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Therapeutic Discovery

Inhibition of ATR-Dependent Signaling by Protoapigenone and Its Derivative Sensitizes Cancer Cells to Interstrand Cross-link–Generating Agents *In Vitro* and *In Vivo*

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Abstract

DNA damage caused during cancer treatment can rapidly activate the ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR)-dependent phosphorylation of Chk2 and Chk1 kinases, which are hallmarks of the DNA damage response (DDR). Pharmacologic inhibition of ATR causes a synthetic lethal effect on ATM- or p53-defective cancers, suggesting that such inhibition is an effective way to improve the sensitivity of cancers to DNA-damaging agents. Here, both the natural compound protoapigenone (WYC02) and its synthetic derivative WYC0209 exhibited cytotoxic effects on various cancer cell lines. WYC02 causes chromosomal aberration in the mitotic spreads of Chinese hamster ovary cells. Interestingly, cancer cells did not exhibit typical DDR markers upon exposure to WYC02 and WYC0209 (WYCs). Further investigation into the molecular mechanisms of WYCs function revealed that they have a potential ability to inhibit DDR, particularly on activation of Chk1 and Fanconi anemia group D2 protein (FANCD2), but not Chk2. In this way, WYCs inhibited ATR-mediated DNA damage checkpoint and repair. Furthermore, when combined with the DNA cross-linking agent cisplatin, treatment with WYCs resulted in increased tumor sensitivity to interstrand cross-link–generating agents both *in vitro* and *in vivo*. Our results therefore especially implicate WYCs in enhancing tumor chemosensitivity when the ATR checkpoint is constitutively active in states of oncogene-driven replicative stress or tolerance to DNA-interfering agents. *Mol Cancer Ther; 11(7); 1443–53. ©2012 AACR.*

Introduction

Ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) are 2 members of the phosphoinositide 3-kinase–related protein kinase family that play a central role in DNA damage response (DDR) coordination; they also function in the signaling machinery of cell-cycle arrest, DNA repair and transcription, and cell death. Although ATM is predominantly activated in response to DNA strand breaks, ATR is activated in response to damage arising from UV rays or replication block; both kinases activate signaling cascades that involve 2 checkpoint kinase effectors, Chk1 and Chk2, whose roles were previously suggested to be redundant (1). In contrast to ATM, ATR has been reported to be indispensible for cell growth and for life. ATR knockout mouse embryos died early due to mitotic catastrophe characterized by incomplete DNA replication and chromosomal fragmentation (2, 3). Moreover, ATR gene mutations are rarely found in humans. The only mutation variants that can survive are heterozygous or hypomorphic variants. Furthermore, cells derived from patients with Seckel syndrome exhibit cellular features associated with ATR signaling defects. Consistent with this phenotype, Seckel-like mouse embryonic cells showed accelerated aging due to replicative stress, exhibiting an accumulation of lethal chromosomal breaks (4, 5). However, with regard to its role in regulating the replication checkpoint, ATR is activated by most cancer chemotherapeutic agents that target DNA in replicating cells. Therefore, inhibition of ATR signaling is a valid and promising strategy that can improve efficiency of chemotherapy or

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radiotherapy (6, 7). Thus far, several inhibitors of DDR-related kinases, including Chk1 and Chk2, have been successfully used alone or in combination with each other in clinical trials (8, 9). Recently, several chemicals that inhibit ATR kinase activity *in vitro* were used to support the hypothesis that ATR kinase can be targeted to improve cancer therapy (10–14). Because most of these studies are in their initial stages, it is imperative to focus more efforts toward investigating strategies to inhibit ATR signaling.

WYC02 (Fig. 1A) is a flavonoid that we previously isolated and identified from the whole plant extract of *Thelypteris torresiana*, a fern species native to Taiwan. This compound was originally screened for cytotoxicity function; using a colorimetric cytotoxicity assay, WYC02 showed therapeutic effects and was a lead compound for potential anticancer drug development (15–17). In previous studies, WYC02 and its more potent analog WYC0209 (Fig. 1A) were shown to induce oxidative stress, consequently activating the p38 and c-*jun*-NH_2-kinase (JNK) 1/2 mitogen-activated protein kinase (MAPK) pathways following cell-cycle arrest and apoptosis in several cancer cell types. These compounds were also found to reduce the size of tumor xenografts in nude mice without exerting toxic effects on the recipient (18–22). Recently, WYCs were found to induce chromosomal breakage through oxidative stress (18, 20), implicating a role for WYCs in interfering with DNA metabolism. To date, the biomolecular actions and implications of this WYC-mediated interference are mostly undetermined. Here, we found that WYCs are capable of inhibiting DNA damage–induced activation of ATR targets Chk1 and Fanconi anemia group D2 protein (FANCD2), which then sensitize tumor cells to chemotherapy, and finally result in tumor size reduction in mice. Our results propose that these new flavonoid compounds are of noteworthy potential to treat cancers by inducing replication stress via the inhibition of ATR signaling.

