**CELL BIOLOGY**

A CDC7 inhibitor sensitizes DNA-damaging chemotherapies by suppressing homologous recombination repair to delay DNA damage recovery

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Cell division cycle 7 (CDC7), a serine/threonine kinase, plays important roles in DNA replication. We developed a highly specific CDC7 inhibitor, TAK-931, as a clinical cancer therapeutic agent. This study aimed to identify the potential combination partners of TAK-931 for guiding its clinical development strategies. Unbiased high-throughput chemical screening revealed that the highest synergistic antiproliferative effects observed were the combinations of DNA-damaging agents with TAK-931. Functional phosphoproteomic analysis demonstrated that TAK-931 suppressed homologous recombination repair activity, delayed recovery from double-strand breaks, and led to accumulation of DNA damages in the combination. Whole-genome small interfering RNA library screening identified sensitivity-modulating molecules, which propose the experimentally predicted target cancer types for the combination, including pancreatic, esophageal, ovarian, and breast cancers. The efficacy of combination therapy in these cancer types was preclinically confirmed in the corresponding primary-derived xenograft models. Thus, our findings would be helpful to guide the future clinical strategies for TAK-931.

**INTRODUCTION**

Replication stress (RS), inefficient DNA replication, is a hallmark of cancer; it leads to the accumulation of genomic instability, which is closely associated with aggressiveness and therapy resistance in cancer cells (1–9). Conventional chemotherapeutic drugs target this cancer hallmark to generate RS in various cancer types, including solid and hematological cancers (6, 10). The serine/threonine kinase cell division cycle 7 (CDC7; also known as DBF4-dependent kinase) is an attractive target for novel drugs, which is activated by binding to its regulatory protein, DBF4; activated CDC7 kinase phosphorylates minichromosome maintenance 2 (MCM2) at Ser40 to initiate DNA synthesis (11–13). Because of these central roles of CDC7 kinase in DNA replication (14–20), many CDC7 inhibitors are being developed as next-generation RS-inducing cancer drug candidates (11, 12, 21, 22). We successfully developed the first orally active CDC7-selective inhibitor, TAK-931, which exhibited a broad spectrum of antitumor efficacy and showed unique mechanisms of action in various preclinical cancer models (22, 23). Our preclinical study findings strongly suggest the therapeutic potential of TAK-931 as a next-generation anticancer drug.

In the past two decades, cancer therapeutics has been remarkably improved along with the substantial advancement in molecular-targeting therapy based on pathogenic oncogenes, cancer vulnerability, and synthetic lethality. However, further improvement in these therapies is needed for patients with cancer, especially late-stage patients, in whom cancers would be refractory or poorly adapted to currently available molecular-targeted therapies. The therapeutic windows can be expanded, and the complete potential of anticancer drugs can be exerted by identifying the best possible combination partners, such as regulatory board–approved cancer drugs and irradiation (IR). For example, in the case of cyclin-dependent kinase 4/6 (CDK4/6) inhibitors, combination therapies of CDK4/6 inhibitors with estrogen blockers (e.g., tamoxifen) or selective estrogen receptor (ER) degraders (e.g., fulvestrant) expand the therapeutic window of these anti-ER drugs: Highly improved efficacy of combination therapies has been clinically confirmed in patients with ER-positive breast cancer, whereas treatment with CDK4/6 inhibitors alone exhibited only modest responses in clinical trials (24, 25). Thus, combination treatment with CDK4/6 inhibitors and anti-ER therapies has become a standard therapeutic protocol in patients with ER-positive breast cancer. Similarly, identification of prospective combination partner(s) would provide a remarkable advantage, especially for novel drug candidates such as TAK-931, to design a clinical pathway: The tumor types for which combination partners are already identified could be first targeted for improving the clinical efficacy of the combination treatment.

This study aimed to identify the potential combination partners of TAK-931 to guide its clinical development strategies. We performed in vitro combination screening with currently used chemotherapeutic drugs and found that DNA-damaging agents, such as topoisomerase inhibitors and platinum compounds, had the highest
The in vitro double-agent screening of TAK-931 and small-molecule agents in cancer cell lines

To identify the chemical agents that enhance the antiproliferative activity of TAK-931, we used a completely automated system for unbiased in vitro combination screening of TAK-931 and 50 chemicals, termed double-agent study, in the indicated six cancer cell lines (Fig. 1A and fig. S1A). The chemicals tested in the double-agent study included DNA-damaging agents, tubulin binders, mitotic inhibitors, cell-signaling modulators, proteasome inhibitors, and some reference agents (Fig. 1B). Combination effects with TAK-931 were classified on the basis of the combination index as synergistic (0 to 0.7), additive (0.7 to 1.3), sub-additive (1.3 to 2.0), or antagonistic (2 or more; Fig. 1, C and D). Combination performance was ranked as the most frequent occurrence of synergy across the cell lines (Fig. 1E). The top-ranking combinations included DNA-damaging agents such as topoisomerase inhibitors (teniposide, topotecan, etoposide, and SN-38), nucleoside and nitrogenous base analogs (decitabine), DNA-alkylating agents (melphalan and mitoxantrone), cross-linking agents (mitomycin C), or platinum compounds (carboplatin and cisplatin). On the contrary, the microtubule inhibitors (docetaxel, paclitaxel, and vincristine sulfate), controls for non-DNA damage agents, exhibited relatively mild combination effects with TAK-931 (fig. S1B). These findings suggested that the antiproliferative activity of certain types of DNA-damaging chemotherapeutic drugs is enhanced in combination with TAK-931 in a broad spectrum of cancer cell lines.

