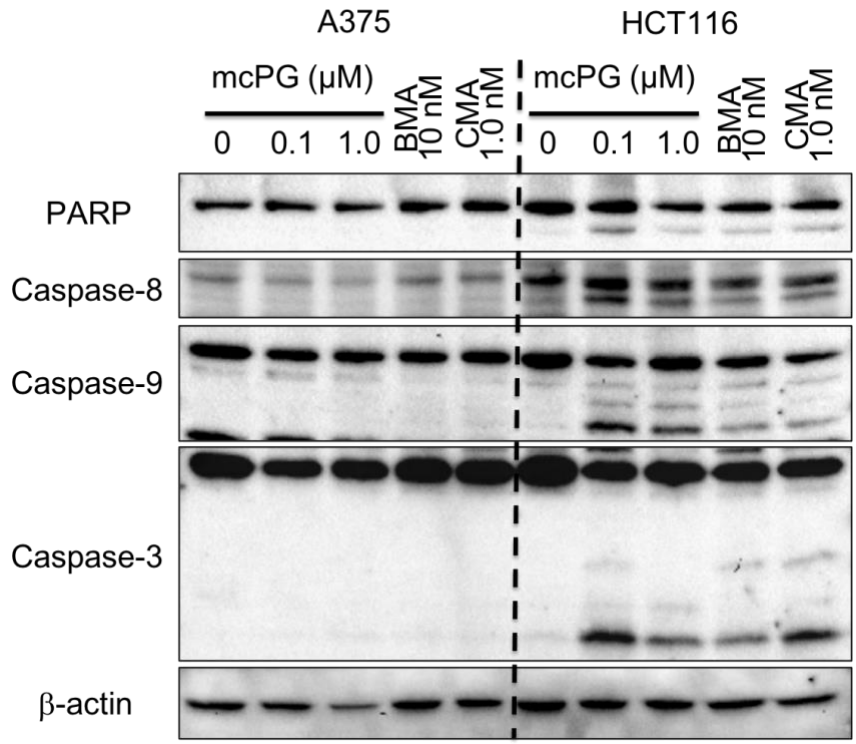

Figure 2-11. Caspase activation by V-ATPase inhibitors treatment.

A375 and HCT116 cells were treated with the indicated concentrations of mcPG, BMA and CMA for 24 h. Cell lysates were prepared for western blot analysis to detect PARP, caspase-8, -9, -3.

2.1.5 DR4-mediated death signal is involved in V-ATPase inhibitors-induced apoptosis.

Caspase-8 is well characterized as an initiator of death receptor-mediated apoptosis, and the death receptor-mediated extrinsic apoptosis pathway is initiated when the extracellular tumor necrosis factor (TNF) superfamily death ligands including TNF-α, Fas ligand/CD95L, and TNF-related apoptosis-inducing ligand (TRAIL) bind to specific cell surface death receptors[60]. Among TNF superfamily death ligands, TRAIL has been shown to induce apoptosis in cancer cells through binding to its receptors DR4 and DR5, but not in normal cells[61,62]. Therefore, the author first investigated the possibility whether DR4 and/or DR5 was involved in V-ATPase inhibitors-induced apoptosis in HCT116 cells. As shown in Figure 2-12, when HCT116 cells and A375 cells were treated with V-ATPase inhibitors for 12 h, expression levels of cell surface DR4 were dramatically increased in HCT116 cells, but not in A375 cells. On the contrary, expression levels of cell surface DR5 were not affected in both cells following V-ATPase inhibitor treatment. Next the author examined the effect of DR4 or DR5 siRNA on V-ATPase inhibitors-induced apoptosis. As shown in Figure 2-13, the successful knockdown of DR4 and DR5 by its siRNA was confirmed by western blot analysis. V-ATPase inhibitors-induced apoptosis was suppressed by the knockdown of DR4 in HCT116 cells. On the other hand, V-ATPase inhibitors still induced apoptosis in DR5 siRNA-transfected HCT116 cells (Figure 2-14). Neither inactivation of TNF-α signal nor Fas/FasL system by using neutralizing antibodies suppressed V-ATPase inhibitors-induced apoptosis in HCT116 cells (Figure 2-15).

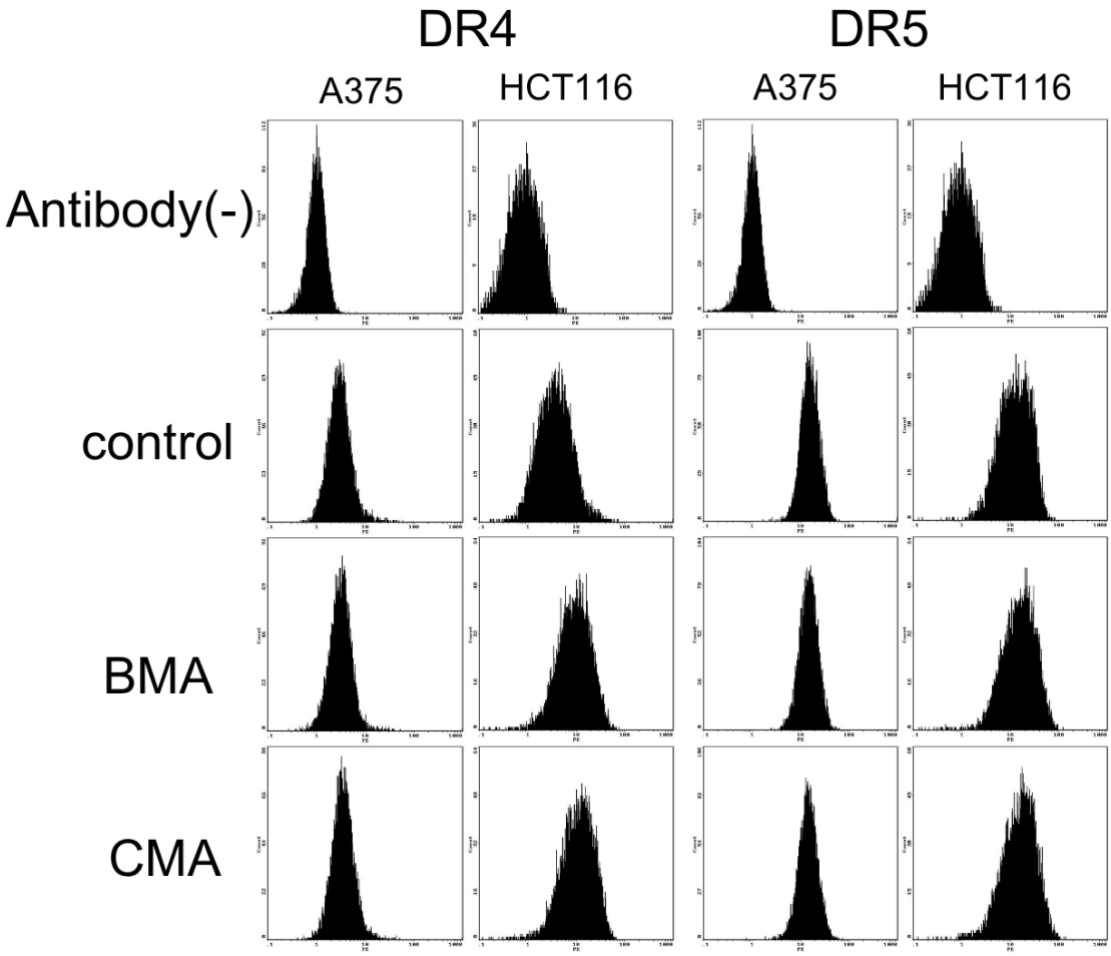

Figure 2-12. The effect of V-ATPase inhibitors on DR4/5 cell surface expression levels.

A375 and HCT116 cells were treated with BMA and CMA for 12 h. Cell surface expression of DR4/5 was determined by flow cytometer.

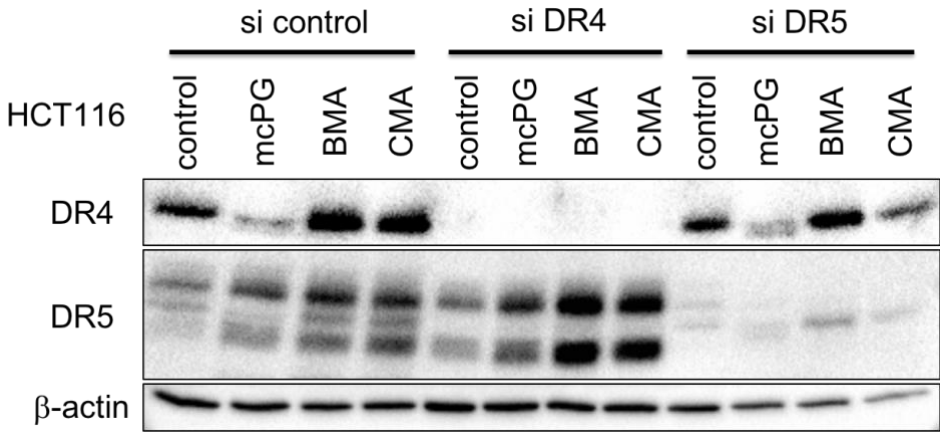

Figure 2-13. Expression levels of DR4/5 after V-ATPase inhibitors treatment.

HCT116 cells were transiently transfected with negative control, DR4 or DR5 siRNA. 48 h after, cells were treated with V-ATPase inhibitors. After 24 h treatment, cell lysates were prepared for western blot analysis to detect the expression of DR4, DR5.

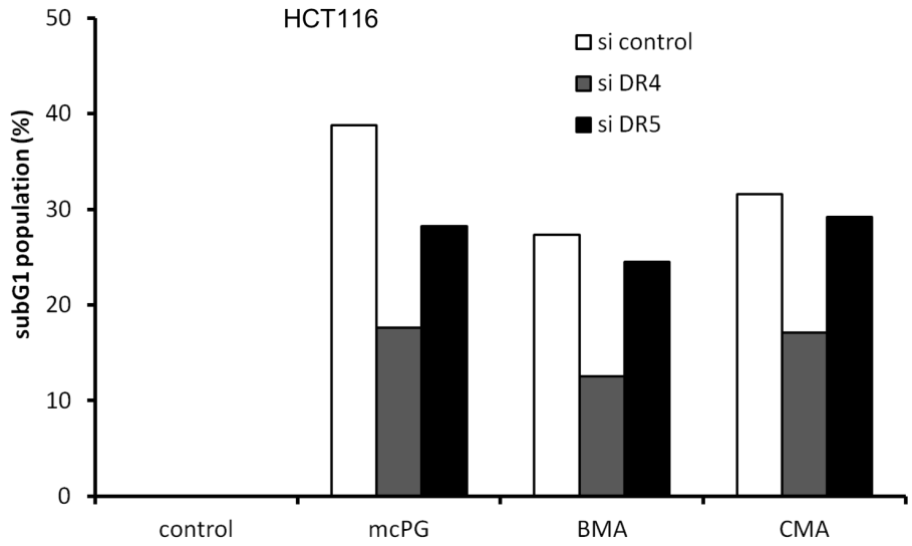

Figure 2-14. Involvement of DR4 in V-ATPase inhibitors-induced apoptosis.

HCT116 cells were transiently transfected with negative control, DR4 or DR5 siRNA. 48 h after, cells were treated with V-ATPase inhibitors. After 48 h treatment, subG1 populations were measured by flow cytometer.

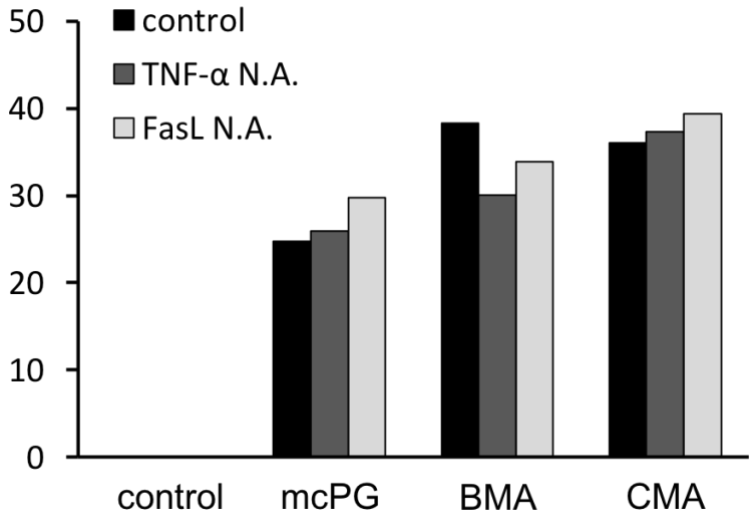

Figure 2-15. Involvement of TNF/Fas signaling pathway in V-ATPase inhibitors-induced apoptosis.

HCT116 cells were treated with V-ATPase inhibitors in the presence or the absence of 100 ng/mL TNF-α neutralizing antibody or 1.0 μg/mL FasL neutralizing antibody for 48 h. SubG1 populations were measured by flow cytometer.

2.2 Discussion

β-catenin is a main component of Wnt signaling pathway playing an important role in tumor cell proliferation. Activating β-catenin mutations are frequently seen in human cancer. Numerous downstream signaling pathways have been shown to be deregulated by β-catenin mutations. Therefore, several small molecules targeting Wnt signaling pathway have been developed for the patients with mutant β-catenin. However, to date there are still no effective therapeutic compounds. Therefore, development of a new type of small molecule that can exhibit synthetic lethality with β-catenin mutation by a distinct mode of action from other inhibitors that target Wnt signaling pathway itself is required.