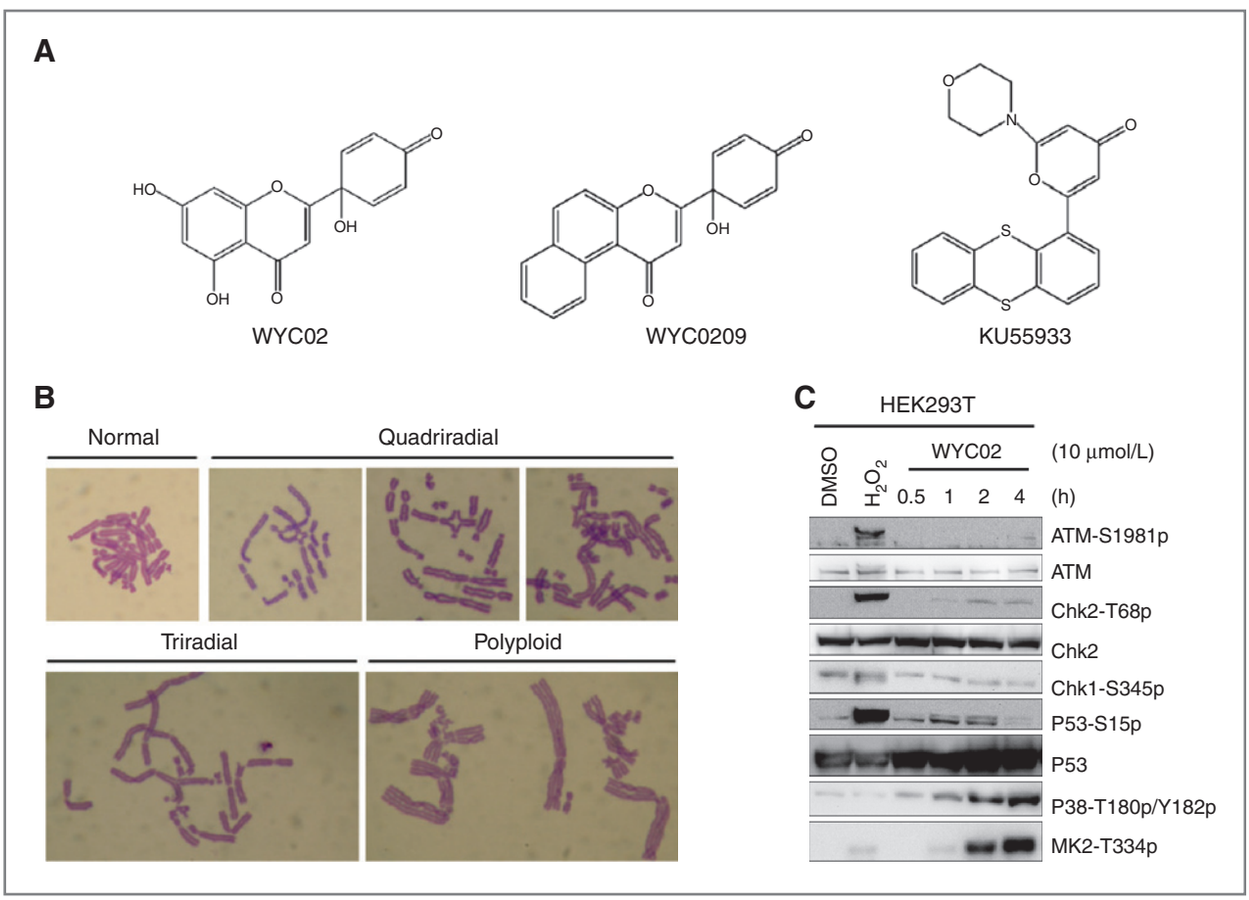

Figure 1. WYC02 induces chromosomal aberration with no marked DDR. A, illustration of the chemical structure of selective ATM inhibitor KU55933, WYC02, and its derivative WYC0209. B, mitotic spreads were prepared from WYC02-treated CHO cells at the indicated concentrations. The representative architecture of normal and aberrant chromosomes is shown. The numbers for different types of aberrations were counted from 200 mitotic cells in 2 independent slides is shown in Table 1. C, immunoblots showing DDR by detecting phosphorylation of ATM Ser^{1981} (ATM-S1981p), Chk1 Ser^{345} (Chk1-S345p), Chk2 Thr^{68} (Chk2-T68p), P53 Ser^{15} (P53-S15p), P38MAPK Thr^{180}/Tyr^{182} (P38-T180p/Y182p), or MAPKAPK2 Thr^{334} (MK2-T334p) following exposure of HEK293T cells to 10 μmol/L WYC02 for the indicated times. Cells treated with 0.1 mmol/L H_2O_2 for 30 minutes served as the positive control.

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Table 1. WYC02 induces chromosomal aberration in CHO cells

Treatment	Concentration (μmol/L)	Type of structural aberrations (numbers)									Average aberrant metaphases (%)[a]
		TB	TD	TR	QR	R	CR	DC	PP	PC	
DMSO control	0.00	0	0	0	0	0	0	0	1	0	0.5
WYC02	2.17	2	0	4	3	1	0	0	3	0	6.5
WYC02	4.35	1	1	13	9	2	0	0	1	1	14
Mitomycin C	2.00	2	3	42	29	0	2	1	0	5	42

NOTE: Two hundred cells per treatment were analyzed for chromosomal aberration. The total numbers of chromatid break (TB), chromatid deletion (TD), triradial (TR), quadriradial (QR), ring (R), complex rearrangement (CR), dicentric (DC), polyploid (PP), and pulverized cell (PC) were indicated. Others, chromosome gap, chromosome break, chromosome deletion, and chromatid gap, were not observed in this experiment.

[a]Indicated statistic significantly for tested versus control group by *t* test, respectively.

Materials and Methods

Cell culture and treatment

MDA-MB-231 [breast adenocarcinoma; ATCC HTB-26, BCRC 60425] and A549 (lung adenocarcinoma; ATCC CCL-185, BCRC 60074) human cell lines were purchased from Bioresource Collection and Research Center (BCRC) and were authenticated by American Type Culture Collection (ATCC). U2OS (osteosarcoma), HeLa (cervical adenocarcinoma), and HEK 293T (embryonic kidney cells) human cell lines were provided by Dr. Sheau-Yann Shieh (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). Cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% FBS (Gibco). For DDR induction, freshly diluted H_2O_2 (Merck) was added to the culture medium 1 hour before the cells were harvested. For UV irradiation treatment, the cells were irradiated for 10 J/m^2 by a cross-linker (UVP CL-1000) 1 hour before analysis. WYC02 and WYC0209 were isolated and synthesized as described previously (15–17).

In vitro chemosensitization assay

To evaluate *in vitro* chemosensitization, cells were seeded in 6-well plates 1 day before the experiment at a density of 100 to 400 cells per well. The drugs were incubated with the cells for 6 hours, after which the medium was replaced with fresh drug-free FBS-containing medium. The colonies became visible and were counted 7 to 10 days later with 0.1% crystal violet staining following image capture by a CCD camera (LAS 4000 mini; Fujifilm).

Flow cytometry

To evaluate the effect of DNA damage checkpoint activation on cell-cycle distribution, the cells were harvested at indicated time points and fixed with methanol for at least 2 hours. The DNA was then stained with a solution containing propidium iodide (PI) and RNase A (Sigma-Aldrich). Fluorescently labeled cells were subsequently analyzed by the flow cytometer (LSR II; BD Biosciences) with a suitable selection of excitation and emission wavelengths. The percentages of different fluorescent cell populations were analyzed by WinMDI version 2.9 (The Scripps Research Institute).

In vitro chromosome aberration test

In brief, 5×10^5 Chinese hamster ovary (CHO) cells were seeded in 60-mm dishes 1 day before the experiment. WYC02-induced structural chromosomal changes after 20 hours were compared with that of the cells cultured in 2 μmol/L mitomycin C (MMC). At 18 hours after WYC02, 0.1 μg/mL colchicine was added for 2 hours, and metaphase cells were collected by shaking them off the dishes. Mitotic cells were treated with 0.5% KCl for 10 minutes and fixed with a 3:1 mixture of methanol:glacial acetic acid. The cells were then spread on slides for chromosome staining with 5% Giemsa solution. We then analyzed the chromosome structure of 200 well-spread metaphase cells (100 metaphase cells per experiment) under a ×100 oil immersion objective.

Plasmids and siRNAs

The plasmids ATR, ATRIP, and claspin were kindly provided by Dr. X. Wu (The Scripps Research Institute, La Jolla, CA), and TopBP1 was provided by Dr. J. Chen (University of Texas MD Anderson Cancer Center, Houston, Texas). The siRNA sequences of the target ATM (5′-AAGCGCCTGATTCGAGATCCT-3′), ATR (5′-CCTCCGTGATGTTGCTTGATT-3′), DNA-PKcs (5′-GATCGCACCTTACTCTGTTGA-3′), and the random sequence that served as the control (5′-AAGTCAATATGCGACTGATGG-3′) were synthesized by Sigma-Proligo (23, 24). All transfections in HEK293T cells were carried out by the calcium phosphate precipitation method.

Western immunoblotting

Cell lysate preparations, gel electrophoresis, and immunoblotting were carried out as previously described (23). The binding of primary antibodies were detected by horseradish peroxidase–coupled secondary antibodies

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(Jackson ImmunoResearch) followed by enhanced chemiluminescence (Millipore). The images of nonsaturated bands were captured by a luminescent image analyzer (LAS-4000 mini; Fujifilm). The antibodies used in this study are listed in Supplementary Materials.

DNA homologous recombination repair assay

DNA constructs of the recombination substrate pHPRT-DRGFP, in which the I-*Sce*I site lies within 1 copy of 2 mutated tandem repeated *GFP* genes, and the I-*Sce*I endonuclease expression vector pCBASceI, were originally constructed by Dr. M. Jasin (25). In brief, we generated a stable pHPRT-DRGFP construct in HeLa cells and evaluated the chromosomal breaks generated by I-*Sce*I endonuclease expression. Six hours after pCBASceI was delivered into the cells, complete medium with or without WYC02 or WYC0209 was replaced onto the cells. Forty-eight hours after delivery, the efficiency of chromosomal homologous recombination repair (HRR) was obtained as the percentage of GFP-positive cells, which was assessed by flow cytometry.