The in vivo combination studies: Pharmacokinetics, pharmacodynamics, and antitumor efficacy

The pharmacological combination effects were also confirmed by conducting in vivo combination studies in a COLO205 xenograft mouse model, which is well characterized for its usability and relatively high take and growth rates (26). The in vivo studies were performed with the representative combination of TAK-931 and topoisomerase inhibitor CPT-11. First, to exclude the possibility of adverse effects by drug–drug interactions (DDIs), we evaluated the pharmacokinetic (PK) profile of each compound in the combination (Fig. 2A). The COLO205-xenografted mice were administrated a single dose of TAK-931 orally, CPT-11 intraperitoneally, and their combination, and then the tumors were collected at the indicated time points to measure each drug concentration. TAK-931 exposures in tumors were equivalent between the single-agent and combination treatments (Fig. 2B, left, and fig. S2A). Similarly, CPT-11 exposures also had equivalent profiles between single-agent and combination treatments (Fig. 2B, right). Pharmacodynamic (PD) modulation in the combination, an index to monitor biological exposure, was also performed. Phosphorylated MCM2 at Ser⁴⁰ (pMCM2), which is directly phosphorylated by CDC7 kinase, was used as a target engagement PD marker for TAK-931 (22). The COLO205-xenografted mice were administrated pMCM2 at the indicated regimens, and then time-dependent changes in pMCM2 level were measured using immunohistochemistry. The pMCM2 in tumors was similarly modulated between single-agent TAK-931 and combination treatments: pMCM2 level decreased from 2 hours to the least value at 8 hours and then increased by 24 hours up to the vehicle control levels (Fig. 2, C and D, and fig. S2B). The PK and PD studies in COLO205-xenografted models detected less effect of DDIs between TAK-931 and CPT-11.

The in vivo antitumor efficacy of the combination with TAK-931 and CPT-11 was confirmed in the COLO205-xenografted model at the indicated regimens. The antitumor activity (% T/C) on day 15 was 53, 63, and 21% in TAK-931, CPT-11, and the combination treatments, respectively (Fig. 2E and fig. S3). Bodyweight loss (BWL) was not significantly different from that in the vehicle-treated control mice: The nadir of BWL was 5.1% in vehicle, 0.7% in TAK-931, −4.3% in CPT-11, and −7.0% in the combination. Combination of IR and TAK-931 also induced potent antitumor efficacy, delaying tumor regrowth even after the treatment was terminated (Fig. 2F): % T/C on day 14 was 52, 17, and −27% in TAK-931, IR, and combination treatments, respectively. These in vivo combination studies indicated that the combination with TAK-931 enhances the antitumor activity of DNA-damaging chemotherapies and IR.

Phosphoproteomic analyses of the combination of TAK-931 and IR

We performed in vitro phosphoproteomic analyses to identify the signaling networks associated with the TAK-931–mediated enhancement of the antiproliferative activity of DNA-damaging agents. The combination of TAK-931 and IR was used for the phosphoproteomic analyses. Since IR physically induces DNA damages immediately after the treatment, this might be more feasible to assess the time-dependent dynamics of DNA damages. The in vitro cell viability and 6-day colony formation assays revealed that the combinational effects of TAK-931 and IR induced significant antiproliferation in H460 lung cancer cells (Fig. 3A and fig. S4, A and B). Stable isotopic labeling of amino acid in cell culture (SILAC)–labeled H460 cells were irradiated at 4 grays (Gy) with or without TAK-931 and then subjected to phosphoproteomic analysis at 4 and 24 hours after IR treatment (Fig. 3B and fig. S4C). Mass spectrometry detected 4890 phosphorylation sites, which were used in the following analyses (table S1).