Previously Kiga *et al*. have reported that MEK inhibitor, SMK-17, induced G1 cell cycle arrest in BRAF-mutant tumor cells, but it induced apoptosis in tumor cell lines harboring β-catenin mutations[50]. Moreover, tumor regression in response to multiple daily oral administration of SMK-17 was observed only in active β-catenin mutant xenograft models, without significant body weight loss. On the other hand, the maximum effect of SMK-17 was just growth inhibition of tumor cells expressing wild type β-catenin. These results demonstrated that MEK inhibitor, SMK-17, exhibited synthetic lethality with β-catenin mutation. Unfortunately, when they found this effect of SMK-17, development of SMK-17 in pharmaceutical company has already dropped out. Therefore, the author conducted the screening for other compounds than MEK inhibitor, and found that inhibitors of V-ATPase preferably induced apoptosis in β-catenin-mutated tumor cells. Moreover, the author demonstrated that apoptosis induction by V-ATPase inhibitors in β-catenin-mutated tumor cells was closely correlated to their V-ATPase inhibitory activities. V-ATPase is a complex multisubunit protein devoted to the transport of protons from the

cytoplasm towards intracellular compartments and from inside to outside of the cell through the cytoplasmic membrane[63,64]. V-ATPase contributes to lower extracellular pH thus activating extracellular metalloproteinases that promote tumor cell survival, motility and invasion, resulting in enhanced malignancy ability. In addition, upregulation or overexpression of V-ATPase is frequently observed in several types of solid tumors and the causative acidic microenvironment offers an advantage in tumor progression, chemoresistance, and metastatic behavior[65,66]. Therefore, the diverse functions of the V-ATPase in tumor survival and metastasis make it an attractive potential target in the development of anticancer drugs. Indeed, several papers have reported that V-ATPase inhibitors showed antitumor effects *in vitro* and *in vivo*. Sasazawa *et al*. previously reported that V-ATPase inhibitors potentiated the cytotoxicity of anticancer drugs in Bcl-2/Bcl-xL-overexpressing tumor cells[67], and Yoshimoto *et al*. reported that V-ATPase inhibitors induced apoptosis in EGF receptor-overexpressing tumor cells only when the cells were stimulated with EGF[68]. However, so far, selective apoptosis inducing ability in mutant β-catenin tumor cells by V-ATPase inhibitors have not been reported; therefore, this is the first report to find that V-ATPase inhibitors exhibited synthetic lethality with active β-catenin mutation in tumor cells. Synthetic lethality of V-ATPase inhibitors with active β-catenin mutation was confirmed by the author's finding that V-ATPase inhibitors failed to induce apoptosis in HEK-293T cells, but they induced apoptosis in HEK-293T cells by the overexpression of the active form of β-catenin (Figure 2-8). These results indicated that expression of active form of β-catenin is required for the induction of apoptosis by V-ATPase inhibitors.

The author also demonstrated the apoptotic pathway to reveal the mechanisms of V-

ATPase inhibitors-induced apoptosis in tumors harboring active β-catenin mutations. Apoptosis involves the activation of caspases, which orchestrate all of the morphological changes that characterize this form of cell death. The author found that mcPG-induced apoptosis pathway was initiated by activation of caspase-8 that leads to the activation of downstream caspases, caspases-9 and -3. Caspase-8 activation is initiated through death receptors/ligands interaction, for example, Fas, TNF and DR4/5 are well-known as death receptors[69,70]. Among these receptors, the author found that V-ATPase inhibitors induced the increase in the expression levels of cell surface DR4, but not DR5, selectively in β-catenin mutant HCT116 cells. Numerous compounds have been reported to upregulate both DR4 and DR5, or activate DR5 but not DR4. On the other hand, very few compounds have been reported to upregulate DR4, but not DR5. One example is andrographolide, a diterpenoid lactone isolated from a traditional herbal medicine *Andrographis paniculata*[71]. Andrographolide increased the cell surface expression of DR4 but not DR5 in a p53-dependent manner. V-ATPase inhibitors are reported to induce upregulation of expression levels of DR4 and DR5[72], however, they induced predominantly upregulation of cell surface expression of DR4 but not DR5 in this study. Therefore, V-ATPase inhibitors are second example that activated DR4 signaling. Intracellular V-ATPase is important for receptor-mediated endocytosis and intracellular trafficking, protein processing and degradation, coupled transport of small molecules and ions. Therefore, it is likely that impairment of these events is closely related to the increase in the expression of DR4 on cell surface, because these death receptors are degraded in lysosome via endocytosis. However, at present the author still does not know why it occurred selectively in tumor cells carrying active β-catenin mutations, or why expression levels of DR5 on cell surface

was not affected following V-ATPase inhibition.

Furthermore, DR4 knockdown was found to suppress V-ATPase inhibitors-induced apoptosis, suggesting the involvement of DR4 activation in V-ATPase inhibitors-induced apoptosis (Figure 2-14). Apoptosis mechanism induced by V-ATPase inhibitors is cell type dependent. In EGF receptor-overexpressing cells, V-ATPase induced apoptosis only when cells were stimulated with EGF. This EGF-dependent apoptosis induced by V-ATPase inhibitors was mediated by Fas/FasL signaling[68]. In this case, V-ATPase inhibitors caused enhancement of EGF-induced cell surface expression of FasL, thereby inducing apoptosis through Fas/FasL system. Indeed, a monoclonal antibody to FasL (NOK2) that neutralizes the cytotoxic effect of FasL, inhibited EGF dependent V-ATPase inhibitors-induced apoptosis. On the other hand, in β-catenin mutant tumor cells, NOK2 failed to inhibit V-ATPase inhibitors-induced apoptosis (Figure 2-15). Moreover, TNF-α receptor neutralizing antibody neither inhibited V-ATPase inhibitors-induced apoptosis. These results demonstrated that V-ATPase inhibitors induced the increase in cell surface expression levels of DR4, thereby causing activation of caspase-8 that leads to the activation of downstream caspases, including caspases-9 and -3.

In conclusion, this study interestingly found new roles for V-ATPase inhibitors, indicating the possibility of V-ATPase inhibitors as new therapeutic strategies against tumors carrying active β-catenin mutations.

2.3 Materials and Methods

2.3.1 Cell lines and cell culture

Human malignant melanoma A375 cells, human colorectal carcinoma HCT116, HT29, SW48, Colo-205, DLD-1, LoVo, LS-174T, SW620 cells, human hepatocellular carcinoma HepG2 cells, human prostate carcinoma PC-3 cells and human lung carcinoma A549 cells were maintained in Rosewell Park Memorial Institute medium (Nissui) supplemented with 10% fetal bovine serum. Human embryonic kidney HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum. Cells were passaged every 2-3 days to maintain exponential growth.

2.3.2 Compounds and materials

Metacycloprodigiosin was isolated from actinomycetes strain. Bafilomycin A1, propidium iodide (PI), z-VAD-FMK, z-IETD-FMK and z-LEHD-FMK were from Sigma. Acridine orange was from Merck. Ac-DEVD-CHO was from Peptide Institute. Concanamycin A was kindly provided by Prof. Hiroyuki Osada (Riken CSRS Chemical Biol. Res. Group). Mutated β-catenin (S37A, S45A) plasmid vectors were kindly provided by Daiichi Sankyo Co. Ltd.

2.3.3 Propidium iodide (PI) staining

Cells were treated with each compound for 48 h. After adherent and detached cells were combined, the cells were fixed with 70% ice-ethanol. After ethanol was removed, cells

were treated with 10 μg/mL RNase for 20 min, and stained with propidium iodide. Finally, subG1 population within 10,000 cells was measured by flow cytometer.

2.3.4 Acridine orange staining

Cells grown on coverslips were treated with indicated compounds for 4 h. Then, they were incubated with 3 μM acridine orange for 30 min. After washing with PBS–, the coverslips were examined with a confocal laser scanning microscope.

2.3.5 Western blotting

Antibodies recognizing PARP, caspase-8, caspase-9, caspase-3 and cleaved PARP (Asp214) were from Cell Signaling Technology. β-actin antibody was from Sigma-Aldrich. β-catenin antibody was from BD Biosciences. DR4 and DR5 antibodies were from Millipore. Cells were lysed with RIPA buffer (25 mM HEPES, 1.5% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 1 tablet/50 mL protease inhibitor cocktail cOmplete (Roche Applied Science): pH7.8). Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. After probing with specific antibodies, the immune complexes were detected with the Immobilon Western kit (Millipore) and ChemiDoc Imaging system (Bio-Rad).

2.3.6 Transfection

Cells were transfected with control vectors or mutated β-catenin (S37A, S45A) plasmid vectors via forward transfection using Lipofectamine 3000 (Life Technologies), according to the manufacturer's protocol.

2.3.7 Detection of cell surface protein expression

Cells were treated with each compound for 12 h. After adherent and detached cells were combined, the cells were incubated with FCM buffer (0.1%BSA, 0.1%NaN_3/PBS_-) with the indicated antibodies. After washed twice, fluorescence was measured by flow cytometer.

2.3.8 RNA interference

Cells were transfected with Stealth RNAi Negative Control (Invitrogen), siDR4 (HSS112945) and siDR5 (HSS112939) reverse transfection using Lipofectamine RNAiMAX (Invitrogen), according to the protocol provided by the manufacturer.

Chapter 3:
Miclxin, a novel MIC60 inhibitor, induces apoptosis via mitochondrial stress response in β-catenin mutant tumor cells

3.1 Result

3.1.1 Miclxin induced β-catenin-dependent cell death.

The author screened for compounds that induced cell death in β-catenin-mutated HCT116 cells but not in β-catenin wild-type A375 cells. More than 25,000 compounds provided by Daiichi-Sankyo Pharmaceutical Company were screened, and six compounds showed selective cell death in HCT116 cells (Figure 3-1). Among these six hit compounds, only DS37262926 (miclxin; Figure 3-2) failed to induce cell death in β-catenin-knockdown HCT116 cells (Figure 3-3), indicating that miclxin induced cell death in β-catenin-mutated HCT116 cells in a β-catenin-dependent manner.

The author's research group previously reported that MEK1/2 inhibitors (e.g., SMK-17) and V-ATPase inhibitors (e.g., metacycloprodigiosin) also induced cell death selectively in β-catenin-mutated tumor cells[50,73]. On the other hand, miclxin inhibited neither MEK activity as judged from the phosphorylation of ERK1/2 (Figure 3-4) nor V-ATPase activity as judged from the intracellular pH (Figure 3-5). These results indicate that miclxin induces cell death selectively in β-catenin-mutated HCT116 cells through a different mechanism from previously reported other compounds.

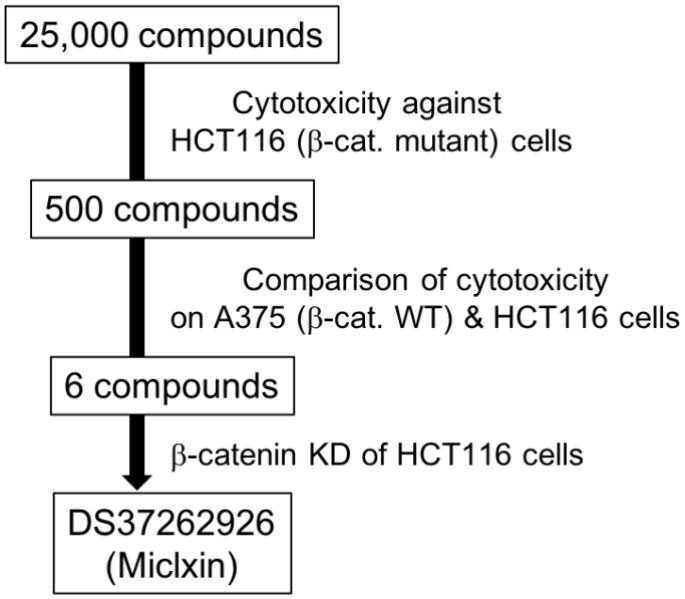

Figure 3-1. Schematic view of the screening.

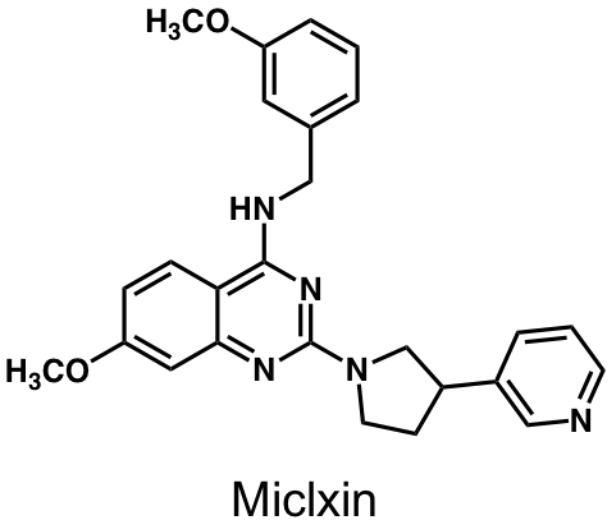

Figure 3-2. Structure of miclxin.

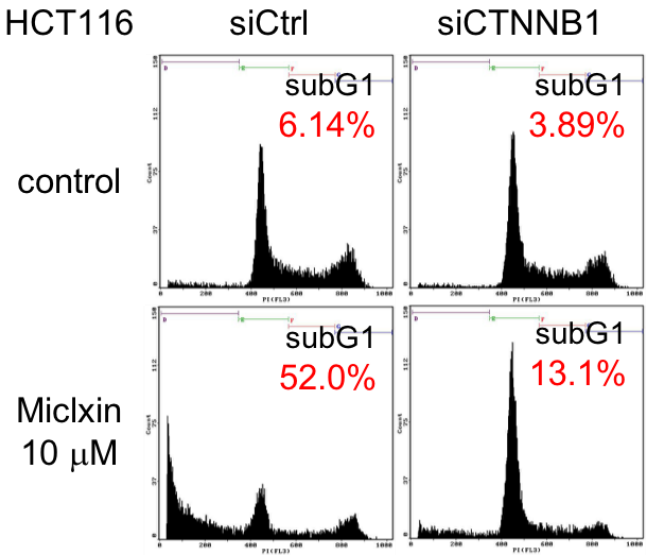

Figure 3-3. Miclxin induced β-catenin-dependent cell death.

Control and β-catenin-knockdown HCT116 cells were treated with miclxin for 48 h. Subsequently, the subG1 populations were counted through flow cytometer.

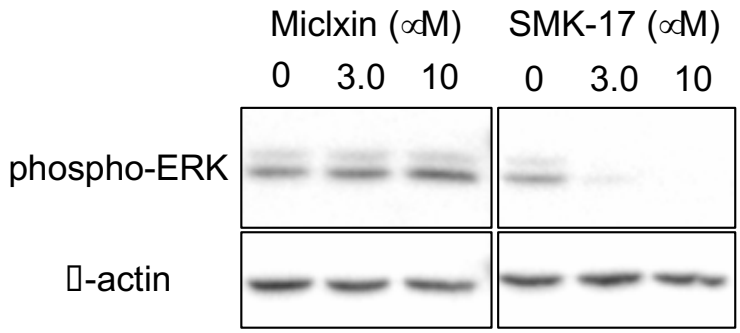

Figure 3-4. Miclxin did not inhibit MEK activity.

HCT116 cells were treated with miclxin or SMK-17, as MEK inhibitor positive control, for 24 h. Cell lysates were immunoblotted with the indicated antibodies.