Human xenograft tumors in nude mice

Human breast cancer MAD-MB-231 cells were harvested from the culture, resuspended in medium without serum at 1×10^8 cells/mL, and 0.1 mL of this suspension was subcutaneously injected into the right flank of female nude mice (BALB/cAnN-Foxn1nu/Crl Narl; purchased from the National Science Council Animal Center). Tumor-injected mice that successfully developed tumors that grew to approximately 50 to 100 mm^3 in volume were randomly sorted into groups and used for the experiments. Control vehicle or 2 mg/kg of cisplatin with or without 0.2 mg/kg of WYC0209 was administered intraperitoneally every 4 days throughout the experiment. The drug formulations are described in Supplementary Materials.

Results

WYC02 induces chromosomal aberrations but does not produce marked DDR

Previously, WYC02 and WYC0209 were shown to cause DNA strand breaks and apoptosis in lung and prostate cancers (18, 20), suggesting that inducing DNA damage may be the potential mechanism underlying the anticancer effect of WYCs. To test this hypothesis, we investigated the cytogenetic effect of WYC02 on CHO cells (Fig. 1B). Low WYC02 concentrations produced dose-dependent increases in chromosomal structural changes, such as breakages, radials, and chromosomal polyploidy, similar to the effects seen with MMC treatment; however, the complete mitotic chromosome could not be obtained upon high-dose WYC02 treatment. Because MMC can induce DDR in many cancers, we investigated what kind of DDR signaling was activated by WYC02. Surprisingly, high doses of WYC02 in HEK293T cells did not induce noticeable changes in the putative DDR signaling, which we measured by analyzing the phosphorylation of the ATM-dependent Chk2 Thr^{68} residue and the ATR-dependent Chk1 Ser^{345} residue (Fig. 1C). We did observe that WYC02 treatment caused slight accumulation of the p53 protein, which could have been the result of several posttranslational modifications. However, phosphorylation of the p53 Ser^{15} residue did not contribute to this WYC02-induced p53 protein accumulation, suggesting that WYC02 does not directly damage DNA because DNA damage normally stimulates ATM/ATR-dependent p53 Ser^{15} phosphorylation. Our result is similar to previous reports that p38 MAPK is activated by WYC02 (19, 21, 22), as its downstream target MAPKAPK2 was found to be phosphorylated starting as early as 2 hours after WYC02 exposure (Fig. 1C). We repeated the WYCs experiment on lung and breast carcinoma cell lines A549 and MDA-MB-231 cells, respectively, and obtained similar results. Consistently, no marked changes in Chk1 and Chk2 phosphorylation signaling were detected even at high doses of either drug for as long as 8 hours after drug treatment (Supplementary Fig. S1A). The cytotoxic effect by WYCs on cancer cells was determined by MTT assay at 48 hours of incubation (Supplementary Fig. S1B); our data indicated that the IC_{50} value range for cytotoxicity was similar to those in previous reports, confirming that WYCs are stable compounds that do not directly cause DNA damage.

WYC02 and WYC0209 inhibit Chk1 phosphorylation after DNA damage

Understanding the mechanism by which the WYCs compounds cause chromosomal breakages and other abnormalities might aid in identifying their targets. We hypothesized that genes with functions associated with DNA damage checkpoints and/or DNA repair might be targeted by WYCs. To test this hypothesis, we assessed the effects of WYCs on DDR induced by H_2O_2. WYC02 was found to inhibit Chk1, but promote Chk2 phosphorylation in A594 cells treated with 0.1 mmol/L H_2O_2 for 2 hours; however, ATM autophosphorylation was not affected (Fig. 2A). Pretreatment of cells with okadaic acid (OA; a phosphatase inhibitor) or MG132 (a proteasome inhibitor) could not reverse the WYC02-induced inhibition of Chk1 phosphorylation, indicating that the inhibition does not occur due to phosphatase activation or proteasome degradation by other regulatory factors (Fig. 2B). Furthermore, we investigated other sources of DNA stimuli specific for ATR activation; our results show that UV-induced Chk1 phosphorylation was dose dependently inhibited by WYCs within different cells (Fig. 2C). In response to DNA double-strand breaks (DSB), FANCD2 is known to be monoubiquitinated on K561 (FANCD2-Ub) in an ATR-dependent manner to stimulate repair (26, 27). We showed that FANCD2-Ub was also inhibited by WYCs (Fig. 2A and C); furthermore, ATR inhibition by WYCs was also observed in cells treated with currently prescribed chemotherapeutic agents (Supplementary Fig. S2). Collectively, these findings indicate that WYCs can modify ATR signaling after various types of DNA

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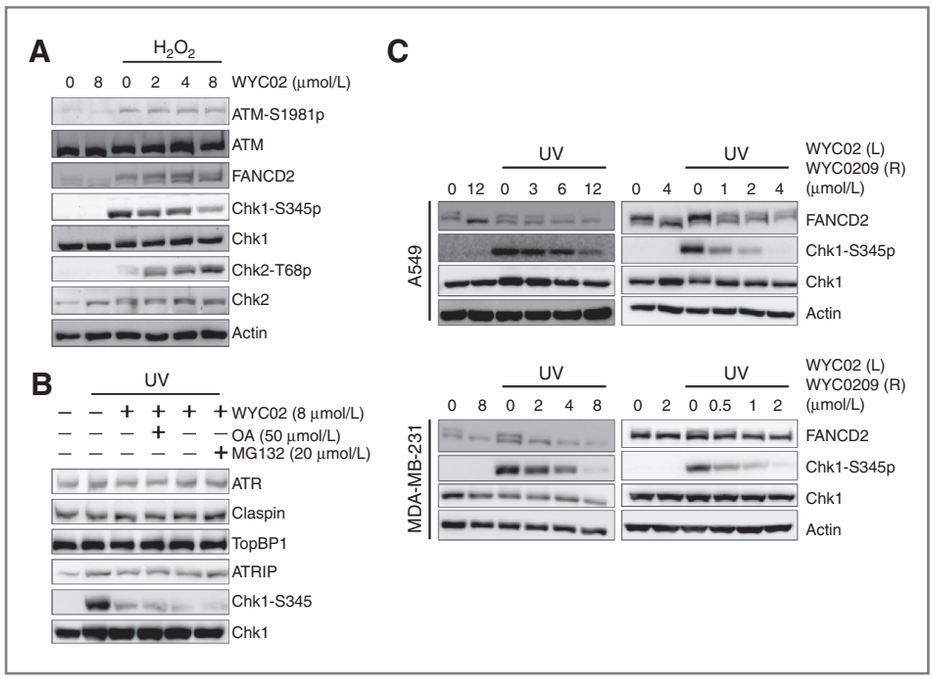

Figure 2. WYCs inhibit DNA damage-induced Chk1 phosphorylation. A, immunoblots showing DDR by detecting phosphorylation of ATM, Chk1, Chk2, and monoubiquitination of FANCD2 following exposure of A549 cells to 0.1 mmol/L H_2O_2 for 2 hours with or without pretreatment of the indicated doses of WYC02. B, A549 cells pretreated with 8 μmol/L WYC02 combined with 50 μmol/L OA or 20 μmol/L MG132 for 30 minutes and subjected to 10 J/m^2 UV for 1 hour to induce DDR. C, dose-dependent effect of WYC02 (left) and WYC0209 (right) on the inhibition of UV-induced Chk1 phosphorylation in A549 (top) and MDA-MB-231 (bottom) cells. Cells were pretreated with chemicals for 30 minutes and subjected to 10 J/m^2 UV for 1 hour to induce DDR.

damage. Interestingly, WYC0209 was more potent than WYC02 in inhibiting Chk1 phosphorylation and cytotoxicity (Fig. 2C and Supplementary Fig. S1B). We speculate that the replacement of 2 hydroxyl groups on WYC02 with an additional benzene ring contributes positively to this ATR inhibition; however, the definite pharmacophores need to be further investigated when the ATR protein structure is resolved.