First, the phosphorylation sites were compared between IR alone (IR) and the combination (Combo) at each time point. The phosphorylation spectrums at 4 hours of treatment were relatively identical between IR and Combo [coefficient of determination (R²) = 0.72; Fig. 3C, left], in which 40 and 68 phosphopeptides were detected as Combo-specific up-regulated (red dots; log₂ [Combo/Cont] > 0.5) and down-regulated (blue dots; −0.5 > log₂ [Combo/Cont] > log₂ [IR/Cont]), respectively. For instance, phosphorylated ATM (pATM) at Ser¹⁸⁸ and phosphorylated Chk1 (pChk1) at Ser²⁰⁶, Ser⁵¹⁶, and Thr²⁰⁷ were similarly elevated in both treatments, whereas pMCM2 at Ser²⁶² was down-regulated and phosphorylated CDC6 (pCDC6) at Ser⁴⁵ was up-regulated as the Combo-specific
Fig. 1. The in vitro combination effect of TAK-931 with chemotherapeutic agents. (A) Experimental schemes for double-agent studies. The indicated compounds were assessed for viability by using the 72-hour ATP-based assay. Inward and outward curving isobolograms indicate Loewe synergy and antagonism, respectively. (B) Compound list used for the double-agent study. (C) Representative response surface in the double-agent study. A response surface in SW620 treated with TAK-931 and cisplatin. (D) Representative fitted isobolograms in the double-agent study. Isobolograms for cisplatin in SW620 cells (top left), mitomycin C in SW620 cells (top right), SN-38 in A549 cells (bottom left), and topotecan in A549 cells (bottom right). The combination indices (CIs) are shown in the panels. (E) Summary of CIs in the top 10 combinations tested. Combinations are ordered on the basis of the occurrence of synergy across cell lines. Green, orange, and red indicate synergy, additive, and sub-additive effects, respectively. n.a., not available.
modulations, which should be attributed to TAK-931 treatment effects (22). In contrast, at 24 hours of treatment, 248 phosphopeptides were specifically up-regulated by Combo, thereby decreasing the correlation coefficient ($R^2 = 0.54$; Fig. 3C, right), suggesting that stress response signaling pathways were highly up-regulated in the 24-hour Combo-treated cells.

The parametric analysis of gene set enrichment revealed that 32, 34, 14, and 22 pathways were enriched in the 4-hour IR, 4-hour Combo, 24-hour IR, and 24-hour Combo treatments, respectively. At 4 hours, most of the enriched pathways were shared between the IR and Combo treatments (31 of 35; Fig. 3D, left, and fig. S4D). In contrast, at 24 hours, few shared pathways (13 of 23) were noted, with greater number of the Combo-specific enriched pathways (9 of 23) (Fig. 3D, right, and fig. S4E). In the categories of gene ontology (GO) biological process (BP), DNA repair, double-strand break (DSB) repair, and E2F transcription factor (E2F TF) were significantly enriched in both treatments at 4 hours ($P < 0.05$; enclosed with green dotted line, Fig. 3E), whereas these network pathways were continuously enriched in Combo ($P < 0.05$), but not in IR, at 24 hours (enclosed with green dotted line, Fig. 3E). Supporting the scatter-plotting data, the heatmap also showed that protein phosphorylation of these pathways was continuously up-regulated in Combo until 24 hours, maintaining equivalent levels at 4 hours, whereas they were remarkably down-regulated in IR at 24 hours (Fig. 3F and fig. S4F). Changes in pCHEK2 at Ser260, pATM at Ser1981, pBRCA1 at Ser1524, and pBARD1 at Ser186 are shown as representatives in Fig. 3G. These findings suggest that combination with TAK-931 prolongs activation of IR-induced DNA damage response (DDR)

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**Fig. 2. PK, PD, and efficacy studies in combination with TAK-931 and CPT-11 in the COLO205 xenograft models.** (A) Experimental schemes of PK, PD, and efficacy studies. (B) Time-dependent tumor PK of TAK-931 and CPT-11. Left and right panels indicate tumor PKs of TAK-931 and CPT-11, respectively. Blue, green, and red lines indicate TAK-931, CPT-11, and the combination treatments, respectively. (C) Time-dependent PD modulations of TAK-931. Tumor pMCM2 was used as a PD marker of TAK-931. (D) Quantitative PD modulations in the combination. The pMCM2 intensity was quantified using the results shown in (C). Blue, green, and red lines indicate TAK-931, CPT-11, and the combination, respectively. (E) Efficacy studies for combination with TAK-931 and CPT-11 in COLO205 xenograft models. Efficacy data as the mean tumor volume (left, cubic millimeters ± SEM; $n = 6$) and body weight data (right, grams ± SEM; $n = 6$) are plotted in vehicle control (black), TAK-931 (blue), topotecan (green), and the combination (red). (F) Efficacy studies for combination with TAK-931 and IR in the COLO205 xenograft models. Tumor size was continuously measured for the indicated periods after the termination of drug treatment.
Fig. 3. Phosphoproteomic analysis in combination with IR and TAK-931. (A) Combination effects of IR and TAK-931. H460 cells were treated with DMSO (black) or TAK-931 at 1000 nM for 1 hour, followed by IR at the indicated dose (gray). (B) Experimental schemes for phosphoproteomic analysis. (C) Comparison of quantified phosphorylation sites. The x and y axes indicate the log2-scaled mean ratio of each phosphorylation site of IR alone and the combination treatments. Red and blue dots indicate the out-of-range phosphopeptides of y = x ± 1 at the positive and negative sides, respectively. The 4-hour (left) and 24-hour (right) treatments are shown. (D) Venn diagram of the pathway analyses. Red and blue circles indicate the IR- and combination-modulated GO terms, respectively. (E) Pathway comparison. The log10-scaled P values of the GO terms in IR (x axis) and the combination (y axis) are plotted. The green dotted line area indicates the combination-specific modulated pathways (P < 0.05 in combination and P > 0.05 in IR). The GO terms of DNA repair, DSB repair, and E2F TF are shown in orange, red, and blue, respectively. (F) The phosphorylation heatmaps. Red and blue indicate “up-regulated” and “down-regulated,” respectively. (G) Time-dependent phosphorylation changes in IR- and combination-treated cells. Each dot indicates the quantified phosphorylation changes as described in (C). pCHEK2 at Ser260, pATM at Ser1981, pBRCA1 at Ser1524, and pBARD1 at Ser186 are highlighted as representatives.