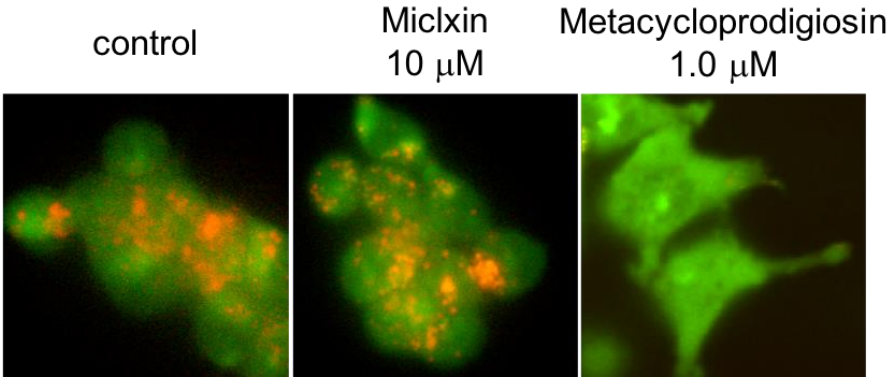

Figure 3-5. Miclxin did not inhibit V-ATPase activity.

HCT116 cells on coverslips were treated with miclxin or metacycloprodigiosin, as V-ATPase inhibitor positive control, for 4 h, followed by staining with 3 μM acridine orange for 30 min. After washing, the cells were observed under fluorescence microscope.

3.1.2 Miclxin directly bound to the mitochondrial inner membrane complex protein MIC60.

The author attempted to identify the target protein of miclxin to reveal the mechanism of miclxin-induced cell death. For this purpose, the author used miclxin-immobilized agarose beads prepared through the photocross-linking method[74]. The cytosolic, nuclear, and mitochondrial fractions of HCT116 cells were incubated with control beads or miclxin beads for 3 h. The proteins bound to each set of beads were separated via SDS-PAGE and stained using the silver staining method. As shown in Figure 3-6, the author detected one protein band in the cytosolic fraction, namely heat shock cognate 71 kDa protein (HSPA8). In the mitochondrial fraction, the author detected four protein bands, namely MIC60, translocase of outer mitochondrial membrane 40 (TOM40), voltage-dependent anion channel (VDAC), and TOM22. It was previously reported that MIC60 co-localizes with the TOM complex and VDAC[75,76]. Therefore, the author focused on this protein. The binding of miclxin to MIC60 was confirmed through western blotting using an anti-MIC60 antibody (Figure 3-7). The author performed an *in vitro* binding assay using GST-tagged MIC60 mutants to determine whether miclxin could directly bind to MIC60. Owing to the insolubility of full-length MIC60[77], the author conducted this assay using C-terminal deletion mutant GST-tagged MIC60(1-200) or N-terminal deletion GST-tagged MIC60(65-758). As shown in Figure 3-8, unlike GST-MIC60(65-758), GST-MIC60(1-200) was co-precipitated with miclxin beads, indicating that miclxin directly bound to the N-terminal region (1-64) of MIC60.

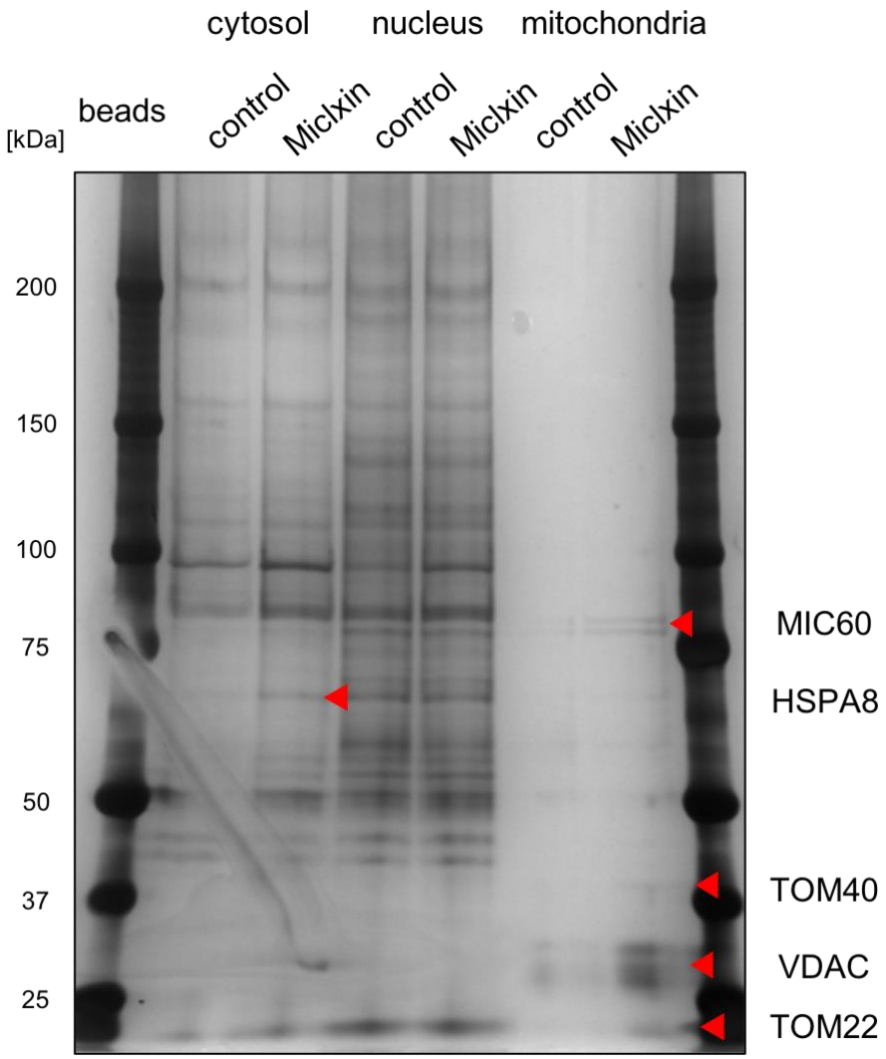

Figure 3-6. Identification of miclxin-binding proteins.

Subcellular fractionated HCT116 lysates were incubated with control or miclxin beads for 3 h. The co-precipitated proteins were eluted and analyzed using the silver staining method. Detected bands were identified using LC-MS/MS.

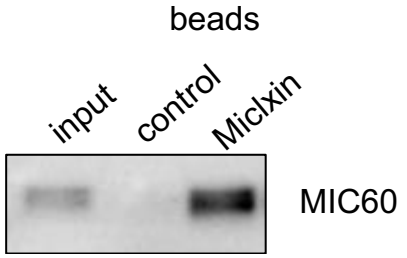

Figure 3-7. Miclxin bound to MIC60.

Subcellular fractionated HCT116 lysates were incubated with control or miclxin beads for 3 h. The co-precipitated proteins were eluted and immunoblotted with an anti-MIC60 antibody.

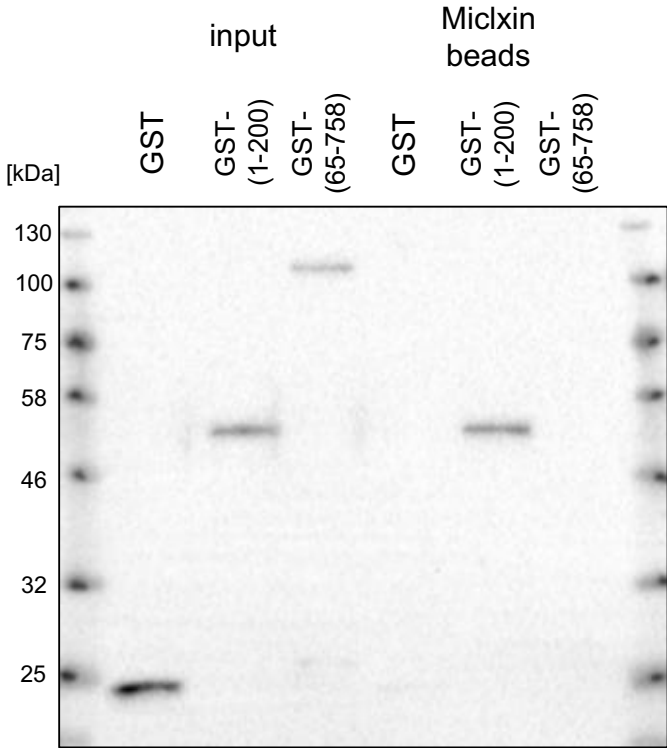

Figure 3-8. Miclxin directly bound to MIC60 N-terminal region.

Purified GST, GST-MIC60(1–200), and GST-MIC60(65–758) were incubated with miclxin beads overnight. The co-precipitated proteins were eluted and immunoblotted with an anti-GST antibody.

3.1.3 Miclxin induced MIC60 dysfunction, leading to mitochondrial stress.

MIC60 is one of the major components of the mitochondrial contact site and cristae organizing system (MICOS) complex, and plays an important role in the maintenance of mitochondrial function, structure, and homeostasis[78,79]. As miclxin directly bound to MIC60, the author next examined whether miclxin inhibited the function of MIC60. As shown in Figure 3-9, 12 h treatment with miclxin or 3 days treatment with MIC60 siRNA significantly reduced MMP, judged from the observed decrease in DilC1(5) fluorescence. In addition, the production of ROS was observed by determining the levels of CM-H_2DCFDA fluorescence after treatment with miclxin or knockdown of MIC60 (Figure 3-10). Furthermore, pre-treatment with the antioxidant α-tocopherol prevented miclxin-induced cell death (Figure 3-11). These results suggested that miclxin bound to MIC60 and inhibited its function, leading to mitochondrial damage, ROS production, and subsequent cell death.

The eukaryotic translation initiation factor 2α (eIF2α)-activating transcription factor 4 (ATF4)-C/EBP homologous protein (CHOP) pathway is upregulated in response to various intracellular stresses, especially ER stress[80] and mitochondrial stress[81–83]. Therefore, the author examined whether dysfunction of MIC60 could induce mitochondrial stress response. The author found that treatment with miclxin or knockdown of MIC60 induced an increase in the expression levels of phosphorylated eIF2α, ATF4, and CHOP (Figure 3-12). This finding indicated that dysregulation of MIC60 by miclxin or MIC60 siRNA contributed to the activation of mitochondrial stress response.

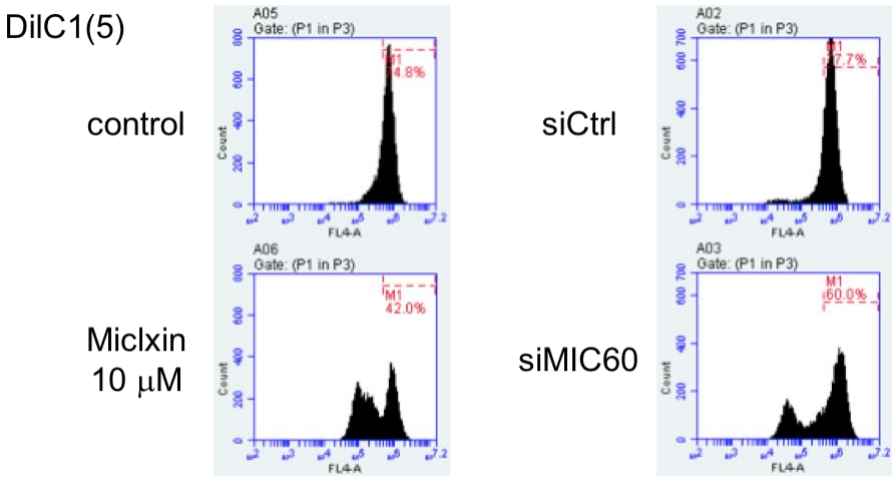

Figure 3-9. Miclxin induced MMP loss.

After treatment with miclxin for 12 h or transfection with MIC60 siRNA for 3 days, the MMP of HCT116 cells was analyzed via staining with DilC1(5) using flow cytometer.

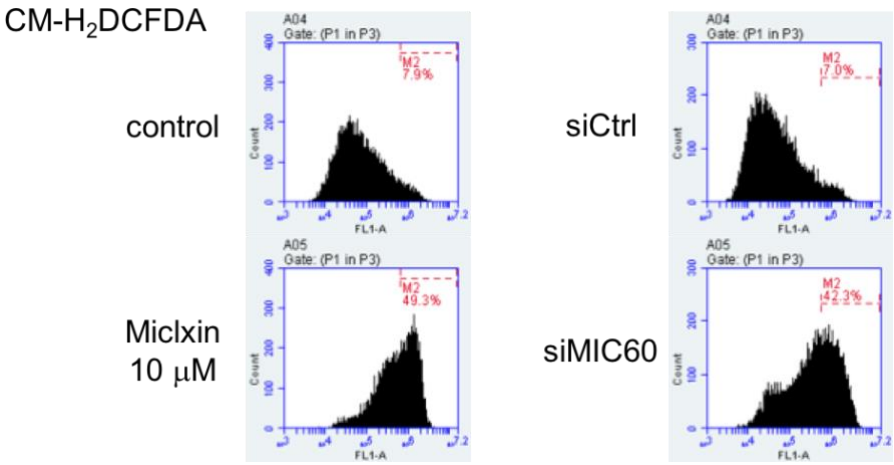

Figure 3-10. Miclxin induced ROS production.

After treatment with miclxin for 12 h or transfection with MIC60 siRNA for 3 days, the production of ROS in HCT116 cells was analyzed through flow cytometer by staining with CM-H2DCFDA.

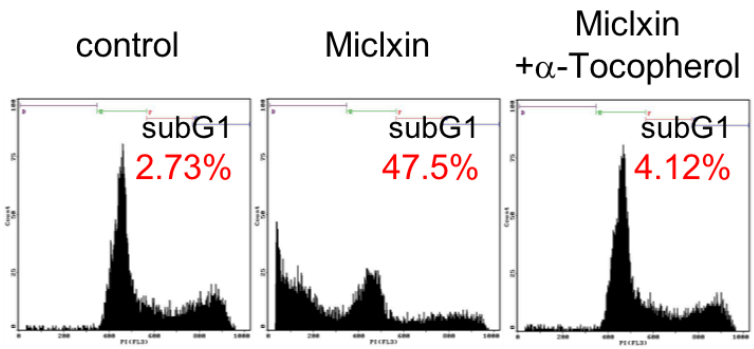

Figure 3-11. Miclxin induced ROS-mediated apoptosis.