Target specificity of WYC02 and WYC0209 for ATR-mediated signaling inhibition

To elucidate the specificity of the WYC inhibition on ATR-mediated signaling, we compared the change between cells treated with WYCs or the ATM-specific inhibitor KU55933 (Fig. 1A) before the induction of DDR. After H_2O_2 damage, ATM is thought to be the principal responder, and KU55933 treatment strongly inhibited ATM-mediated Chk2 phosphorylation specifically, but its effect on ATR-mediated Chk1 phosphorylation was small (Fig. 3A, top). In contrast, after hydroxyurea (HU; a replication blocker) damage, ATR is thought to be the principal responder, and WYCs treatment significantly inhibited Chk1 phosphorylation, but only slightly inhibited Chk2 phosphorylation (Fig. 3A, bottom). Using these pharmacologic methods, we showed that the specificity of DDR inhibition between WYCs and KU55933 was completely different. To strengthen the argument that WYCs specifically inhibit ATR signaling, small inhibitory RNAs against ATM, ATR, and the catalytic subunit of DNA protein kinase (DNA-PKcs) were introduced into HEK293T cells before exposure to UV or H_2O_2. Our results showed that WYCs completely inhibited UV- or H_2O_2-induced Chk1 phosphorylation in a manner similar to siRNA knockdown of ATR, but not ATM or DNA-PKcs (Fig. 3B and Supplementary Fig. S3). The siRNAs against ATM and DNA-PKcs decreased the UV- or H_2O_2-induced Chk2 phosphorylation, which were not altered by the addition of WYC02, but were increased by WYC0209 treatment. Interestingly, neither siRNA targeted to ATM or ATR nor DNA-PKcs affected the WYC0209-mediated increase in Chk2 phosphorylation. Because a high dose of WYC0209 itself slightly induces Chk2 activation (Supplementary Fig. S1), the increased Chk2 phosphorylation was likely a synergistic effect due to DNA damage. To further identify the specific mediator that contributes to the effect of WYC02 on the initiation of ATR kinase activation, we tested whether TopBP1, ATRIP, and claspin were involved, as they have been identified as mediators of ATR kinase activation (28). Our results showed that overexpression of ATRIP or TopBP1 did not reverse the inhibitory effect of WYC02 on Chk1 phosphorylation, whereas overexpression of claspin or ATR did (Fig. 3C), suggesting that WYC02 might affect the function of ATR or claspin contributing to ATR signaling inhibition.

WYC02 and WYC0209 impair the functions of DNA damage checkpoints and DNA repair

Previously, it has been shown that S–M and G_2–M checkpoints are activated by ATR in response to different types of DNA damage (6, 7). Of these, the G_2–M checkpoint involves ATM and ATR in collaboration, whereas the S–M checkpoint is mediated solely by ATR. To maintain genetic integrity, ATR can prevent premature mitotic entry in the event of incomplete DNA replication or unrepaired DNA damage (6). To evaluate the effect of WYCs on these ATR-associated DNA damage

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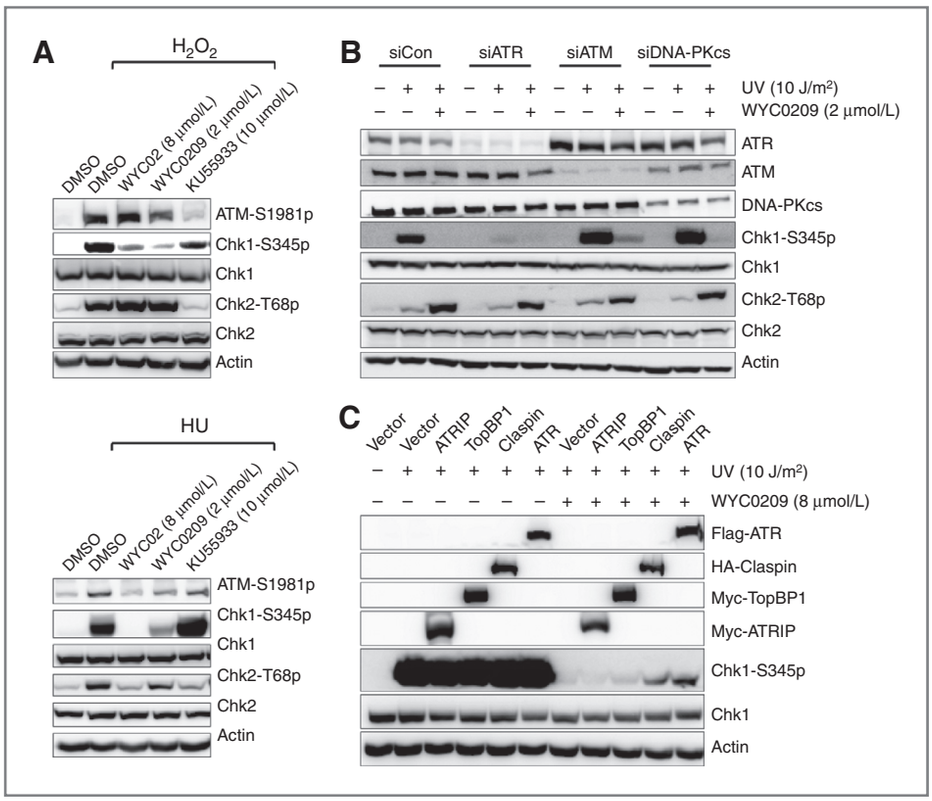

Figure 3. WYCs specifically inhibit ATR-dependent Chk1 phosphorylation. A, DDR induced by either 0.1 mmol/L H_2O_2 (top) or 2 mmol/L HU (bottom) for 1 hour in MDA-MB-231 cells pretreated with DMSO, 8 μmol/L WYC02, 2 μmol/L WYC0209, and 10 μmol/L KU55933 for 30 minutes. B, effects of WYC0209 on Chk1 and Chk2 phosphorylation were assayed 1 hour after 10 J/m^2 UV irradiation on HEK293T cells, and this exhibited decreased expression of ATM, ATR, or DNA-PKcs following the RNA interference method for 48 hours. C, effects of WYC02 on Chk1 phosphorylation were assayed 1 hour after 10 J/m^2 UV irradiation on HEK293T cells overexpressing ATRIP, TopBP1, claspin, or ATR following delivery of tagged full-length cDNA constructs for 48 hours.

checkpoints, we observed the effect of WYCs on mitotic entry following HU or cisplatin treatment. In MDA-MB-231 cells, HU and cisplatin significantly decreased the number of mitotic cells, indicating that the S–M and G_2–M checkpoints are intact in MDA-MB-231 cells (Fig. 4A). WYCs or KU55933 treatment increased the percentage of mitotic cells in cisplatin-treated cells, suggesting that all of these compounds inhibited the damage-induced G_2–M checkpoint. However, WYCs, but not KU55933, significantly increased the HU-induced mitotic entry that is specific for the S–M checkpoint, indicating that WYCs specifically impaired this distinctive checkpoint mediated solely by ATR (Fig. 4A).