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signaling networks, presumably owing to the recovery delay from DNA damages.

**TAK-931 suppresses HRR-mediated DNA damage recovery**

To determine the effects of TAK-931 on the recovery delay, DNA damage recovery assays were conducted in combination with TAK-931 and IR, monitoring foci formation for DSBs and/or single-strand breaks (SSBs) after the treatments. Foci formation of 53BP1, a DSB marker, was prominently detected 8 hours after IR treatment in both the presence and absence of TAK-931 (Fig. 4, A and B). At 48 hours after IR treatment, 53BP1 foci–positive cells had drastically decreased in the IR-alone treatment, suggesting that DNA repair of IR-induced DSBs was completed in 48 hours after IR treatment. In contrast, in the combination treatment, 53BP1 foci formation was detected in numerous cells even after 48 hours of treatment, suggesting that TAK-931 significantly delays recovery from IR-induced DSBs. Cells positive for the BRCA1 foci, which is a marker for both DSBs and SSBs, were greater in the 48-hour combination treatment than in the 48-hour TAK-931 or IR single treatment (Fig. 5A, A and B). SSBs appear to be generated by DNA R5, which is the direct effect of CDC7 inhibition by TAK-931 (22). Comet assays also revealed that DNA tails in the combination were significantly longer than those in the single-agent treatment with IR or TAK-931, suggesting that DSBs and SSBs are more prominently accumulated in the combination treatment (Fig. 4, C and D). Consistent with the results of phosphoproteomic analysis, immunoblotting also revealed that pCHK2 and pATM were more abundant in the combination than in each single-agent treatment at 24 hours (Fig. 4E).

In the DSB repair GO term of Combo (Fig. 3F), phosphorylation levels of HRR-associated proteins were significantly higher than those of other proteins ($P < 0.01$; Fig. 4F). Next, we investigated the effects of TAK-931 on HRR by using the SceGFP-based HRR reporter system (Fig. 4G). To exclude the adverse effects of replication-coupled repair modulated by CDC7 inhibition, we performed HRR assay using transiently transfected SceGFP reporter plasmids (27, 28), which should not be autonomously replicated in mammalian cells. The HRR assay revealed that TAK-931 treatment significantly reduced green fluorescent protein (GFP)–positive cells, where I–Sce I–induced DSBs in SceGFP plasmids were repaired by HRR but not by nonhomologous end joining (NHEJ); $P < 0.01$; Fig. 4, H and I). Thus, although the DDR proteins such as 53BP1 and BRCA1 were recruited to the damage site, TAK-931 significantly suppressed HRR activity independently of DNA replication, and this attenuated HRR was likely involved in the DSB recovery defects and combinational antiproliferative effects.

Considering that TAK-931 potently suppresses HRR activity, it could elicit a chemically induced “BRCAness,” a phenocopy of BRCA1 or BRCA2 mutation (29, 30). BRCA1- or BRCA2-deficient tumors exhibit hypersensitivity against PARP inhibitors (31). To determine the effects of TAK-931 on BRCAness, we conducted in vivo combination studies of TAK-931 and a PARP inhibitor (niraparib) in breast cell line–derived xenograft, breast DPD, and ovarian PDX models, i.e., MDA-MB-231, PHTX-147B, and PHTXS-130, respectively (Fig. 4J and Fig. S5C). MDA-MB-231 and PHTX-147B have wild-type BRCA1 and BRCA2, and PHTXS-130 has mutant BRCA2 at M784V, which is a variant of unknown significance (32). In all three xenograft models, the combination treatment of TAK-931 and niraparib significantly improved the antitumor efficacy, suggesting that TAK-931 could chemically induce BRCAness in preclinical tumor models, leading to PARP inhibitor–induced synthetic lethality.

**DDR-focused siRNA library screening for caspase-3/7 activation**

To determine the sensitivity-modulating molecules in the combination treatment, first, we performed focused siRNA screening of the DDR pathway library. We used a lung cancer cell line H460 in the combination treatment of TAK-931 and the topoisomerase inhibitor SN-38 (Fig. S6A). According to the combination with IR, this combination treatment also activated the DDR-associated pathway: up-regulation of pATM, pChk1, pChk2, γH2AX, pTP53, TP53, and p21 (Fig. S6B). Furthermore, the combination treatment exhibited the intense caspase-3/7 activity in apoptosis assay, as well as the enhanced antiproliferation in clonogenic assay (Fig. 5, A and B, and Fig. S6C). Caspase-3/7 screening of 104 DDR pathway–focused siRNAs was conducted in H460 cells treated with dimethyl sulfoxide (DMSO), TAK-931, SN-38, and the combination (Fig. 5C and Fig. S7); the ranges of relative activity were 0.78 to 2.83 (average: 1.47), 1.16 to 6.72 (average: 2.71), 0.84 to 18.1 (average: 3.76), and 0.94 to 13.23 (average: 5.94), respectively (Fig. 5D). Compared to the siNS values in each treatment (green point, Fig. 5D), 13, 29, and 31 siRNAs suppressed caspase-3/7 activities in the DMSO–, TAK-931–, SN-38–, and combination-treated cells, respectively.