HCT116 cells were pre-treated with 100 μM antioxidant α-tocopherol for 1 h and subsequently treated with miclxin for 48 h. SubG1 populations were counted using flow cytometer.

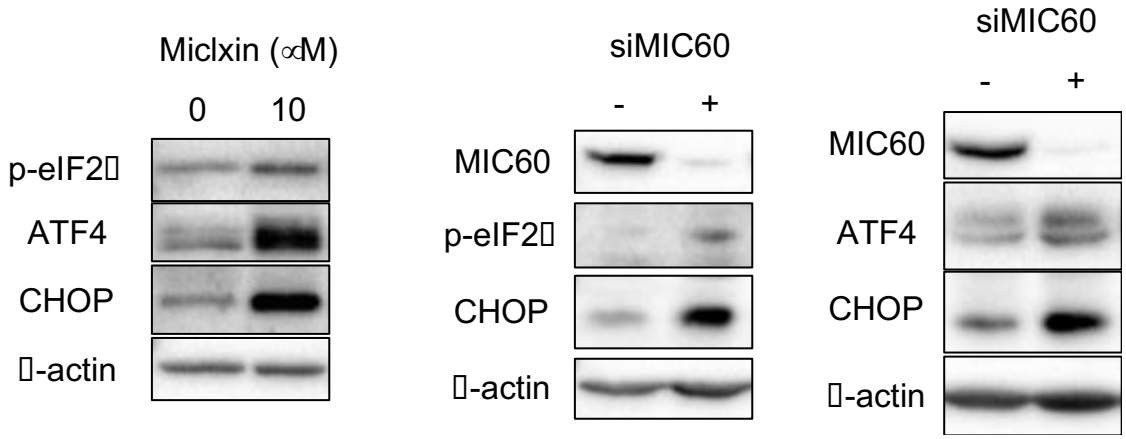

Figure 3-12. Miclxin induced mitochondrial stress.

HCT116 cells were treated with miclxin for 24 h or transfected with MIC60 siRNA for 3 days. Cell lysates were immunoblotted with the indicated antibodies.

3.1.4 Miclxin-induced mitochondrial stress response caused late-stage MMP loss.

The author examined the effect of CHOP knockdown on the loss of MMP by MIC60 dysfunction to determine the role of mitochondrial stress response on mitochondrial damage. As shown in Figure 3-13, loss of MMP was slightly induced by knockdown of CHOP. Miclxin-mediated loss of MMP was observed in both CHOP-knockdown HCT116 cells and control cells at 12 h. At 24 h, further loss of MMP was observed in control cells but not in CHOP-knockdown cells. These results indicated that miclxin induces MMP loss in two stages: 1) inhibition of MIC60 function triggers early-stage MMP loss; and 2) expression of CHOP in response to mitochondrial stress is responsible for late-stage MMP loss.

It has been shown that CHOP regulates the expression levels of proteins belonging to the Bcl family[84,85]. As shown in Figure 3-14, miclxin induced the downregulation of Bcl-2, and this downregulation was cancelled by the knockdown of CHOP. Similar results were obtained following the knockdown of MIC60. These results indicated that expression of CHOP in response to MIC60 dysfunction-mediated mitochondrial stress is responsible for the downregulation of Bcl-2, leading to late-stage MMP loss.

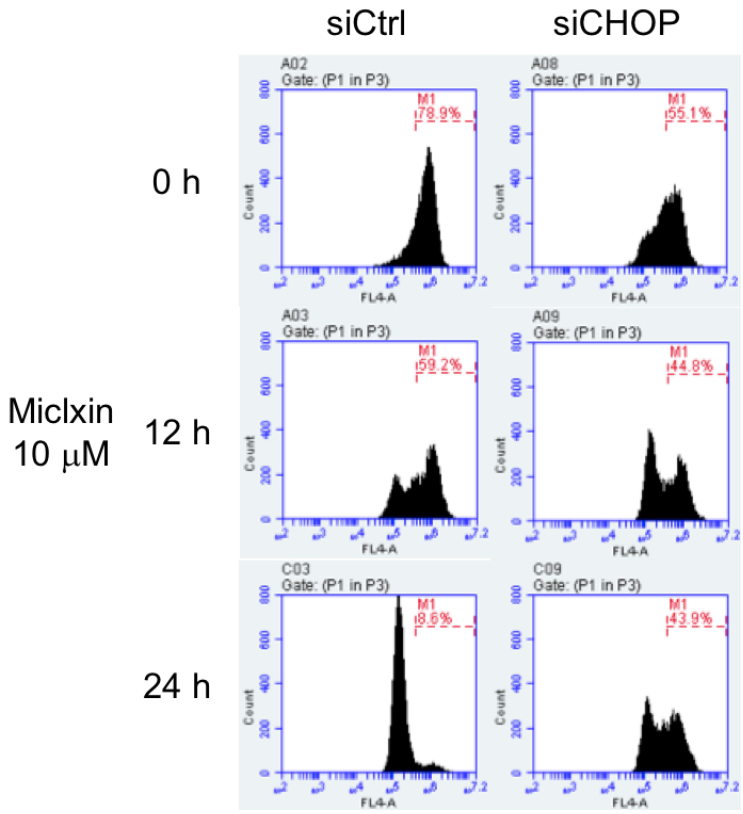

Figure 3-13. Miclxin caused the two-stage MMP loss by inducing mitochondrial stress.

Following treatment with miclxin for 12 h and 24 h, the MMP of HCT116 cells transfected with CHOP siRNA was analyzed via staining with DilC1(5) using flow cytometer.

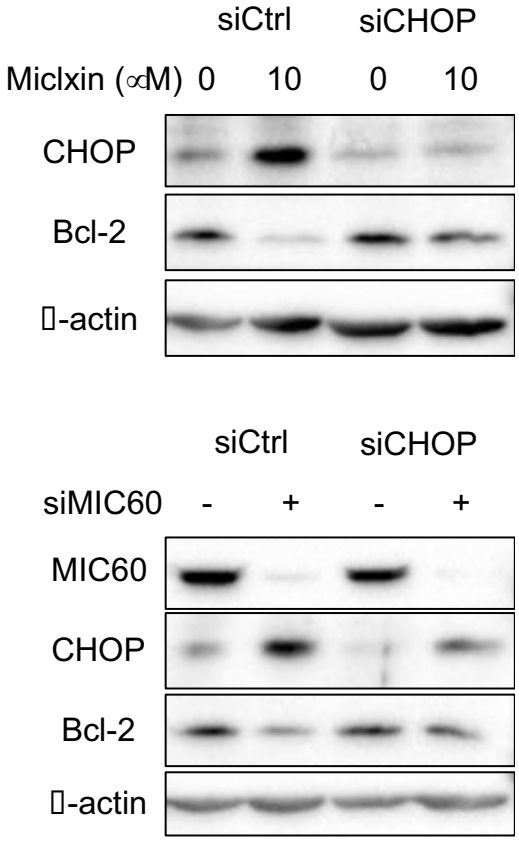

Figure 3-14. Mitochondrial stress induced by miclxin caused Bcl-2 downregulation. HCT116 cells transfected with CHOP siRNA were treated with miclxin for 24 h. Cell lysates were immunoblotted with the indicated antibodies.

3.1.5 Miclxin induced mitochondrial stress-mediated apoptosis.

It is established that caspases mediate apoptosis, therefore, the author tested the effect of the pan-caspase inhibitor z-VAD-FMK on miclxin-induced apoptosis. As shown in Figure 3-15, pre-treatment of HCT116 cells with z-VAD-FMK failed to inhibit the miclxin-induced increase in the subG1 population detected through flow cytometry analysis. These results indicated that miclxin induced caspase-independent apoptosis. Normally, AIF is localized in mitochondria. However, various cellular stresses (e.g., oxidative stress) cause the release of AIF to the cytosol and its translocation into the nucleus, resulting in caspase-independent apoptosis[19,67]. Therefore, the author next examined whether dysregulation of MIC60 by miclxin or MIC60 siRNA could result in the translocation of AIF into the nucleus. HCT116 cells were treated with miclxin or MIC60 siRNA and fractionated into the cytosol and nucleus. According to the results, the expression levels of AIF in the nucleus and cytosol were significantly increased under the condition of pharmacological and genetical inhibition of MIC60 (Figure 3-16). These results strongly suggested that miclxin induced caspase-independent, AIF-dependent apoptosis.

Owing to the induction of mitochondrial stress response by miclxin, the author assessed whether mitochondrial stress was involved in miclxin-induced apoptosis using CHOP siRNA. Following the knockdown of CHOP, miclxin failed to induce apoptosis in HCT116 cells (Figure 3-17). Increased levels of AIF were detected in both the cytosolic and nuclear fractions following treatment with miclxin or MIC60 siRNA. In contrast, AIF was not detected in either of the fractions following knockdown of CHOP even after treatment with miclxin or MIC60 siRNA (Figure 3-18). These results indicated that the

activation of mitochondrial stress response by MIC60 dysfunction contributed to the nuclear translocation of AIF, leading to apoptosis.

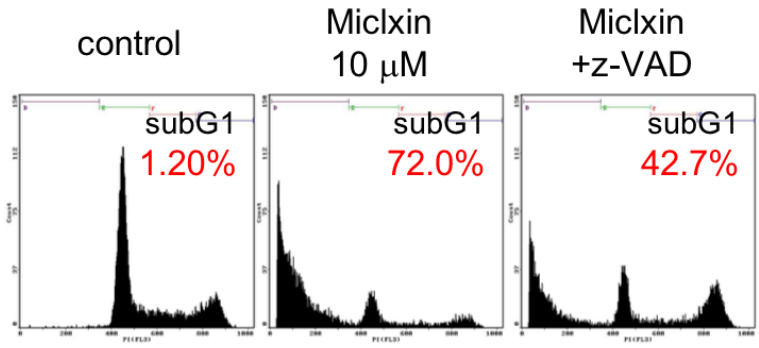

Figure 3-15. Caspase inhibitor failed to inhibit miclxin-induced apoptosis.

HCT116 cells were pre-treated with 50 μM caspase inhibitor z-VAD-FMK for 1 h, and subsequently treated with miclxin for 48 h. SubG1 populations were counted using flow cytometer.

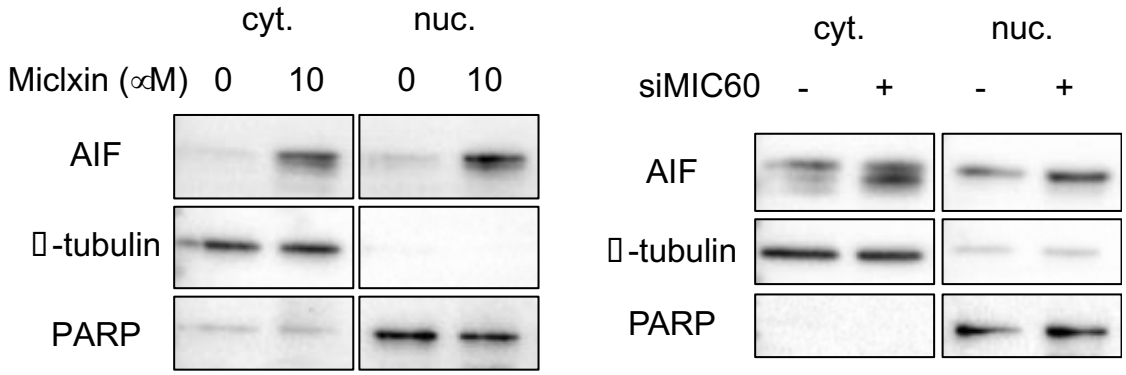

Figure 3-16. Miclxin induced AIF-dependent apoptosis.

HCT116 cells were treated with miclxin for 24 h or transfected with MIC60 siRNA for 3 days and divided into cytosolic and nuclear fractions. The fractionated lysates were immunoblotted with the indicated antibodies.

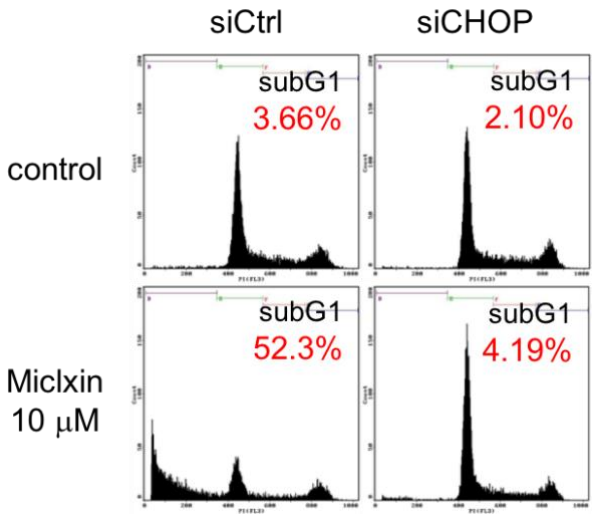

Figure 3-17. Miclxin induced mitochondrial stress-mediated apoptosis.

HCT116 cells transfected with CHOP siRNA were treated with miclxin for 48 h. SubG1 populations were counted using flow cytometer.

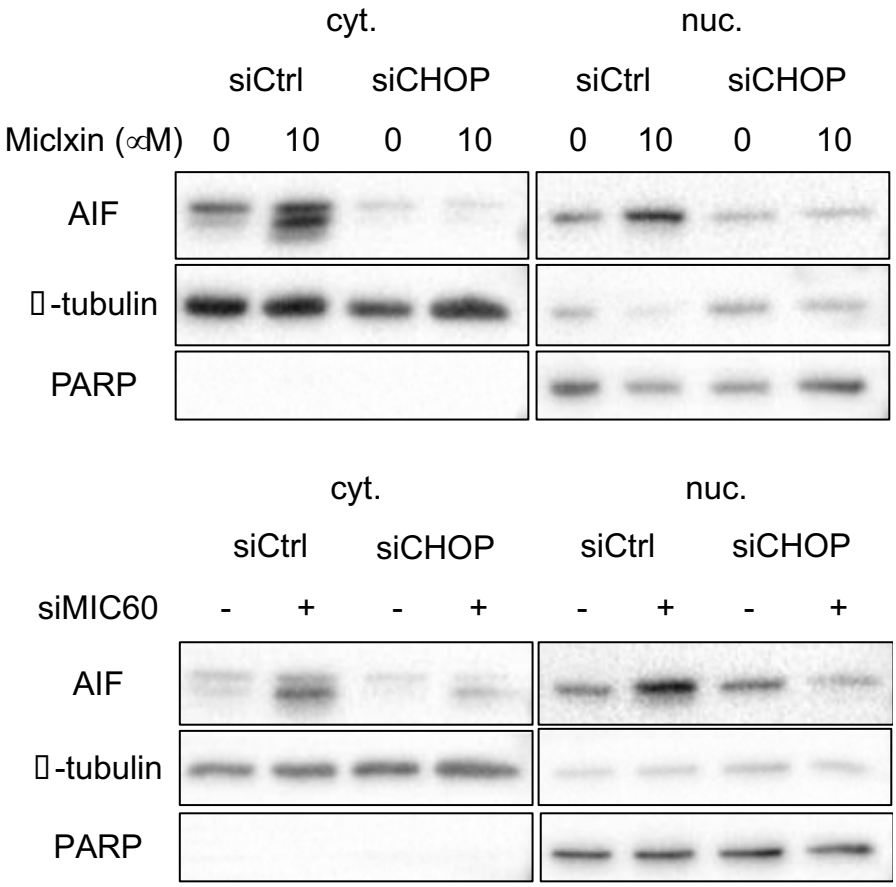

Figure 3-18. Mitochondrial stress triggered AIF-dependent apoptosis induced by miclxin.