ATR function is also linked to DNA repair via its coupled targets (29). To examine the effect of WYCs treatment on DNA repair, we carried out an HRR assay in HeLa cells. Our result showed that chromosomal breaks normally repaired by HRR were dose dependently inhibited by WYC02 at low concentrations. WYC0209 produced similar effects at doses that were 10-fold lower than that of WYC02 (Fig. 4B). From these results, we assumed that the cells carrying unrepaired DNA would enter into mitosis following DNA damage. To verify this assumption, we analyzed the DNA-damage marker γH2AX on mitotic cells using immunofluorescence staining. As expected, the numbers of large γH2AX foci were increased upon addition of WYCs in both unperturbed and perturbed mitotic cells (Fig. 4C and Supplementary Fig. S8), suggesting that WYCs increase DNA damage in mitotic cells. The chromosomes became flat and aggregated after WYC treatment, differing from the 3-dimensional and hair-like appearance of normal chromosomes at metaphase.

WYC02 and WYC0209 enhance chemosensitivity

Inhibition of the checkpoint and repair mechanisms leads to chemosensitization in cancers. We questioned whether WYCs could function as sensitizers for the chemotherapeutic drug cisplatin that has been shown to induce ATR activation as well as FANCD2 monoubiquitination, which is the vital step for DNA crosslink repair (26, 30, 31). We found that WYCs treatment decreased the cisplatin-induced Chk1 phosphorylation and FANCD2 monoubiquitination in A549, MDA-MB-231, and U2OS cells (Fig. 5A). Using the same doses, WYC0209 not only inhibits monoubiquitination of FANCD2 but also affects FANCD2 protein stability; these data emphasize that WYC0209 has more potent inhibitory effects as than WYC02. We further treated individual cells with cisplatin in combination with several varying doses of WYCs and counted survival colonies to determine their ability to survive after cisplatin-induced damage. Our results showed that WYCs effectively decreased the clonogenic survival in cisplatin-treated MDA-MB-231 and A549 cells in the nanomolar dose range (Fig. 5B). To investigate the chemosensitization effect of low-dose WYCs *in vivo*, we established a tumor xenograft in nude mice with human MDA-MB-231 tumor cells, which are considered to be more resistant to cisplatin and are also sensitive to

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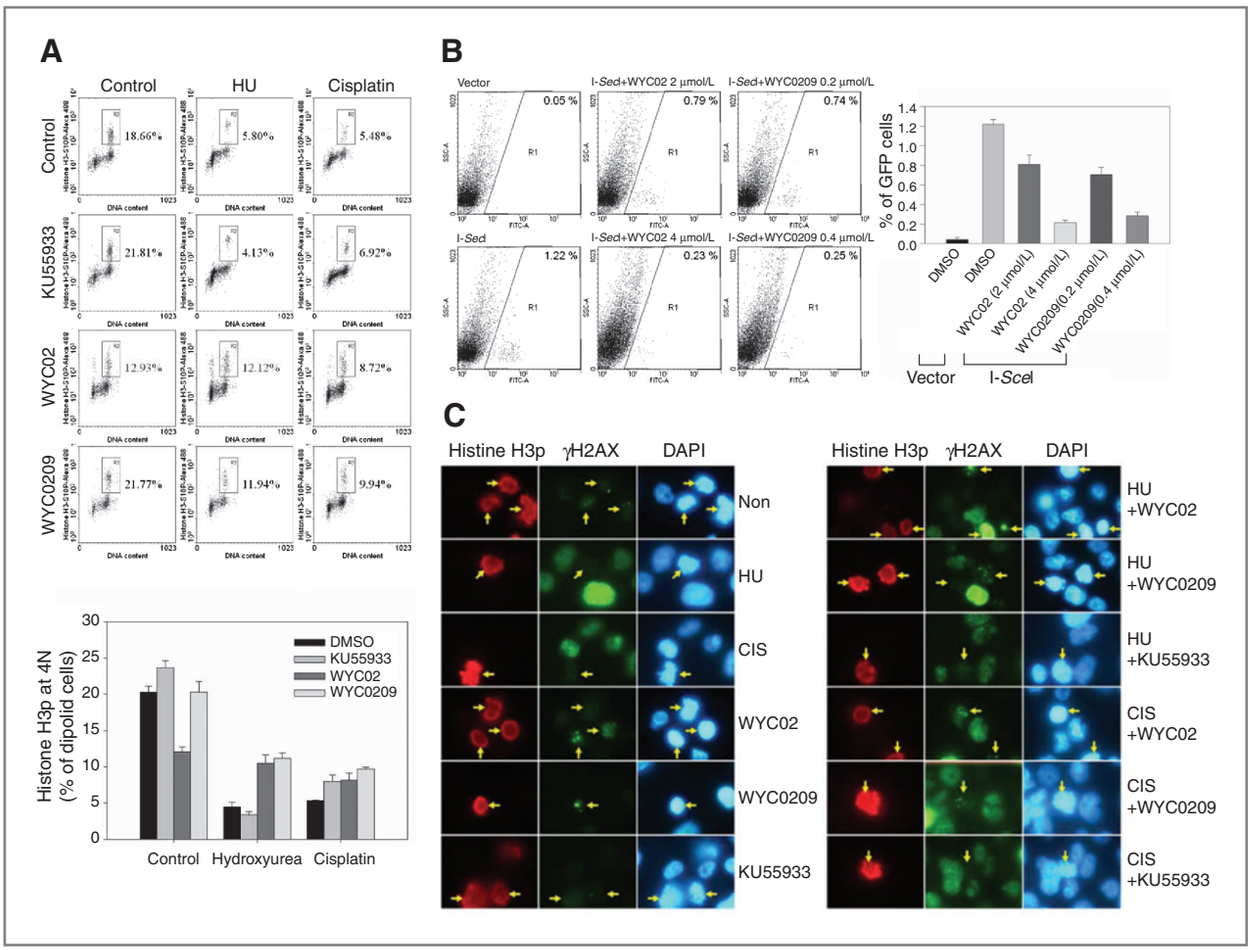

Figure 4. WYCs inhibit DNA damage checkpoint and repair. A, fluorescence-activated cell sorting dot blot analysis for mitotic markers. HU (1 mmol/L) or cisplatin (80 μmol/L) was incubated with or without 10 μmol/L KU55933, 4 μmol/L WYC02, or 0.4 μmol/L WYC0209 for 16 hours in presence of 70 nmol/L nocodazole. Cells were stained with phosphohistone H3 and PI, and the percentages of the mitotic marker for diploid cells were analyzed. B, fluorescence-activated cell sorting dot blot for analyzing the percentage of GFP cells denoting the HRR frequency, as described in Materials and Methods. C, immunohistochemical staining for the mitotic marker phosphohistone H3 (red), DNA damage marker γ-H2AX (green), and nucleus DAPI (blue) of cultured MDA-MB-231 cells was conducted using the same experimental design as in A. Microscopic evaluation was carried out with fluorescence microscopy with a ×100 oil immersion objective (Axiovert40; Zeiss). The yellow arrow indicates colocalization of the 3 markers in the same location, indicating a mitotic cell. The quantification result is shown in Supplementary Fig. S8.

treatment with WYCs, at least as compared with A549 cells (Supplementary Fig. S1B and S4). When the mice were treated with 0.2 mg/kg WYC0209 in combination with 2 mg/kg cisplatin, the tumor inhibitory effect was greater than that of cisplatin treatment alone (Fig. 5C). However, WYC02 unexpectedly did not affect the cisplatin sensitivity of MDA-MB-231 tumors when a higher dose of 2 mg/kg was used in our experiments (data not shown). The pharmacokinetic data of WYC02 and WYC0209 needs to be compared in future studies to determine the differences in the chemical effects of these 2 compounds *in vitro* and *in vivo*.