The siRNAs suppressing caspase-3/7 in each treatment are shown in Fig. 5E; many of these down-regulator genes (17 of 29) were commonly noted across SN-38 and the combination (Fig. 5E): BRCA1 complex (BRCA1 and BARD1), base excision repair (XRCC1, POLB, LIG1, LIG3, PARP3, and RAD9A), Fanconi anemia (FANCB and FANCN), HR (RAD51C and RIM2), NHEJ (XRCC6 and NHEJ1), and others (MCM2 and XPA). Among these, especially, BRCA1 and BARD1 were ranked first and second in both SN-38 and the combination, the siRNAs of which potently inhibited PARP cleavage and caspase-3/7 activation (Fig. 5, F and G). Previous studies have shown apoptosis induction by BRCA1 and BARD1 in response to genotoxic stresses (33–35); thus, the BRCA1/BARD1 complex might play an important role in the activation of the apoptosis pathway in the case of the combination as well. Comparison analyses also revealed that the spectrum of siRNA effects in the combination was more similar to that in SN-38 rather than that in DMSO or TAK-931: The values of $R^2$ were 0.09, 0.20, and 0.58 for comparison of DMSO versus Combo, TAK-931 versus Combo, and SN-38 versus Combo, respectively (Fig. 5H). Thus, in the combination, TAK-931 may play as a sensitizer to enhance the biological activities of DNA-damaging agents.

**Genome-wide siRNA library screening in the combination**

To more comprehensively identify other sensitivity-modulating pathways in the combination, we performed genome-wide siRNA library screening on caspase-3/7 activation by using 64,773 siRNAs, which cover 21,591 genes (3 distinct siRNAs for each gene), in the H460 cells treated with TAK-931 and SN-38 (Fig. 6A). The screening revealed hit genes based on (i) four or more of mean absolute deviation, (ii) one or more next-generation sequencing (NGS) value (fragments per kilobase of exon per million reads mapped), and (iii) unique genes selected (fig. S8A). The genome-wide screening identified 153 and 44 hit genes as caspase-3/7 down-regulators and up-regulators, respectively (fig. S8B).