HCT116 cells transfected with CHOP siRNA were treated with miclxin for 24 h or transfected with MIC60 siRNA for 3 days and divided into cytosolic and nuclear fractions. Fractionated lysates were immunoblotted with the indicated antibodies.

3.1.6 β-catenin regulated the mitochondrial stress induced by inhibition of MIC60.

Miclxin was originally discovered as a β-catenin-dependent apoptosis inducer. Therefore, the author next examined the mechanism through which β-catenin is responsible for miclxin-induced apoptosis. As shown in Figure 3-19, knockdown of β-catenin could not inhibit miclxin-induced early-stage MMP loss, however, it suppressed late-stage MMP loss. Furthermore, eIF2α phosphorylation as well as ATF4 and CHOP expression in response to mitochondrial stress induced by treatment with miclxin or MIC60 siRNA were suppressed by β-catenin knockdown (Figure 3-20, 3-21). These results indicated that β-catenin played a critical role in mitochondrial stress response, resulting in late-stage MMP loss.

Miclxin-induced apoptosis in HCT116 cells harboring an activating mutation of β-catenin (*CTNNB1* +/Δ45) is dependent on β-catenin. Thus, the author next compared the efficacy of miclxin in an isogenic HCT116 (*CTNNB1* +/–) cell line with knockout of the mutated β-catenin allele and an isogenic HCT116 (*CTNNB1* Δ45/–) cell line with knockout of the wild-type β-catenin allele[86]. Based on the subG1 population, miclxin induced apoptosis in HCT116 *CTNNB1* Δ45/– cells, but not in +/– cells (Figure 3-22). These results indicated that miclxin induced apoptosis in HCT116 cells in a mutant β-catenin-dependent manner.

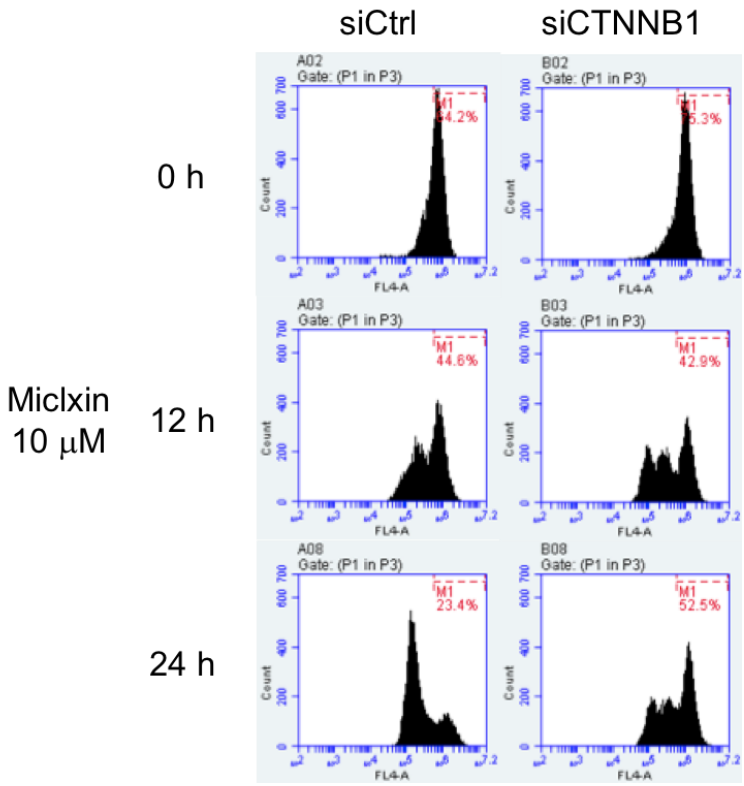

Figure 3-19. β-catenin initiated the late-stage MMP loss.

The mitochondrial membrane potential of HCT116 cells transfected with β-catenin siRNA was analyzed using flow cytometer by staining with DilC1(5) after treatment with miclxin for 12 h and 24 h.

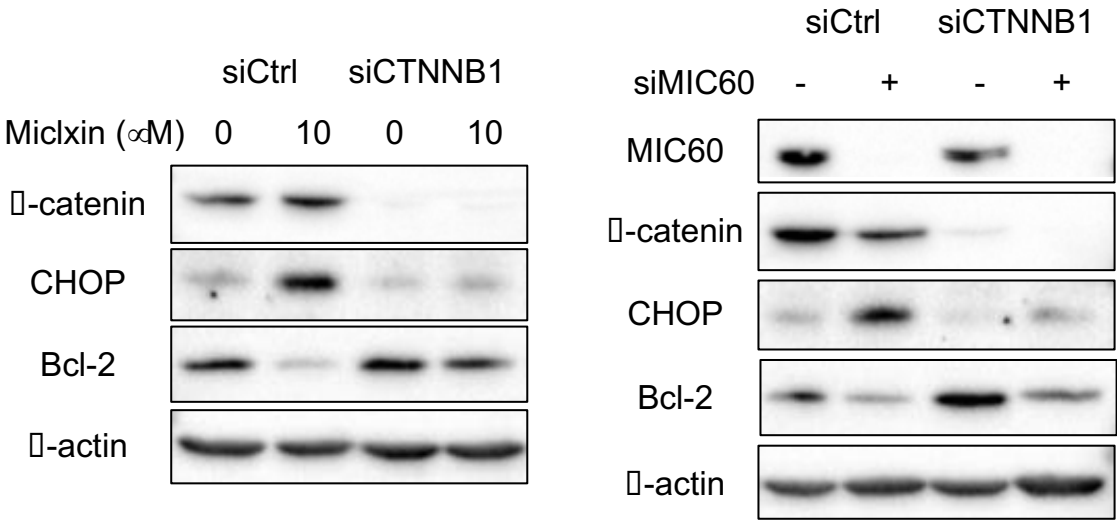

Figure 3-20. β-catenin knockdown recovered Bcl-2 protein expression levels.

HCT116 cells transfected with β-catenin siRNA were treated with miclxin for 24 h or transfected with MIC60 siRNA for 3 days. Cell lysates were immunoblotted with the indicated antibodies.

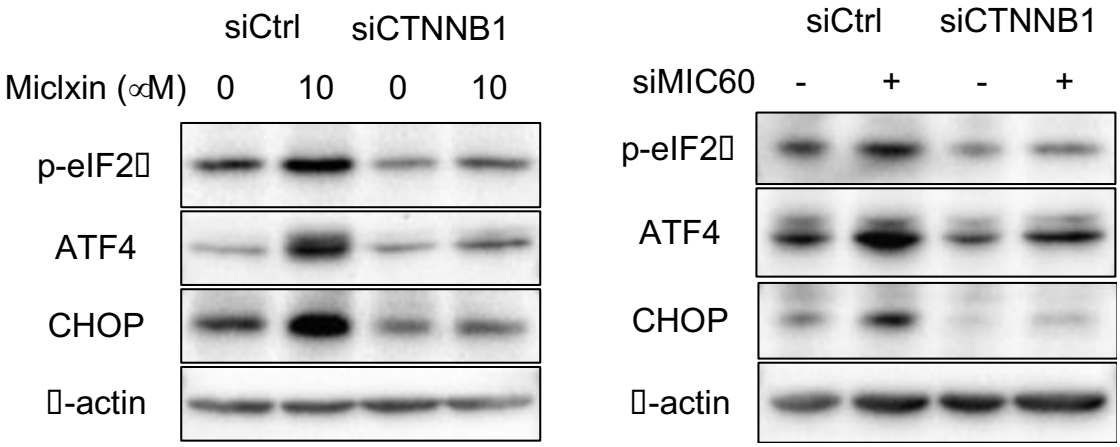

Figure 3-21. β-catenin initiated mitochondrial stress response.

HCT116 cells transfected with β-catenin siRNA were treated with miclxin for 24 h or transfected with MIC60 siRNA for 3 days. Cell lysates were immunoblotted with the indicated antibodies.

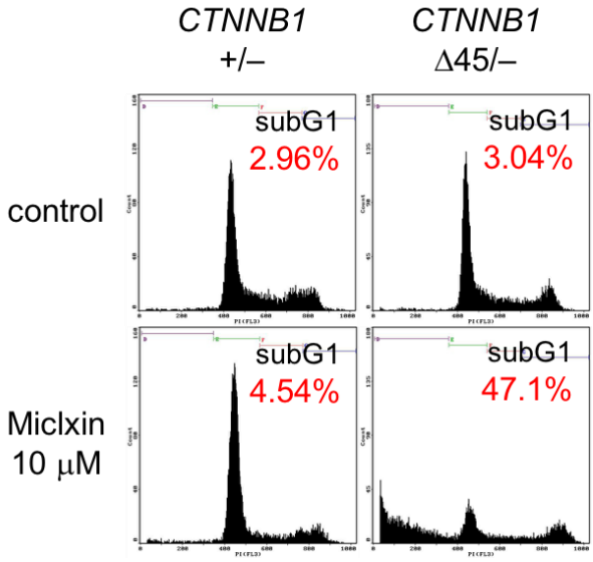

Figure 3-22. Miclxin induced apoptosis selectively in β-catenin-mutated HCT116 cells.

HCT116 *CTNNB1* +/– and Δ45/– cells were treated with miclxin for 48 h. Subsequently, subG1 populations were counted using flow cytometer.

3.2 Discussion

β-catenin is a main component of the Wnt signaling pathway, playing an important role in the proliferation of tumor cells. Park *et al*. concluded that nuclear localization of β-catenin is significantly correlated with shorter survival time and is the most important prognostic factor for survival[87]. β-catenin is normally degraded by the destruction complex to control Wnt signaling activity. Nevertheless, mutations in β-catenin result in the stabilization and nuclear accumulation of β-catenin protein. These previous reports suggest that β-catenin is a potential therapeutic anti-tumor target, however, there have been no promising agents against tumors harboring β-catenin mutations so far[88]. In this study, the author identified miclxin while screening for compounds that induced apoptosis selectively in β-catenin-mutated HCT116 cells and failed to induce apoptosis following the knockdown of β-catenin in these cells (Figure 3-3). Treatment with miclxin induced apoptosis in β-catenin-mutated HCT116 (*CTNNB1* Δ45/–) cells, but not in β-catenin wild-type HCT116 (*CTNNB1* +/–) cells (Figure 3-22). Hence, this apoptosis appears to be dependent on mutant β-catenin. These results suggest that miclxin may be a potential candidate for the development of drugs against β-catenin-mutated tumors.

Next, the author looked into the mechanism of miclxin-induced apoptosis in HCT116 cells. The initial step was to identify MIC60 as the protein binding to miclxin. MIC60 is a major component of the MICOS complex, which consists of at least nine subunits and plays critical roles in mitochondrial function[89]. Madungwe *et al*. previously reported that knockdown of MIC60 impaired the MMP and increased the production of ROS, resulting in AIF-dependent apoptosis[90]. Loss of MMP and increase in ROS, followed by cell death via the AIF mechanism, were also observed in miclxin-treated HCT116 cells, suggesting

that MIC60 was the molecular target through which miclxin induces apoptosis in HCT116 cells. The C-terminal site of MIC60 is located in the mitochondrial intermembrane space, which consists of the MICOS complex and other components (e.g., MIC19 and MIC25), and interacts with outer membrane proteins (e.g., VDAC, TOM, and the sorting and assembly machinery (SAM) complex)[75,76,91,92]. On the other hand, the N-terminal site of MIC60 contains a mitochondria-targeting sequence which contributes to its localization to the mitochondrial inner membrane[91]. *In vitro* binding assay using miclxin-immobilized beads revealed that miclxin directly bound to the N-terminus (1-64) of MIC60 (Figure 3-8). This result suggests that miclxin does not dissociate the MICOS complex or MICOS outer membrane protein complex. However, it affects the connection to the inner membrane of MIC60, which induced dysfunction of MIC60. Suzuki *et al*. previously reported that the synthetic compound MA-5, mitochonic acid 5 (4-(2,4-difluorophenyl)-2-(1H-indole-3-yl)-4-oxobutanoic acid), bound to MIC60 lacking the N-terminal transmembrane domain (1-120) and enhanced the mitochondrial function and cell viability[77,93]. Therefore, to the best of the author's knowledge, miclxin is the first compound that binds to MIC60 and inhibits its function.

Mitochondrial stress response has recently attracted attention as a form of intracellular stress, in which the eIF2α-ATF4-CHOP pathway is upregulated. The author found that treatment of HCT116 cells with miclxin or MIC60 siRNA upregulated the eIF2α-ATF4-CHOP pathway, indicating that dysfunction of MIC60 caused mitochondrial stress response. The author further demonstrated that this mitochondrial stress response played an important role in late-stage MMP loss. Miclxin-mediated dysfunction of MIC60 induced loss of MMP at two stages. Early-stage moderate loss of MMP was observed

following 12 h treatment with miclxin. Late-stage severe loss of MMP was observed after 24 h treatment. This effect was determined to be mediated by mitochondrial stress response because late-stage MMP loss was not observed after knockdown of CHOP. This finding proposed a new function of mitochondrial stress response.