Discussion

Many chemotherapeutic agents kill cancers by interfering with DNA metabolism and lethally damaging the DNA. ATM and ATR are defined as the key checkpoint sensors in the DDR. Before the recent discovery of ATR inhibitors (10–14), ATM and Chk1/2 inhibitors were proven to efficiently enhance the sensitivity of cancers to IR therapy or chemical agents (8). Because the effects of flavonoids on ATR signaling have not yet been investigated, we consider the findings of our study to be novel. The synthetic derivative of WYC02, WYC0209, was found to have similar, yet more potent, inhibitory effects on ATR signaling that further validate our findings.

Low doses of WYCs significantly slowed cancer growth and caused S-phase delay and inhibition of DNA synthesis (Supplementary Fig. S5); these events are similar to previously reported ATR defects (32). WYCs treatment exhibited selective inhibition of ATR signaling, which we determined experimentally by different types of DNA-

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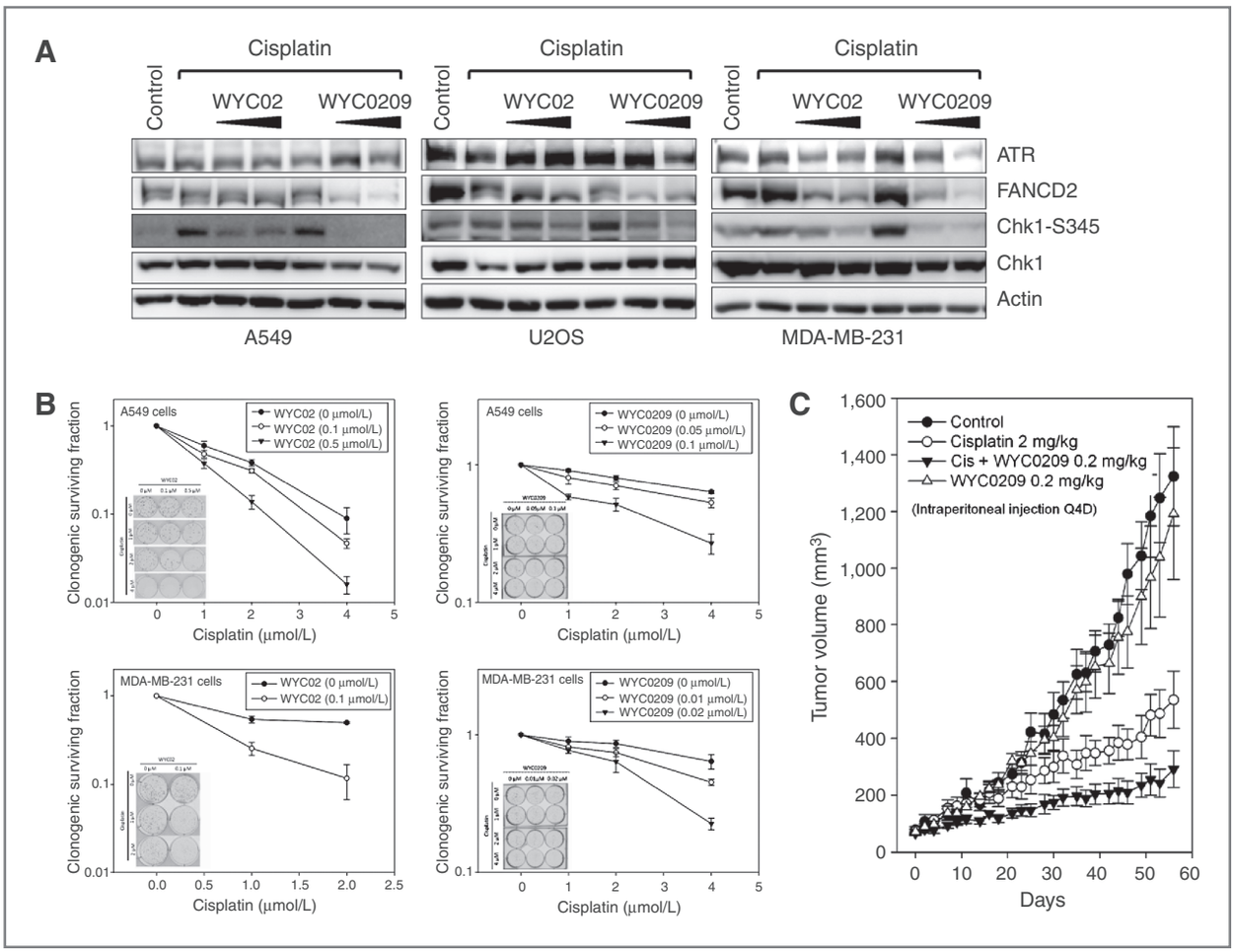

Figure 5. WYCs enhance sensitivity of cancer cells to cisplatin. A, Effect of WYCs on cisplatin-induced DDR in A594, U2OS, and MDA-MB-231 cells. Cisplatin (5 μmol/L) was incubated with or without 4 or 8 μmol/L of WYC02 or WYC0209 for 4 h. Whole-cell lysates were subjected to immunoblot analyses for phosphorylation of Chk1 and FANCD2. B, *In vitro* clonogenic survival for A549 and MDA-MB-231 cells. Cells were incubated with the indicated doses of cisplatin and WYC02 or WYC0209 for 6 h. After drug exposure, medium was replaced with drug-free medium for an additional 7–10 d. C, Effect of WYC0209 on MDA-MB-231 xenograft tumor *in vivo*. Mice with developed tumors were randomly sorted into 4 groups (n = 8–11per group) and received intraperitoneal injections of control vehicle or 2 mg/kg cisplatin combined with or without 0.2 mg/kg WYC0209 every 4 d throughout the experimental period. The width and length of the tumor were measured 3 times per week, and the volume was calculated as $(\text{width})^2 \times \text{length}/2$.

damage inducers. We clearly indicated that KU55933 specifically inhibits the ATM-mediated phosphorylation of Chk2 and WYCs specifically inhibit the ATR-mediated phosphorylation of Chk1 (Figs. 2A and 3A). Because the ATM activation that normally follows replication fork stalling or UV-induced irradiation damage depends on ATR kinase activity (33), the WYCs-mediated reduction in ATM activation upon HU, but not H_2O_2, treatment might result from ATR kinase dysfunction (Figs. 2A and 3A). This specificity was also supported by our observation that the WYC-mediated inhibition of DNA damage-induced Chk1 phosphorylation was dependent on ATR but not on ATM and DNA-PKcs (Fig. 3B and Supplementary Fig. S3). Furthermore, radial changes in chromosome structure are characteristics of cells derived from Fanconi anemia patients but not from patients with Ataxia telangiectasia (AT) or AT-like disease (34, 35); therefore, the WYC02-mediated inhibition of ATR-dependent FANCD2 activation links this effect to radial changes in chromosome structure from FA patients (Fig. 1B).