Metascape enrichment analysis of the GO category BP and comprehensive resource of mammalian protein complexes (CORUM)
Fig. 4. TAK-931 suppresses HRR activity and enhances the antitumor activity of a PARP inhibitor. (A) Representative images of 53BP1 IF. HeLa cells were collected 8 and 48 hours after the treatment. Red and blue indicate 53BP1 and DAPI (4′,6-diamidino-2-phenylindole) (DNA), respectively. (B) Quantitative analysis of 53BP1 foci formation. n.s., not significant. The graphs show the percentage of cells with >10 53BP1 foci. Blue and red bars indicate 8 and 48 hours after the treatment, respectively. Statistical analysis was performed using Student’s t test (n = 3). (C) Representative images of comet assay. HeLa cells were collected 48 hours after the treatment. The white bars indicate DNA tails. (D) Quantification of DNA tails using KEYENCE BZ-II. Statistical analysis was performed using Student’s t test. (E) Immunoblotting of DDR proteins. HeLa cells were collected 4 and 24 hours after the treatment. (F) Phosphorylation heatmaps of the DSB repair pathway. HR-associated genes are highlighted in red. Statistical analysis was performed using nonparametric Wilcoxon-Mann-Whitney test. (G) Experimental schemes of HR assays. (H) Representative images of HR assays. (I) Quantitative data of HR activity after TAK-931 treatment. Statistical analysis was performed using Student’s t test (n = 3). (J) Efficacy studies in combination with TAK-931 and the PARP inhibitor. Breast cell line–derived xenograft (CDX), breast PDX, and ovarian PDX were used. Efficacy data are plotted as the mean tumor volume (n = 8) in vehicle control (black), TAK-931 (blue), niraparib (green), and the combination (red) treatments.
Fig. 5. DDR-focused siRNA library screening. (A) Antiproliferative effects of SN-38 and TAK-931. H460 cells were treated with DMSO (black), 100 nM TAK-931 (blue), or 1000 nM TAK-931 (red) in combination with SN-38. (B) Caspase-3/7 activation in combination. H460 cells were treated with DMSO (black) or TAK-931 (red) in combination with SN-38 for 48 hours. The bars represent means ± SD (n = 3). (C) Experimental schemes of siRNA screening. (D) Effects of 104 DDR-focused siRNA. Each dot indicates the relative caspase activity in TAK-931 (left), SN-38 (middle), and the combination (right). Green, blue, and red indicate siNS, siBARD1, and siBRCA1 as representatives. (E) Venn diagram of the caspase-3/7–down-regulating siRNAs. Gray, blue, green, and red circles indicate the caspase-3/7–down-regulating siRNAs in DMSO, TAK-931, SN-38, and the combination, respectively. The orange area encircled by the dotted line indicates the 19 shared siRNAs between SN-38 and the combination. (F) Effects of siBRCA1 and siBARD1. Black, red, and blue bars indicate siNS, siBRCA1, and siBARD1, respectively. (G) Effects of siBRCA1 on PARP1 cleavage in combination. Cleaved PARP1 (cPARP) was detected by long exposure. (H) Comparison of siRNA effects. Each dot indicates the relative caspase activity of each siRNA treatment. The left, middle, and right panels indicate comparison of the combination with DMSO, TAK-931, and SN-38, respectively. The siRad51L1 in SN-38 and siSMC3 in Combo were excluded as the outliers.
Fig. 6. Genome-wide siRNA library screening in the combination treatment. (A) Experimental schemes of the genome-wide caspase-3/7 siRNA screening in the combination-treated H460. (B and C) Enrichment analysis in GO BPs and CORUM structural complex. The 153 down-regulated (B) and 44 up-regulated genes (C) were used for the GO and CORUM term enrichment analysis. Enrichment analysis was performed using Metascape. The P values were calculated using Fisher’s exact test. The P values at <0.05 are considered as significantly enriched terms. (D and E) Subextractor network analysis with the down-regulators and up-regulators. The network is visualized using Cytoscape (v3.1.2) by using the 153 down-regulating genes (D) and 44 up-regulating genes (E). (F) Cross-network analysis between the down- and up-regulators. The network is visualized using Cytoscape (v3.1.2) by using the merged genes of the 153 down-regulators and 44 up-regulators. Red and blue pie sectors represent the down- and up-regulators, respectively. (G) Heatmaps of the pathways in the down-regulators and up-regulators. The phosphorylation heatmaps of DNA repair, DSB repair, and E2F TF are shown. Red and blue indicate down-regulated and up-regulated, respectively. The heatmap cells are colored according to their P values: White cells indicate the lack of enrichment for that term in the corresponding gene list.
revealed that 17 and 12 terms were significantly enriched in the down-regulators and up-regulators, respectively ($P < 0.01$; Fig. 6, B and C). Network analyses of the down-regulators and up-regulators also revealed a number of various significant network modules (Fig. 6, D and E, and fig. S9, A and B); BRCA1-associated pathway was also detected in the genome-wide siRNA screening and the DDR-focused screening.

Cross-analyses of the down-regulators (red) and up-regulators (blue) showed that the enrichment clusters were mostly composed of a single collar but not of mixed collars; for instance, BRCA1 C complex is in red, whereas RNA splicing is in blue (Fig. 6F and fig. S9, C and D). Heatmap comparison of the enrichment terms between the down- and up-regulators also revealed that, although only 2 enrichment terms—regulation of protein ubiquitination and mRNA catabolic process—appeared commonly, the other 18 enrichment terms exclusively appeared in either one or the other (Fig. 6G). These findings suggest that, although the detailed mechanisms are still not clear, caspase-3/7 activity in the combination appears to be controlled by multiple and complex signaling pathways, which are not limited to the DDR-associated pathways. Furthermore, up-regulation and down-regulation of caspase-3/7 in the combination appear to be regulated by the independent or distinct signaling pathways. These comprehensive and parametric views of the sensitivity-modulating networks could offer a new direction not only in deeply understanding the molecular mechanisms of the combination effects but also in developing the therapeutic strategies to determine the target indications for the combination treatment.

**Experimental prediction of potential target cancers by using the genome-wide siRNA screening hit genes**

To identify the target cancer types for the combination, we further performed bioinformatic analyses by using public databases for the hit genes identified by genome-wide screening as the experimental predictive markers (Fig. 7A). First, we selected experimental resistant markers from hit genes identified by siRNA screening, by reranking the down-regulators in order of caspase-3/7 suppressive activity by using two siRNAs showing higher suppressive activity to exclude the outlier siRNAs (fig. S10A). Two-peaked histogram of caspase-3/7 suppressive activity was composed of a strong-effect ($\geq$70) and moderate-effect population ($<$70; fig. S10B). Thirty-nine genes from the strong-effect population were selected as the experimental resistant markers (fig. S10C). The up-regulators were not used because of their limited numbers of strong-effect genes (fig. S10D). Second, we extracted the expression data of these 39 markers from 10,071 cases from The Cancer Genome Atlas (TCGA) database (www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). We named “resistant marker—positive cancer” if the case exhibited low expression of 1 or more of the 39 markers (threshold: $\leq$one-fifth of the median value). Third, we sorted the cancer types in the order of percentage “resistant marker positive” in each cancer type (Fig. 7B, red bars, resistant marker—positive cancers and blue bars, others). Bioinformatic analyses predicted that resistant marker rate was lower in PAAD, LU (AD and SC), UCEC, BRCA, KIRC, PRAD, THCA, STAD, OV, and ESCA whereas higher in UVM, DLBC, GBM, LAML, SARC, SKCM, LGG, PCPG, THYM, KICH, and LIHC, suggesting that the former cancer types may have more sensitivity to the combination treatment (Fig. 7B and fig. S10E).