Subsequently, the author investigated the mechanism through which mitochondrial stress response induces late-stage MMP loss. It has been shown that CHOP regulates the expression levels of certain downstream proteins, including proteins belonging to the Bcl family and death receptors[84,85]. Among these proteins, the author demonstrated that Bcl-2 may be the key regulator of late-stage MMP loss in response to mitochondrial stress response. Bcl-2 is established as an anti-apoptotic protein that interacts with mitochondria to prevent the loss of MMP and programmed cell death[94,95]. Therefore, it is suggested that downregulation of Bcl-2 by CHOP may be an important event in late-stage MMP loss, which is responsible for AIF-dependent apoptosis. On the other hand, mitochondrial stress response induced an increase in the expression of Bim and death receptor 5 (DR5). However, knockdown of DR5 or Bim did not suppress miclxin-induced apoptosis, indicating that these two factors do not play an important role in miclxin-induced apoptosis (Figure 3-23).

What had remained unclear is how mutant β-catenin contributed to miclxin-induced apoptosis in HCT116 cells. In this study, the author demonstrated that mutant β-catenin contributed to the activation of mitochondrial stress response, leading to apoptosis. This conclusion is supported by the following findings: 1) Miclxin induced apoptosis only in HCT116 cells harboring mutant β-catenin; 2) knockdown of β-catenin inhibited miclxin-mediated apoptosis in HCT116 cells; 3) knockdown of β-catenin inhibited the miclxin-

mediated upregulation of the eIF2α-ATF4-CHOP axis; and 4) knockdown of β-catenin inhibited late-stage MMP loss, but not early-stage MMP loss. However, at present, the mechanism involved in the activation of mitochondrial stress by mutant β-catenin in response to MIC60 dysfunction and subsequent late-stage MMP loss remains unclear. In mitochondrial stress response, kinase GCN-2 phosphorylates eIF2α, resulting in the upregulation of stress response genes, such as CHOP and ATF4[96,97]. Thus, the author cannot rule out the possibility that mutant β-catenin may affect this phosphorylation step. The oncogenic role of mutant β-catenin in the pathogenesis of a subset of tumors has been demonstrated. However, several reports have described an additional role in the induction of apoptosis. Raab *et al*. previously reported that the PKC inhibitor enzastaurin elevated the expression of CHOP via accumulation of β-catenin, while depletion of β-catenin inhibited this increase[98]. In addition, they also showed that accumulated β-catenin triggered c-Jun-dependent induction of p73, thereby resulting in apoptosis. Miclxin also induced an increase in c-Jun expression in a β-catenin-dependent manner (Figure 3-24). However, knockdown of c-Jun did not affect miclxin-induced apoptosis (Figure 3-25). Furthermore, treatment with miclxin did not lead to the accumulation of β-catenin (Figure 3-20). Based on these results, further investigations are required to reveal the mechanism of β-catenin-mediated apoptosis with respect to mitochondrial stress.

In conclusion, the author's findings suggest that the novel MIC60 inhibitor miclxin primarily induces early-stage mitochondrial impairment, which leads to downregulation of Bcl-2 expression through a β-catenin-mediated mitochondrial stress response. This effect results in late-stage mitochondrial damage, nuclear translocation of AIF, and eventually the induction of apoptosis (Figure 3-26). Thus, the author demonstrated that

MIC60 may be a potential therapeutic target for tumor cells harboring β-catenin mutations.

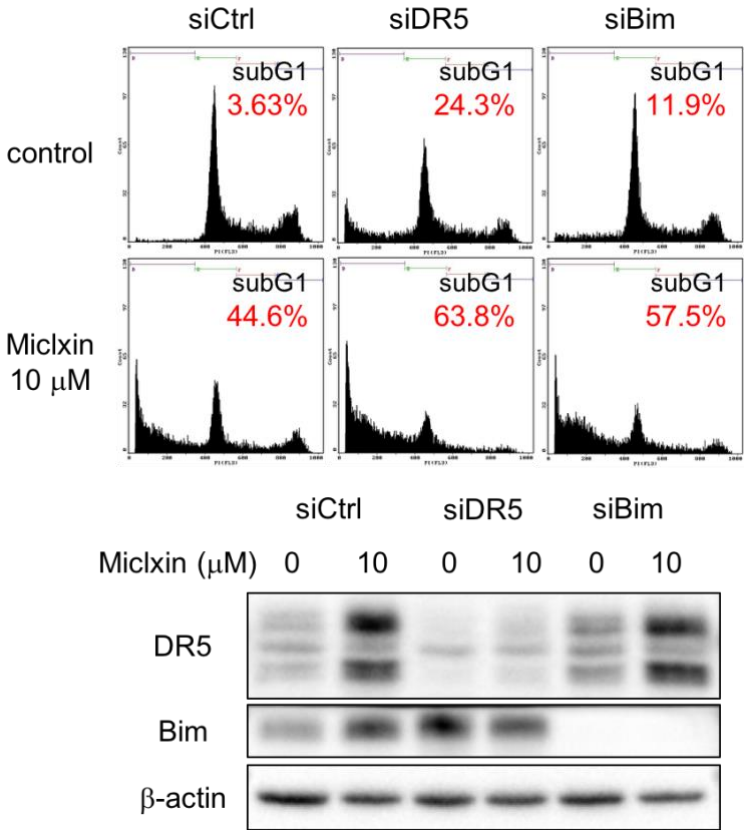

Figure 3-23. DR5 and Bim were not involved in miclxin-induced apoptosis.

HCT116 cells transfected with DR5 or Bim siRNA were treated with miclxin for 24 h or 48 h. Cell lysates were immunoblotted with the indicated antibodies, and subG1 populations were counted using flow cytometer.

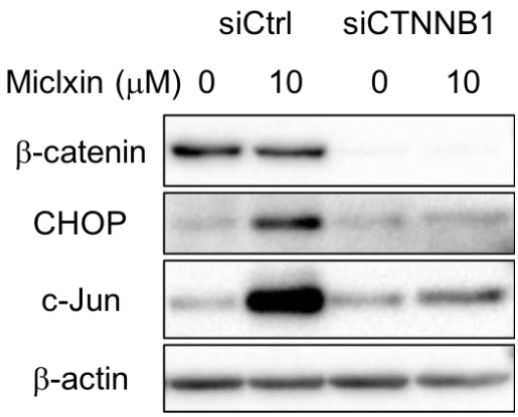

Figure 3-24. Miclxin increased c-Jun protein expression levels.

HCT116 cells transfected with β-catenin siRNA were treated with miclxin for 24 h. Cell lysates were immunoblotted with the indicated antibodies.

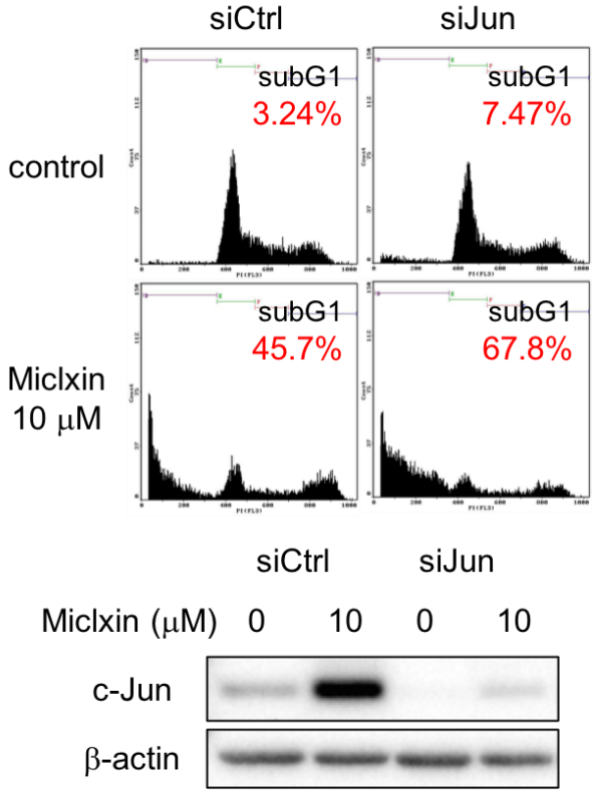

Figure 3-25. c-Jun was not involved in miclxin-induced apoptosis.

HCT116 cells transfected with c-Jun siRNA were treated with miclxin for 24 h or 48 h. Cell lysates were immunoblotted with the indicated antibodies, and subG1 populations were counted using flow cytometer.

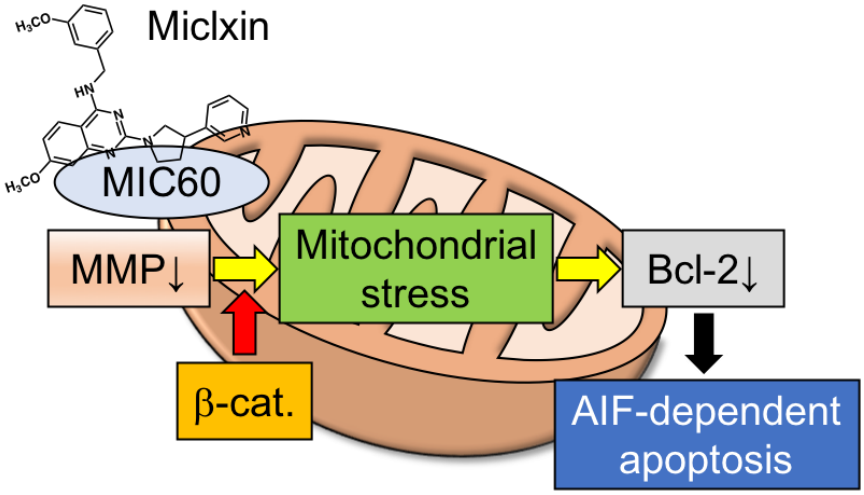

Figure 3-26. Illustrated abstract.

3.3 Materials and Methods

3.3.1 Reagents and cell lines

The compound DS37262926 (named “miclxin”) was provided by Daiichi-Sankyo Pharmaceutical Company. Metacycloprodigiosin was isolated from actinomycetes strain as previously described[73]. Z-VAD-FMK and α-tocopherol were purchased from Sigma-Aldrich. The parental HCT116 (*CTNNB1* +/Δ45), isogenic HCT116 (*CTNNB1* +/–) cells, and isogenic HCT116 (*CTNNB1* Δ45/–) cells were purchased from Horizon Discovery, and maintained in RPMI-1640 (Nissui) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G (Sigma-Aldrich) and 0.1 mg/mL kanamycin (Sigma-Aldrich) at 37ºC in a humidified atmosphere containing 5% CO_2.

3.3.2 RNA interference

Cells were transfected with Stealth RNAi Negative Control (Invitrogen), siCTNNB1 (HSS102460), siMIC60 (HSS116992), siCHOP (5′-CCUCACUCUCCAGAUUCCAGUCAGA-3′), siDR5 (HSS112939), siBim (HSS145413), or siJun (HSS105641) reverse transfection using Lipofectamine RNAiMAX (Invitrogen), according to the protocol provided by the manufacturer.

3.3.3 Propidium iodide (PI) staining

Cells were collected and fixed with 70% ethanol at 4ºC. After removal of the ethanol, cells were treated with 10 μg/mL RNase for 20 min and stained with 50 μg/mL PI (Sigma-Aldrich). Finally, PI fluorescence was measured using flow cytometer.

3.3.4 Western blotting

Cells were lysed with RIPA buffer [25 mM HEPES, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 M Na_3VO_4, and a protease inhibitor cocktail tablet (Roche); pH 7.8]. The lysates were centrifuged at 13,000 rpm for 15 min to remove the insoluble fraction. Equal amounts of total protein were separated using SDS-PAGE, transferred to a PVDF membrane, and probed with the indicated antibodies. The chemiluminescence signal was detected using an ECL Select Western Blotting Detection Reagent (GE Healthcare) and ChemiDoc XRS+ System (Bio-Rad). The primary antibodies used were as follows: anti-MIC60 (Abcam), anti-GST, anti-ATF4, anti-AIF (Santa Cruz Biotechnology), anti-phospho-eIF2α, anti-Bcl-2, anti-PARP, anti-phospho-ERK1/2, anti-Bim, anti-c-Jun (Cell Signaling), anti-CHOP (Thermo Fisher Scientific), anti-β-actin, anti-α-tubulin (Sigma-Aldrich), anti-β-catenin (BD Biosciences), anti-DR5 (Millipore). The secondary antibodies were horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (GE Healthcare).

3.3.5 Cell fractionation

Cell fractionation was performed as previously described[67]. Briefly, cells were resuspended in buffer A [20 mM HEPES-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, and a protease inhibitor cocktail tablet; pH 7.5]. Cell homogenates were centrifuged at 600 g for 10 min at 4ºC to obtain nucleus-enriched pellets and supernatants containing cytosol and mitochondria. The pellets were washed twice with buffer A and lysed with buffer A supplemented with 500

mM NaCl for 10 min. This lysate was subsequently cleared through centrifugation at 13,000 g for 5 min, and the resulting supernatant was designated as the nuclear fraction. The supernatants, which included cytosol and mitochondria, were centrifuged at 15,000 g for 60 min, and the resulting supernatants were collected and designated as the cytosolic fraction. The mitochondria-enriched pellets were lysed with IP buffer [50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1% CHAPS, and a protease inhibitor cocktail tablet; pH 7.5].

3.3.6 Detection of miclxin beads binding proteins

Miclxin beads were prepared as previously described[74]. HCT116 cells were collected and divided into cytosolic, nuclear, and mitochondrial fractions. Cell lysates were incubated with miclxin beads for 3 h at 4ºC. The reacted beads were washed with IP buffer, and the bound proteins were eluted with SDS-PAGE sample buffer, separated through SDS-PAGE, and visualized via silver staining. Identification of the proteins was performed using LC-MS/MS, as previously described[99].

3.3.7 *Invitro* miclxin beads pull-down assay

MIC60 was subcloned into pGEX-2T (GE Healthcare), and GST-fused MIC60 mutants (1-200, 65-758) were generated through PCR using pGEX-2T-MIC60 as a template. GST-fusion proteins, which were expressed in the *E.coli* BL21 strain and purified using Glutathione Sepharose 4B (GE Healthcare), were incubated with miclxin beads in IP buffer overnight. The beads were washed with IP buffer and eluted with SDS-PAGE sample buffer. The eluted proteins were subsequently subjected to SDS-PAGE.

3.3.8 Detection of the MMP

The MMP was detected using DilC1(5) (Thermo Fisher Scientific). Cells were collected and stained with 50 nM DilC1(5) for 30 min at 37ºC. Finally, DilC1(5) fluorescence was measured using flow cytometer.