UV-induced Chk1 phosphorylation can be restored by overexpression of claspin and ATR but not TopBP1 or ATRIP (Fig. 3C). These data suggest that WYC02 might block the function of ATR kinase itself or the ATR–claspin–Chk1 interactions that then affect Chk1 activation. However, we could not exclude the possibility that WYCs target other mediators involved in ATR kinase activation. For example, FANCM has been shown to be necessary for both ATR activation and FANCD2 monoubiquitination (36). It remains unclear as to whether WYCs actually inhibit the ATR kinase enzyme activity or just abort the process of activation, and further investigations are required to clarify the

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precise mechanism(s) of action by WYCs. We screened a human kinome set for WYC02 involving 58 diverse *in vitro* kinase assays (Kinome Diversity Screen; MDS Pharma Services) and found that only PLK1 and SGK2 kinases were moderately inhibited by a high dose of WYC02 (Supplementary Fig. S6); therefore, it is clear that WYC02 does not target any of the kinases tested in this assay.

Our results supported 2 possible mechanisms by which WYCs could enhance cancer cell sensitivity to cisplatin: first, inhibition of the ATR checkpoint could lead cells that carry erroneous DNA into the next generation; second, inhibition of the FA repair pathway could result in elevated DNA DSB formation and error accumulation. We showed that WYCs compromise the cisplatin-induced checkpoint and lead to DSB accumulation within mitotic cells (Fig. 4A and C), suggesting that mitotic catastrophe might be induced in WYC-treated cells, whose DNA replication was not completed before entering into mitosis (37). Although ATM inhibition by KU55933 treatment rapidly accumulates damage in the late S and G_2-phase cells rather than in the M-phase cells (38), and induces cell death via apoptosis (39), WYC02 treatment mainly causes cells to undergo necrosis-like death rather than apoptosis in our system (Supplementary Fig. S7). These results indicate that the inhibition of ATM or ATR differentially cause accumulation of DNA lesions in cells undergoing diverse fates via different death pathways.

Previous data suggest that ATR and ATM play different roles in preventing DSB formation through HRR (40). ATR and its targets, such as Chk1, FANCD2, and WRN, are required to repair cells damaged during the S-phase via HRR (26, 29, 40, 41). We found that low doses of WYCs almost completely inhibited the HRR-mediated repair of chromosomal breaks, even in cells that possessed ATM function (Fig. 4B). Although ATM-deficient cells did not show reduced frequency of HRR to repair introduced DSBs (42), ATM-dependent FANCD2 Ser^{222} phosphorylation is required for the intra-S checkpoint, whereas the ATR-dependent FANCD2 Lys^{561} monoubiquitination is necessary for HRR (41). In the specific case of recovering from a stalled replication fork, we propose that ATM and ATR collaborate to induce checkpoint activation and recruit repair proteins for the initial step of HRR, whereas ATR signaling perhaps plays an indispensable role in the entire HRR process.