**Antitumor efficacy of the combination treatment in the experimentally predicted target cancer PDX models**

Last, we conducted in vivo antitumor efficacy studies for combination of TAK-931 with DNA-damaging agents in the selected PDX models. As shown in the breast and ovarian PDX models tested in combination with the PARP inhibitor (Fig. 4), two additional cancer types—pancreatic and esophageal cancers—were subjected to the following in vivo combination studies based on the higher prediction scores. Two PDX models were used for each cancer type: PHTXM-90Es and PHTXM-79Es for esophageal PDXs and PHTX-249Pa and PHTXM-97Pa for pancreatic PDXs. The clinically used combination partners for each cancer type were also selected: CPT-11 and 5-fluorouracil (5-FU) for esophageal cancer and CPT-11, gemcitabine, and IR for pancreatic cancer (Fig. 7, C to F, and figs. S11 to S13).

In combination with CPT-11 in the esophageal PDX PHTXM-90Es model (first top panel from the left, Fig. 7C), the antitumor activities (% T/C) on day 21 were 18.9, 33.8, and $\sim$9.1% in the single-agent TAK-931, single-agent CPT-11, and combination treatments, respectively (Fig. 7C and fig. S11). BWL was not significantly different from that of the vehicle-treated control mice: BWL on day 21 was 7.8% with vehicle, 1.8% with TAK-931, 1.8% with CPT-11, and $\sim$3.8% with the combination. Days to reach 1000 mm$^3$ of tumor volume were 13.6 with vehicle, 42.6 with TAK-931, 25.5 with CPT-11, and >56 with the combination. The preclinical Kaplan-Meier survival analysis was performed using an endpoint tumor volume of 600 mm$^3$ as a surrogate for mortality (bottom panels, Fig. 7C). Survival analysis for PHTXM-90Es showed that the combination provided survival benefit compared with the vehicle, single-agent TAK-931, or single-agent CPT-11 (first bottom panel from the left, Fig. 7C). In the other esophageal and pancreatic PDX models (PHTXM-79Es, PHTX-249Pa, and PHTXM-97Pa), the combination treatment with CPT-11 also exhibited significantly enhanced antitumor activity, providing survival benefit compared with each single-agent treatment (bottom panels, Fig. 7C).

In the combination with a nucleoside analog, gemcitabine, both the pancreatic PDX models—PHTX-249Pa and PHTXM-97Pa—exhibited significantly enhanced antitumor activities: % T/C on day 21 in TAK-931, gemcitabine, and combination treatments were 18.9, 0.8, and $\sim$9.0% in PHTX-249Pa and 2.9, 29.6, and $\sim$6.8% in PHTXM-97Pa, respectively (top panels, Fig. 7D and figs. S12 and S13). Days to reach 1000 mm$^3$ of tumor volume were 18.3 in vehicle, 56 in TAK-931, 58.9 in gemcitabine, and >60 in the combination in PHTX-249Pa and 19.1 in vehicle, 45 in TAK-931, 31.9 in gemcitabine, and 58.7 in the combination in PHTXM-97Pa. Survival analysis also showed that the combination with gemcitabine significantly provided survival benefit in both PDX models (bottom panels, Fig. 7D). Accordingly, antitumor activity was also enhanced in the combination with 5-FU in the esophageal PDX PHTXM-79Es model (Fig. 7E and figs. S12 and S13) and in the combination with IR in the pancreatic PDX PHTX-247Pa model (Fig. 7F and figs. S12 and S13). These findings suggest that chemotherapeutic DNA-damaging drugs could be potential candidates for combination with TAK-931 to expand its activity in these experimentally predicted cancer types.

**DISCUSSION**

In an unbiased chemical screening study, we identified DNA-damaging chemotherapies as potential TAK-931 combination partners. Functional phosphoproteomic analyses revealed that TAK-931...
Select 39 down-regulator genes with higher suppressive activity (termed "experimental resistant markers")

Extract expression data in 10,071 cases from TCGA database

Evaluate expression values of 39 experimental resistant markers in each 10,071 cases

Call "resistant marker positive" for the case exhibited low expression of one or more of the 39 markers (cutoff: ≤1/5 of the median value)

Sort cancer types in order of % "resistant marker–positive cancer"

**Fig. 7.** The in vivo antitumor activity in combination with TAK-931 and the clinically used chemotherapies in the experimentally predicted target cancer PDXs.

(A) Bioinformatic schemes for the target cancer prediction. (B) Summary of the experimentally predicted target cancer types. Red and blue bars indicate the resistant marker–positive and other cancers, respectively. The predicted sensitive, intermediate, and resistant cancer types are described in blue, black, and red, respectively.