3.3.9 Detection of ROS production

The production of ROS was determined using CM-H_2DCFDA (Thermo Fisher Scientific). Cells were collected and stained with 1 μM CM-H_2DCFDA for 30 min at 37ºC. Finally, CM-H_2DCFDA fluorescence was measured using flow cytometer.

Chapter 4:
Conclusion

Cancer has long been feared as an incurable disease, but it has become a curable disease year after year. However, cancer is still the leading cause of death worldwide due to the side effect of classical cytotoxic agents and acquired resistance to molecular targeted drugs. Wnt/β-catenin signaling pathway plays crucial roles in cell proliferation. β-catenin is one of its major components, however, β-catenin is actively mutated in a variety of tumors. COSMIC (Catalogue Of Somatic Mutations In Cancer) website displays that about 8% of every tumor samples tested harbor β-catenin mutation, however, there are still no therapeutic agents targeting β-catenin mutation. In this situation, the author used the chemical biological methods to understand how β-catenin-mutated tumor cells can survive and to reveal how we can induce apoptosis selectively in β-catenin-mutated tumors.

In Chapter 2, the author searched for the compound that selectively induces cell death in β-catenin-mutated HCT116 cells, but not in A375 cells harboring wild-type β-catenin. As a result, the author found that metacycloprodigiosin (mcPG) showed such activity. McPG was shown to inhibit V-ATPase activity, which caused selective cell death in HCT116 cells. The author next found that most of V-ATPase inhibitors-sensitive cell lines carried mutations in β-catenin gene and sensitivity to V-ATPase inhibitors was significantly high in β-catenin-mutated cell lines. Moreover, V-ATPase inhibitors could induce cell death in HEK-293T cells transfected with mutated β-catenin but not in control cells. The author further found that V-ATPase inhibitors induce cell death in HCT116 cells partially via DR4/Caspase-8 death signaling pathway. In conclusion, these results suggest that β-catenin mutation is the key factor of V-ATPase inhibitors-induced cell death and V-ATPase inhibition possibly enhances death signaling via DR4.

In Chapter 3, the author searched for the compound targeting mutant β-catenin, and found DS37262926 (miclxin). Miclxin exhibited β-catenin-dependent apoptosis in β-catenin-mutated HCT116 cells and isogenic HCT116 (*CTNNB1* Δ45/–) cells, however, this effect was not observed in isogenic HCT116 (*CTNNB1* +/–) cells. Using miclxin-immobilized beads, MIC60, one of the major components of the MICOS complex, was identified as a target protein of miclxin. The author revealed that MIC60 dysfunction by miclxin induced mitochondrial stress response in a mutant β-catenin-dependent manner. Activation of the mitochondrial stress response was responsible for the downregulation of Bcl-2, leading to severe loss of mitochondrial membrane potential and subsequent AIF-dependent apoptosis. These findings suggest that targeting MIC60 is a potential strategy with which tumor cells can be killed through induction of severe mitochondrial damage in a mutant β-catenin-dependent manner.

β-catenin is known as a modulator of Wnt/β-catenin signaling pathway, moreover, reported to bind to many types of proteins, which is involved in a lot of cellular events. In Wnt/β-catenin signaling, β-catenin binds to APC, Axin, CK1, GSK3 and β-TrCP; components of destruction complex[23]. Also, β-catenin binds to TCF, Pygopus, Bcl-9 and CBP, resulting in cell growth[27,32]. β-catenin and its family proteins (α-catenin, γ-catenin, p120-catenin) are primary reported to interact with cadherin family proteins[100]. The intracellular domain of cadherin is complexed with and regulated by cytoplasmic catenin to link actin filaments[101]. Furthermore, β-catenin is reported to be involved in stemness[102] and autophagy[103], and so on. In this study, the author found that mutant β-catenin contributes to induce apoptosis. Raab *et al.* previously reported similar results, in which the PKC inhibitor enzastaurin inhibited cell growth and induced apoptosis in a β-catenin-

dependent manner via upregulation of CHOP[98]. The author described that miclxin, MIC60 inhibitor, also upregulated CHOP in a β-catenin-dependent manner. Bafilomycin A1, V-ATPase inhibitor, also previously reported to increase CHOP expression levels[72]. Although these reports support the author's findings, which β-catenin is involved in stress-mediated apoptosis, further investigations are required to reveal the mechanism of mutant β-catenin-mediated apoptosis.

Thus far, it remains a lot of unclear points about β-catenin protein in addition to the difference between mutant and wild-type. The author's findings would provide evidence for the new aspect of mutant β-catenin as a promoter of stress response. Furthermore, these studies show that the successful identification of novel synthetic lethal relationship by chemical biological studies.

References

(1) Hanahan, D. (2014) Rethinking the war on cancer. *Lancet 383*, 558–563.

(2) Hurley, L. H. (2002) DNA and its associated processes as targets for cancer therapy. *Nat. Rev. Cancer 2*, 188–200.

(3) Bodley, A., Liu, L. F., Israel, M., Seshadri, R., Koseki, Y., Giuliani, F. C., Kirschenbaum, S., Silber, R., and Potmesil, M. (1989) DNA Topoisomerase II-mediated Interaction of Doxorubicin and Daunorubicin Congeners with DNA. *Cancer Res. 49*, 5969–5978.

(4) Schiff, P. B., and Horwitz, S. B. (1980) Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. U. S. A. 77*, 1561–1565.

(5) Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J., and Lydon, N. B. (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat. Med. 2*, 561–566.

(6) Paez, J. G., Jänne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E., and Meyerson, M. (2004) EGFR mutations in lung, cancer: Correlation with clinical response to gefitinib therapy. *Science 304*, 1497–1500.

(7) Chapman, P. B., Hauschild, A., Robert, C., Haanen, J. B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M., Hogg, D., Lorigan, P., Lebbe, C., Jouary, T., Schadendorf, D., Ribas, A., O'Day, S. J., Sosman, J. A., Kirkwood, J. M., Eggermont, A. M. M., Dreno, B., Nolop, K., Li, J., Nelson, B., Hou, J., Lee, R. J., Flaherty, K. T., and McArthur, G. A. (2011) Improved survival with vemurafenib in melanoma with BRAF

V600E mutation. *N. Engl. J. Med. 364*, 2507–2516.

(8) Wagle, N., Emery, C., Berger, M. F., Davis, M. J., Sawyer, A., Pochanard, P., Kehoe, S. M., Johannessen, C. M., MacConaill, L. E., Hahn, W. C., Meyerson, M., and Garraway, L. A. (2011) Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J. Clin. Oncol. 29*, 3085–3096.

(9) Iwai, Y., Ishida, M., Tanaka, Y., Okazaki, T., Honjo, T., and Minato, N. (2002) Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A. 99*, 12293–12297.

(10) Brough, R., Frankum, J. R., Costa-Cabral, S., Lord, C. J., and Ashworth, A. (2011) Searching for synthetic lethality in cancer. *Curr. Opin. Genet. Dev. 21*, 34–41.

(11) Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N. J., and Helleday, T. (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature 434*, 913–917.

(12) Farmer, H., McCabe, H., Lord, C. J., Tutt, A. H. J., Johnson, D. A., Richardson, T. B., Santarosa, M., Dillon, K. J., Hickson, I., Knights, C., Martin, N. M. B., Jackson, S. P., Smith, G. C. M., and Ashworth, A. (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature 434*, 917–921.

(13) Kim, G., Ison, G., McKee, A. E., Zhang, H., Tang, S., Gwise, T., Sridhara, R., Lee, E., Tzou, A., Philip, R., Chiu, H. J., Ricks, T. K., Palmby, T., Russell, A. M., Ladouceur, G., Pfuma, E., Li, H., Zhao, L., Liu, Q., Venugopal, R., Ibrahim, A., and Pazdur, R. (2015) FDA approval summary: Olaparib monotherapy in patients with deleterious germline BRCA-mutated advanced ovarian cancer treated with three or more lines of chemotherapy. *Clin. Cancer Res. 21*, 4257–4261.

(14) Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer 26*, 239–257.

(15) Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., and Miller, D. K. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature 376*, 37–43.

(16) Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H., and Peter, M. E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J. 14*, 5579–5588.

(17) Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell 91*, 479–489.

(18) Adams, J. M., and Cory, S. (1998) The Bcl-2 protein family: Arbiters of cell survival. *Science 281*, 1322–1326.

(19) Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Molecular characterization of mitochodrial apoptosis-inducing factor. *Nature 397*, 441–446.

(20) Nusse, R., and Clevers, H. (2017) Wnt/β-Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell 169*, 985–999.

(21) Steinhart, Z., and Angers, S. (2018) Wnt signaling in development and tissue homeostasis. *Development 145*, 1–8.

(22) Hart, M. J., De Los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998) Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β. *Curr. Biol. 8*, 573–581.

(23) Stamos, J. L., and Weis, W. I. (2013) The β-catenin destruction complex. *Cold Spring Harb. Perspect. Biol. 5*, a007898.

(24) Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) β-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J. 16*, 3797–3804.

(25) Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X., and He, X. (2002) Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell 108*, 837–847.

(26) Daniels, D. L., and Weis, W. I. (2005) β-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat. Struct. Mol. Biol. 12*, 364–371.

(27) Städeli, R., Hoffmans, R., and Basler, K. (2006) Transcription under the Control of Nuclear Arm/β-Catenin. *Curr. Biol. 16*, 378–385.

(28) Behrens, J., Von Kries, J. P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996) Functional interaction of β-catenin with the transcription factor LEF- 1. *Nature 382*, 638–642.

(29) Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M., and Bejsovec, A. (1998) Drosophila Tcf and Groucho interact to repress wingless signalling activity. *Nature 395*, 604–608.

(30) He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., Da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Identification of c-MYC as a target of the APC pathway. *Science 281*, 1509–1512.

(31) Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999) The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. U. S. A. 96*, 5522–5527.

(32) Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science 275*, 1787–1790.

(33) Polakis, P. (1999) The oncogenic activation of β-catenin. *Curr. Opin. Genet. Dev. 9*, 15–21.

(34) Polakis, P. (2000) Wnt signaling and cancer. *Genes Dev. 14*, 1837–1851.

(35) Hart, M., Concordet, J. P., Lassot, I., Albert, I., Del Los Santos, R., Durand, H., Perret, C., Rubinfeld, B., Margottin, F., Benarous, R., and Polakis, P. (1999) The F-box protein β-TrCP associates with phosphorylated β-catenin and regulates its activity in the cell. *Curr. Biol. 9*, 207–210.

(36) Kahn, M. (2014) Can we safely target the WNT pathway? *Nat. Rev. Drug Discov. 13*, 513–532.

(37) Krishnamurthy, N., and Kurzrock, R. (2018) Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. *Cancer Treat. Rev. 62*, 50–60.

(38) Liu, J., Pan, S., Hsieh, M. H., Ng, N., Sun, F., Wang, T., Kasibhatla, S., Schuller, A. G., Li, A. G., Cheng, D., Li, J., Tompkins, C., Pferdekamper, A. M., Steffy, A., Cheng, J., Kowal, C., Phung, V., Guo, G., Wang, Y., Graham, M. P., Flynn, S., Brenner, J. C.,

Li, C., Villarroel, M. C., Schultz, P. G., Wu, X., McNamara, P., Sellers, W. R., Petruzzelli, L., Boral, A. L., Seidel, H. M., McLaughlin, M. E., Che, J., Carey, T. E., Vanasse, G., and Harris, J. L. (2013) Targeting Wnt-driven cancer through the inhibition of Porcupine by LGK974. *Proc. Natl. Acad. Sci. U. S. A. 110*, 20224–20229.

(39) Madan, B., Ke, Z., Harmston, N., Ho, S. Y., Frois, A. O., Alam, J., Jeyaraj, D. A., Pendharkar, V., Ghosh, K., Virshup, I. H., Manoharan, V., Ong, E. H. Q., Sangthongpitag, K., Hill, J., Petretto, E., Keller, T. H., Lee, M. A., Matter, A., and Virshup, D. M. (2016) Wnt addiction of genetically defined cancers reversed by PORCN inhibition. *Oncogene 35*, 2197–2207.

(40) Gurney, A., Axelrod, F., Bond, C. J., Cain, J., Chartier, C., Donigan, L., Fischer, M., Chaudhari, A., Ji, M., Kapoun, A. M., Lam, A., Lazetic, S., Ma, S., Mitra, S., Park, I. K., Pickell, K., Sato, A., Satyal, S., Stroud, M., Tran, H., Yen, W. C., Lewicki, J., and Hoey, T. (2012) Wnt pathway inhibition via the targeting of Frizzled receptors results in decreased growth and tumorigenicity of human tumors. *Proc. Natl. Acad. Sci. U. S. A. 109*, 11717–11722.

(41) Le, P. N., McDermott, J. D., and Jimeno, A. (2015) Targeting the Wnt pathway in human cancers: Therapeutic targeting with a focus on OMP-54F28. *Pharmacol. Ther. 146*, 1–11.

(42) Lenz, H. J., and Kahn, M. (2014) Safely targeting cancer stem cells via selective catenin coactivator antagonism. *Cancer Sci. 105*, 1087–1092.

(43) Huang, S. M. A., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., Hild, M., Shi, X., Wilson, C. J., Mickanin, C., Myer, V., Fazal, A., Tomlinson, R., Serluca, F., Shao, W., Cheng, H.,

Shultz, M., Rau, C., Schirle, M., Schlegl, J., Ghidelli, S., Fawell, S., Lu, C., Curtis, D., Kirschner, M. W., Lengauer, C., Finan, P. M., Tallarico, J. A., Bouwmeester, T., Porter, J. A., Bauer, A., and Cong, F. (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature 461*, 614–620.

(44) Waaler, J., Machon, O., Tumova, L., Dinh, H., Korinek, V., Wilson, S. R., Paulsen, J. E., Pedersen, N. M., Eide, T. J., Machonova, O., Gradl, D., Voronkov, A., Von Kries, J. P., and Krauss, S. (2012) A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. *Cancer Res. 72*, 2822–2832.

(45) Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature 411*, 494–498.

(46) Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Hsu, P. D., Wu, X., Jiang, W., and Marraffini, L. A. (2013) Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science 339*, 819–823.

(47) Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., and Church, G. M. (2013) RNA-guided human genome engineering via Cas9. *Science 339*, 823–826.

(48) Schenone, M., Dančík, V., Wagner, B. K., and Clemons, P. A. (2013) Target identification and mechanism of action in chemical biology and drug discovery. *Nat. Chem. Biol. 9*, 232–240.