Our data revealed that WYCs could not only modify cancer cell responses to cisplatin but also to other therapeutic agents that induce Chk1 phosphorylation (Figs. 3A, 5A, and Supplementary Fig. S2), suggesting that WYCs may potentially function as cancer cell sensitizers for chemotherapeutic agents that activate the ATR checkpoint. Inhibition of DNA damage checkpoints and repair factors, such as ATM and BRCA1, are associated with promoting carcinogenesis as a consequence of genomic instability (43). However, there is no known cancer associated with ATR gene alterations. Specific ATR inhibition by expression of kinase-inactive ATR specifically in the skin prevents UV-induced skin carcinogenesis (44), suggesting that ATR inhibition has chemopreventive effects on nontransformed cells. Conversely, ATR has been proposed to play a critical role in directing cell death by senescence in unperturbed cells, where ATR is activated by overexpression of the oncoprotein Mos or the ATR activator TopBP1, respectively (45, 46). One plausible explanation proposed by Kawasumi and colleagues is that chemoprevention by ATR inhibition only works on precancerous cells in which oncogenes are not yet activated (44). In DNA-perturbed cells, the difference in the lesions induced by various DNA-damaging agents could also explain the different consequences of ATR/Chk1 inhibition, such as whether this inhibition promotes or suppresses apoptosis (47). However, while ATR activation clearly protects cells from death via suppression of apoptosis when DNA damage is caused by oncogene-induced replication stress or DNA cross-linking, limited ATR activity selectively kills these cells (48–50), probably mediated by an ATM-dependent mitotic catastrophe (51). Taken together, the genetic and pharmacologic evidence suggests that ATR inhibition potentially plays a dual role by achieving both chemoprevention and chemotherapy. Given that the efficiency of ATR inhibition causes the opposite effect of synthetic lethality or tumorigenesis on *Ras*-transformed cells (52), inhibition of ATR function for anticancer therapy should be carefully evaluated by considering the genetic background and responsiveness of the tumor to this type of therapy; furthermore, the efficiency of ATR inhibition should be cautiously monitored during treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol 2008;9:616–27.
2. de KA, Muijtjens M, van OR, Verhoeven Y, Smit B, Carr AM, et al. Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. Curr Biol 2000;10:479–82.
3. Brown EJ, Baltimore D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 2000;14:397–402.
4. O'Driscoll M, Ruiz-Perez VL, Woods CG, Jeggo PA, Goodship JA. A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet 2003;33:497–501.
5. Murga M, Bunting S, Montana MF, Soria R, Mulero F, Canamero M, et al. A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. Nat Genet 2009;41:891–8.
6. Nghiem P, Park PK, Kim Y, Vaziri C, Schreiber SL. ATR inhibition selectively sensitizes G1 checkpoint-deficient cells to lethal premature chromatin condensation. Proc Natl Acad Sci U S A 2001;98:9092–7.
7. Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL, et al. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J 1998;17:159–69.
8. Lapenna S, Giordano A. Cell cycle kinases as therapeutic targets for cancer. Nat Rev Drug Discov 2009;8:547–66.
9. Bolderson E, Richard DJ, Zhou BB, Khanna KK. Recent advances in cancer therapy targeting proteins involved in DNA double-strand break repair. Clin Cancer Res 2009;15:6314–20.
10. Nishida H, Tatewaki N, Nakajima Y, Magara T, Ko KM, Hamamori Y, et al. Inhibition of ATR protein kinase activity by schisandrin B in DNA damage response. Nucleic Acids Res 2009;37:5678–89.
11. Peasland A, Wang LZ, Rowling E, Kyle S, Chen T, Hopkins A, et al. Identification and evaluation of a potent novel ATR inhibitor, NU6027, in breast and ovarian cancer cell lines. Br J Cancer 2011;105:372–81.
12. Reaper PM, Griffiths MR, Long JM, Charrier JD, Maccormick S, Charlton PA, et al. Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. Nat Chem Biol 2011;7:428–30.
13. Toledo LI, Murga M, Zur R, Soria R, Rodriguez A, Martinez S, et al. A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. Nat Struct Mol Biol 2011;18:721–7.
14. Charrier JD, Durrant SJ, Golec JM, Kay DP, Knegtel RM, MacCormick S, et al. Discovery of potent and selective inhibitors of ataxia telangiectasia mutated and Rad3 related (ATR) protein kinase as potential anticancer agents. J Med Chem 2011;54:2320–30.
15. Hunyadi A, Chuang DW, Danko B, Chiang MY, Lee CL, Wang HC, et al. Direct semi-synthesis of the anticancer lead-drug protoapigenone from apigenin, and synthesis of further new cytotoxic protoflavone derivatives. PLoS One 2011;6:e23922.
16. Lin AS, Nakagawa-Goto K, Chang FR, Yu D, Morris-Natschke SL, Wu CC, et al. First total synthesis of protoapigenone and its analogues as potent cytotoxic agents. J Med Chem 2007;50:3921–7.
17. Lin AS, Chang FR, Wu CC, Liaw CC, Wu YC. New cytotoxic flavonoids from Thelypteris torresiana. Planta Med 2005;71:867–70.
18. Chiu CC, Chang HW, Chuang DW, Chang FR, Chang YC, Cheng YS, et al. Fern plant-derived protoapigenone leads to DNA damage, apoptosis, and G(2)/m arrest in lung cancer cell line H1299. DNA Cell Biol 2009;28:501–6.
19. Chen WY, Hsieh YA, Tsai CI, Kang YF, Chang FR, Wu YC, et al. Protoapigenone, a natural derivative of apigenin, induces mitogen-activated protein kinase-dependent apoptosis in human breast cancer cells associated with induction of oxidative stress and inhibition of glutathione S-transferase pi. Invest New Drugs 2011;29:1347–59.
20. Chen HM, Chang FR, Hsieh YC, Cheng YJ, Hsieh KC, Tsai LM, et al. A novel synthetic protoapigenone analogue, WYC02–9, induces DNA damage and apoptosis in DU145 prostate cancer cells through generation of reactive oxygen species. Free Radic Biol Med 2011;50:1151–62.
21. Chang HL, Wu YC, Su JH, Yeh YT, Yuan SS. Protoapigenone, a novel flavonoid, induces apoptosis in human prostate cancer cells through activation of p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase 1/2. J Pharmacol Exp Ther 2008;325:841–9.
22. Chang HL, Su JH, Yeh YT, Lee YC, Chen HM, Wu YC, et al. Protoapigenone, a novel flavonoid, inhibits ovarian cancer cell growth *in vitro* and *in vivo*. Cancer Lett 2008;267:85–95.
23. Wang HC, Chou WC, Shieh SY, Shen CY. Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining. Cancer Res 2006;66:1391–400.
24. Chou WC, Wang HC, Wong FH, Ding SL, Wu PE, Shieh SY, et al. Chk2-dependent phosphorylation of XRCC1 in the DNA damage response promotes base excision repair. EMBO J 2008;27:3140–50.
25. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev 1999;13:2633–8.
26. Andreassen PR, D'Andrea AD, Taniguchi T. ATR couples FANCD2 monoubiquitination to the DNA-damage response. Genes Dev 2004;18:1958–63.
27. Gregory RC, Taniguchi T, D'Andrea AD. Regulation of the Fanconi anemia pathway by monoubiquitination. Semin Cancer Biol 2003;13:77–82.
28. Lopez-Contreras AJ, Fernandez-Capetillo O. The ATR barrier to replication-born DNA damage. DNA Repair (Amst) 2010;9:1249–55.
29. Sorensen CS, Hansen LT, Dziegielewski J, Syljuasen RG, Lundin C, Bartek J, et al. The cell-cycle checkpoint kinase Chk1 is required for mammalian homologous recombination repair. Nat Cell Biol 2005;7:195–201.
30. Yazlovitskaya EM, Persons DL. Inhibition of cisplatin-induced ATR activity and enhanced sensitivity to cisplatin. Anticancer Res 2003;23:2275–9.
31. Chirnomas D, Taniguchi T, de la Vega M, Vaidya AP, Vasserman M, Hartman AR, et al. Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway. Mol Cancer Ther 2006;5:952–61.
32. Cha RS, Kleckner N. ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science 2002;297:602–6.
33. Stiff T, Walker SA, Cerosaletti K, Goodarzi AA, Petermann E, Concannon P, et al. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. EMBO J 2006;25:5775–82.
34. Taylor AM. Chromosome instability syndromes. Best Pract Res Clin Haematol 2001;14:631–44.
35. Patel KJ, Yu VP, Lee H, Corcoran A, Thistlethwaite FC, Evans MJ, et al. Involvement of Brca2 in DNA repair. Mol Cell 1998;1:347–57.
36. Collis SJ, Ciccia A, Deans AJ, Horejsi Z, Martin JS, Maslen SL, et al. FANCM and FAAP24 function in ATR-mediated checkpoint signaling independently of the Fanconi anemia core complex. Mol Cell 2008;32:313–24.
37. Reinhardt HC, Aslanian AS, Lees JA, Yaffe MB. p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. Cancer Cell 2007;11:175–89.
38. White JS, Choi S, Bakkenist CJ. Irreversible chromosome damage accumulates rapidly in the absence of ATM kinase activity. Cell Cycle 2008;7:1277–84.
39. Ivanov VN, Zhou H, Partridge MA, Hei TK. Inhibition of ataxia telangiectasia mutated kinase activity enhances TRAIL-mediated apoptosis in human melanoma cells. Cancer Res 2009;69:3510–9.
40. Ammazzalorso F, Pirzio LM, Bignami M, Franchitto A, Pichierri P. ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery. EMBO J 2010;29:3156–69.
41. Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, et al. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci U S A 2005;102:1110–5.
42. Sakamoto S, Iijima K, Mochizuki D, Nakamura K, Teshigawara K, Kobayashi J, et al. Homologous recombination repair is regulated by

Published OnlineFirst April 24, 2012; DOI:10.1158/1535-7163.MCT-11-0921

domains at the N- and C-terminus of NBS1 and is dissociated with ATM functions. Oncogene 2007;26:6002–9.

43. Li S, Ting NS, Zheng L, Chen PL, Ziv Y, Shiloh Y, et al. Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. Nature 2000;406:210–5.
44. Kawasumi M, Lemos B, Bradner JE, Thibodeau R, Kim YS, Schmidt M, et al. Protection from UV-induced skin carcinogenesis by genetic inhibition of the ataxia telangiectasia and Rad3-related (ATR) kinase. Proc Natl Acad Sci U S A 2011;108:13716–21.
45. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 2006;444:633–7.
46. Toledo LI, Murga M, Gutierrez-Martinez P, Soria R, Fernandez-Capetillo O. ATR signaling can drive cells into senescence in the absence of DNA breaks. Genes Dev 2008;22:297–302.
47. Myers K, Gagou ME, Zuazua-Villar P, Rodriguez R, Meuth M. ATR and Chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. PLoS Genet 2009;5:e1000324.
48. Murga M, Campaner S, Lopez-Contreras AJ, Toledo LI, Soria R, Montana MF, et al. Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. Nat Struct Mol Biol 2011;18:1331–5.
49. Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL, et al. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J 1998;17:159–69.
50. Schoppy DW, Ragland RL, Gilad O, Shastri N, Peters AA, Murga M, et al. Oncogenic stress sensitizes murine cancers to hypomorphic suppression of ATR. J Clin Invest 2012;122:241–52.
51. Imreh G, Norberg HV, Imreh S, Zhivotovsky B. Chromosomal breaks during mitotic catastrophe trigger gammaH2AX-ATM-p53-mediated apoptosis. J Cell Sci 2011;124(Pt 17):2951–63.
52. Gilad O, Nabet BY, Ragland RL, Schoppy DW, Smith KD, Durham AC, et al. Combining ATR suppression with oncogenic Ras synergistically increases genomic instability, causing synthetic lethality or tumorigenesis in a dosage-dependent manner. Cancer Res 2010;70:9693–702.