(49) Feher, M., and Schmidt, J. M. (2003) Property distributions: Differences between drugs, natural products, and molecules from combinatorial chemistry. *J. Chem. Inf.*

Comput. Sci. 43, 218–227.

(50) Kiga, M., Nakayama, A., Shikata, Y., Sasazawa, Y., Murakami, R., Nakanishi, T., Tashiro, E., and Imoto, M. (2015) SMK-17, a MEK1/2-specific inhibitor, selectively induces apoptosis in β-catenin-mutated tumors. *Sci. Rep. 5*, 1–9.

(51) Shikata, Y., Kiga, M., Futamura, Y., Aono, H., Inoue, H., Kawada, M., Osada, H., and Imoto, M. (2017) Mitochondrial uncoupler exerts a synthetic lethal effect against β-catenin mutant tumor cells. *Cancer Sci. 108*, 772–784.

(52) Wasserman, H. H., Rodgers, G. C., and Keith, D. D. (1969) Metacycloprodigiosin, a Tripyrrole Pigment from Streptomyces longisporus ruber. *J. Am. Chem. Soc. 91*, 1263–1264.

(53) Woo, J. T., Ohba, Y., Tagami, K., Sumitani, K., Kataoka, T., and Nagai, K. (1997) Prodigiosin 25-C and Metacycloprodigiosin Suppress the Bone Resorption by Osteoclasts. *Biosci. Biotechnol. Biochem. 61*, 400–402.

(54) Ohkuma, S., Sato, T., Okamoto, M., Matsuya, H., Arai, K., Kataoka, T., Nagai, K., and Wasserman, H. H. (1998) Prodigiosins uncouple lysosomal vacuolar-type ATPase through promotion of H^+/Cl^- symport. *Biochem. J. 334*, 731–741.

(55) Bowman, E. J., Siebers, A., and Altendorf, K. (1988) Bafilomycins; A class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. U. S. A. 85*, 7972–7976.

(56) Dröse, S., and Altendorf, K. (1997) Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. *J. Exp. Biol. 200*, 1–8.

(57) Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M., and Tashiro, Y. (1991) Bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase, inhibits acidification

and protein degradation in lysosomes of cultured cells. *J. Biol. Chem. 266*, 17707–17712.

(58) Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature 371*, 346–347.

(59) Thornberry, N. A., and Lazebnik, Y. A. (1998) Caspases : Enemies Within. *Science 281*, 1312–1316.

(60) Ashkenazi, A., and Dixit, V. M. (1998) Death receptors: Signaling and modulation. *Science 281*, 1305–1308.

(61) Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., and Dixit, V. M. (1997) The receptor for the cytotoxic ligand TRAIL. *Science 276*, 111–113.

(62) Pan, G., Ni, J., Wei, Y. F., Yu, G. I., Gentz, R., and Dixit, V. M. (1997) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science 277*, 815–818.

(63) Pérez-Sayáns, M., García-García, A., Reboiras-López, M. D., and Gándara-Vila, P. (2009) Role of V-ATPases in solid tumors: Importance of the subunit C (review). *Int. J. Oncol. 34*, 1513–1520.

(64) Maxson, M. E., and Grinstein, S. (2014) The vacuolar-type H+-ATPase at a glance - more than a proton pump. *J. Cell Sci. 127*, 4987–4993.

(65) Torigoe, T., Izumi, H., Ise, T., Murakami, T., Uramoto, H., Ishiguchi, H., Yoshida, Y., Tanabe, M., Nomoto, M., and Kohno, K. (2002) Vacuolar H+-ATPase: Functional mechanisms and potential as a target for cancer chemotherapy. *Anticancer. Drugs 13*, 237–243.

(66) Pérez-Sayáns, M., Somoza-Martín, J. M., Barros-Angueira, F., Rey, J. M. G., and

García-García, A. (2009) V-ATPase inhibitors and implication in cancer treatment. *Cancer Treat. Rev. 35*, 707–713.

(67) Sasazawa, Y., Futamura, Y., Tashiro, E., and Imoto, M. (2009) Vacuolar H+-ATPase inhibitors overcome Bcl-xL-mediated chemoresistance through restoration of a caspase-independent apoptotic pathway. *Cancer Sci. 100*, 1460–1467.

(68) Yoshimoto, Y., and Imoto, M. (2002) Induction of EGF-dependent apoptosis by vacuolar-type H+-ATPase inhibitors in A431 cells overexpressing the EGF receptor. *Exp. Cell Res. 279*, 118–127.

(69) Thorburn, A. (2004) Death receptor-induced cell killing. *Cell. Signal. 16*, 139–144.

(70) Lavrik, I., Golks, A., and Krammer, P. H. (2005) Death receptor signaling. *J. Cell Sci. 118*, 265–267.

(71) Zhou, J., Lu, G. D., Ong, C. S., Ong, C. N., and Shen, H. M. (2008) Andrographolide sensitizes cancer cells to TRAIL-induced apoptosis via p53-mediated death receptor 4 up-regulation. *Mol. Cancer Ther. 7*, 2170–2180.

(72) Jin, H. O., Lee, Y. H., Kim, H. A., Kim, E. K., Noh, W. C., Kim, Y. S., Hwang, C. S., Kim, J. Il, Chang, Y. H., Hong, S. Il, Hong, Y. J., Park, I. C., and Lee, J. K. (2013) Inhibition of vacuolar H+ ATPase enhances sensitivity to tamoxifen via up-regulation of CHOP in breast cancer cells. *Biochem. Biophys. Res. Commun. 437*, 463–468.

(73) Ikeda, H., Shikata, Y., Watanapokasin, R., Tashiro, E., and Imoto, M. (2017) Metacycloprodigiosin induced cell death selectively in β-catenin-mutated tumor cells. *J. Antibiot. (Tokyo). 70*, 109–112.

(74) Kanoh, N., Honda, K., Simizu, S., Muroi, M., and Osada, H. (2005) Photo-cross-linked small-molecule affinity matrix for facilitating forward and reverse chemical

genetics. *Angew. Chemie Int. Ed. Engl. 44*, 3559–3562.

(75) van der Laan, M., Horvath, S. E., and Pfanner, N. (2016) Mitochondrial contact site and cristae organizing system. *Curr. Opin. Cell Biol. 41*, 33–42.

(76) Prasai, K. (2017) Regulation of mitochondrial structure and function by protein import: A current review. *Pathophysiology 24*, 107–122.

(77) Suzuki, T., Yamaguchi, H., Kikusato, M., Hashizume, O., Nagatoishi, S., Matsuo, A., Sato, T., Kudo, T., Matsuhashi, T., Murayama, K., Ohba, Y., Watanabe, S., Kanno, S. I., Minaki, D., Saigusa, D., Shinbo, H., Mori, N., Yuri, A., Yokoro, M., Mishima, E., Shima, H., Akiyama, Y., Takeuchi, Y., Kikuchi, K., Toyohara, T., Suzuki, C., Ichimura, T., Anzai, J. I., Kohzuki, M., Mano, N., Kure, S., Yanagisawa, T., Tomioka, Y., Toyomizu, M., Tsumoto, K., Nakada, K., Bonventre, J. V., Ito, S., Osaka, H., Hayashi, K. I., and Abe, T. (2016) Mitochonic acid 5 binds mitochondria and ameliorates renal tubular and cardiac myocyte damage. *J. Am. Soc. Nephrol. 27*, 1925–1932.

(78) Gieffers, C., Korioth, F., Heimann, P., Ungermann, C., and Frey, J. (1997) Mitofilin is a transmembrane protein of the inner mitochondrial membrane expressed as two isoforms. *Exp. Cell Res. 232*, 395–399.

(79) John, G. B., Shang, Y., Li, L., Renken, C., Mannella, C. A., Selker, J. M. L., Rangell, L., Bennett, M. J., and Zha, J. (2005) The mitochondrial inner membrane protein mitofilin controls cristae morphology. *Mol. Biol. Cell 16*, 1543–1554.

(80) Oyadomari, S., and Mori, M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ. 11*, 381–389.

(81) Zhao, Q., Wang, J., Levichkin, I. V., Stasinopoulos, S., Ryan, M. T., and Hoogenraad, N. J. (2002) A mitochondrial specific stress response in mammalian cells. *EMBO J. 21*,

4411–4419.

(82) Münch, C., and Harper, J. W. (2016) Mitochondrial unfolded protein response controls matrix pre-RNA processing and translation. *Nature 534*, 710–713.

(83) Shpilka, T., and Haynes, C. M. (2018) The mitochondrial UPR: Mechanisms, physiological functions and implications in ageing. *Nat. Rev. Mol. Cell Biol. 19*, 109–120.

(84) Iurlaro, R., and Muñoz-Pinedo, C. (2016) Cell death induced by endoplasmic reticulum stress. *FEBS J. 283*, 2640–2652.

(85) Hu, H., Tian, M., Ding, C., and Yu, S. (2019) The C/EBP homologous protein (CHOP) transcription factor functions in endoplasmic reticulum stress-induced apoptosis and microbial infection. *Front. Immunol. 9*, 1–13.

(86) Chan, T. A., Wang, Z., Dang, L. H., Vogelstein, B., and Kinzler, K. W. (2002) Targeted inactivation of *CTNNB1* reveals unexpected effects of β-catenin mutation. *Proc. Natl. Acad. Sci. U. S. A. 99*, 8265–8270.

(87) Park, W. S., Oh, R. R., Park, J. Y., Kim, P. J., Shin, M. S., Lee, J. H., Kim, H. S., Lee, S. H., Kim, S. Y., Park, Y. G., An, W. G., Kim, H. S., Jang, J. J., Yoo, N. J., and Lee, J. Y. (2001) Nuclear localization of β-catenin is an important prognostic factor in hepatoblastoma. *J. Pathol. 193*, 483–490.

(88) Anastas, J. N., and Moon, R. T. (2013) WNT signalling pathways as therapeutic targets in cancer. *Nat. Rev. Cancer 13*, 11–26.

(89) Kozjak-Pavlovic, V. (2017) The MICOS complex of human mitochondria. *Cell Tissue Res. 367*, 83–93.

(90) Madungwe, N. B., Feng, Y., Lie, M., Tombo, N., Liu, L., Kaya, F., and Bopassa, J. C. (2018) Mitochondrial inner membrane protein (Mitofilin) knockdown induces cell

death by apoptosis via an AIF-PARP-dependent mechanism and cell cycle arrest. *Am. J. Physiol. Cell Physiol. 315*, 28–43.

(91) Li, H., Ruan, Y., Zhang, K., Jian, F., Hu, C., Miao, L., Gong, L., Sun, L., Zhang, X., Chen, S., Chen, H., Liu, D., and Song, Z. (2016) Mic60/Mitofilin determines MICOS assembly essential for mitochondrial dynamics and mtDNA nucleoid organization. *Cell Death Differ. 23*, 380–392.

(92) An, J., Shi, J., He, Q., Lui, K., Liu, Y., Huang, Y., and Sheikh, M. S. (2012) CHCM1/CHCHD6, novel mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae morphology. *J. Biol. Chem. 287*, 7411–7426.

(93) Matsuhashi, T., Sato, T., Kanno, S. ichiro, Suzuki, T., Matsuo, A., Oba, Y., Kikusato, M., Ogasawara, E., Kudo, T., Suzuki, K., Ohara, O., Shimbo, H., Nanto, F., Yamaguchi, H., Saigusa, D., Mukaiyama, Y., Watabe, A., Kikuchi, K., Shima, H., Mishima, E., Akiyama, Y., Oikawa, Y., Hsin-Jung, H. O., Akiyama, Y., Suzuki, C., Uematsu, M., Ogata, M., Kumagai, N., Toyomizu, M., Hozawa, A., Mano, N., Owada, Y., Aiba, S., Yanagisawa, T., Tomioka, Y., Kure, S., Ito, S., Nakada, K., Hayashi, K. ichiro, Osaka, H., and Abe, T. (2017) Mitochonic Acid 5 (MA-5) Facilitates ATP Synthase Oligomerization and Cell Survival in Various Mitochondrial Diseases. *EBioMedicine 20*, 27–38.

(94) Hockenbery, D., Nuñez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature 348*, 334–336.

(95) Shimizu, S., Eguchi, Y., Kamiike, W., Funahashi, Y., Mignon, A., Lacronique, V., Matsuda, H., and Tsujimoto, Y. (1998) Bcl-2 prevents apoptotic mitochondrial

dysfunction by regulating proton flux. *Proc. Natl. Acad. Sci. U. S. A. 95*, 1455–1459.

(96) Baker, B. M., Nargund, A. M., Sun, T., and Haynes, C. M. (2012) Protective coupling of mitochondrial function and protein synthesis via the eIF2α kinase GCN-2. *PLoS Genet. 8*, e1002760.

(97) Melber, A., and Haynes, C. M. (2018) UPR_{mt} regulation and output: A stress response mediated by mitochondrial-nuclear communication. *Cell Res. 28*, 281–295.

(98) Raab, M. S., Breitkreutz, I., Tonon, G., Zhang, J., Hayden, P. J., Nguyen, T., Fruehauf, J. H., Lin, B. K., Chauhan, D., Hideshima, T., Munshi, N. C., Anderson, K. C., and Podar, K. (2009) Targeting PKC: A novel role for beta-catenin in ER stress and apoptotic signaling. *Blood 113*, 1513–1521.

(99) Futamura, Y., Muroi, M., Aono, H., Kawatani, M., Hayashida, M., Sekine, T., Nogawa, T., and Osada, H. (2019) Bioenergetic and proteomic profiling to screen small molecule inhibitors that target cancer metabolisms. *Biochim. Biophys. Acta Proteins Proteom. 1867*, 28–37.

(100) Ozawa, M., Baribault, H., and Kemler, R. (1989) The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J. 8*, 1711–1717.

(101) Aberle, H., Schwartz, H., and Kemler, R. (1996) Cadherin-catenin complex: Protein interactions and their implications for cadherin function. *J. Cell. Biochem. 61*, 514–523.

(102) Kretzschmar, K., and Clevers, H. (2017) Wnt/β-catenin signaling in adult mammalian epithelial stem cells. *Dev. Biol. 428*, 273–282.

(103) Petherick, K. J., Williams, A. C., Lane, J. D., Ordóñez-Morán, P., Huelsken, J., Collard, T. J., Smartt, H. J., Batson, J., Malik, K., Paraskeva, C., and Greenhough, A.

(2013) Autolysosomal β-catenin degradation regulates Wnt-autophagy-p62 crosstalk. *EMBO J. 32*, 1903–1916.

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