(C) Efficacy studies in combination with TAK-931 and CPT-11. Two esophageal PDXs and two pancreatic PDXs were used. Efficacy data are plotted as the mean tumor volume (cubic millimeters ± SEM; n = 8) in vehicle control (black), TAK-931 (blue), CPT-11 (green), and the combination (red). Black bars indicate the treatment period. Preclinical Kaplan-Meier survival curves at 600 mm³ of the endpoint tumor size are also shown (bottom).

(D) Efficacy studies in combination with TAK-931 and gemcitabine. Two pancreatic PDXs were used. Efficacy data are plotted as the mean tumor volume (n = 8) in control (black), TAK-931 (blue), gemcitabine (green), and the combination (red).

(E) Efficacy studies in combination with TAK-931 and 5-FU. The esophageal PDX was used. Efficacy data are plotted as the mean tumor volume (n = 8) in control (black), TAK-931 (blue), 5-FU (green), and the combination (red).

(F) Efficacy studies in combination with TAK-931 and IR. The pancreatic PDX was used. Efficacy data are plotted as the mean tumor volume (n = 8) in control (black), TAK-931 (blue), IR (green), and the combination (red).
significantly suppressed HRR activity to enhance the antitumor activity of a PARP inhibitor or other DNA-damaging agents. Furthermore, siRNA library screening identified the sensitivity-modulating molecules for the combination, which may predict likely sensitive cancer indications for TAK-931 combination treatment, such as pancreatic, esophageal, ovarian, and breast cancers. These preclinical findings could help to guide the future clinical development strategies for TAK-931.

Identification of combination therapies for novel cancer drugs with currently used drugs would greatly increase their therapeutic values. Unbiased high-throughput screening, termed double-agent, is a powerful tool to identify effective drug combinations (36). Our double-agent screening revealed that TAK-931 sensitizes DNA-damaging chemotherapies, such as topoisomerase inhibitors and platinum compounds, in multiple cancer cell lines. Considering that vulnerability against these chemicals has been also reported in cdc7-deficient yeast (37), TAK-931 combination effects could be attributed to the on-target effect of CDC7 inhibition. In addition, TAK-931 intensively suppressed HRR activity, sustainably activated the DDR pathway, remarkably delayed recovery from DSBs, and led to significant accumulation of DNA damages in the combination (Figs. 3 and 4). Thus, the most likely explanation for these combination effects is that TAK-931 impairs the DNA repair process through defective HRR (BRCAness), by which the combination could more effectively induce antiproliferation and/or cell death in cancer cells. Although the detailed molecular mechanisms of how CDC7 kinase controls HRR of DSBs are not yet known, our study showed that DDR proteins such as 53BP1 and BRCA1 are recruited to the IR-induced DSB sites even under CDC7-suppressive conditions by TAK-931 (Fig. 4), suggesting that CDC7 kinase may function during the later events of HRR after the recruitment of effectors to the damage site: D-loop formation, double Holliday junction formation, or resolution of DNA joint molecules (JMs). For instance, in yeast, cdc7 kinase has been reported to regulate JM resolution via Mus81 phosphorylation and meiotic crossover recombination by Msh4 phosphorylation (38, 39). Although these evidences in yeast studies imply the involvement of human CDC7 kinase in the later events of HRR, further mechanistic studies are needed to elucidate the CDC7 kinase function in HRR, and the functions in other repair machineries as well, in cancer cells. In contrast, supporting our hypothesis on BRCAness, the combination of TAK-931 with the PARP inhibitor indicated “chemical-induced synthetic lethality” (Fig. 4). Recent preclinical and clinical evidences revealed several drug-resistant mechanisms against PARP inhibitors in BRCA-mutant tumors: one of the major mechanisms associated with secondary mutations of BRCA1 or BRCA2 to compensate for their function (40–43). Given the synthetic lethal effects of TAK-931 beyond the BRCA1/2-mutant breast and ovarian PDXs (Fig. 4J), the combination of TAK-931 and PARP inhibitor might potentially expand the therapeutic window of the PARP inhibitor, overcoming the secondary mutation-mediated resistant mechanisms against BRCA target therapies.

Whole-genome siRNA library screening identified sensitivity-modulating molecules for the combination (Figs. 6 and 7). By evaluating >10,000 cases on the expression score of these molecules in the TCGA database, we also proposed the experimentally predicted target cancer types for the combination, such as pancreatic, esophageal, ovarian, and breast cancers. Furthermore, the efficacy of combination therapy in these cancer types was preclinically confirmed in the corresponding PDX models by using clinically relevant chemotherapeutic drugs: PARP inhibitor in breast and ovarian PDXs, CPT-11 in esophageal PDXs, and gemcitabine in pancreatic PDXs. In refractory cancers caused by undruggable mutations in oncogenes, such as p53 for esophageal cancers and KRAS for pancreatic cancers, cytotoxic chemotherapies are widely used as standard care, which hinders the development of novel molecular target therapies. Combination therapies with TAK-931 may remarkably improve the therapeutic potential of novel cancer drugs against these refractory tumors and should be evaluated further in future clinical trials.

In conclusion, we identified potential combination partners for TAK-931 to guide future clinical combination strategies, the concept of which was experimentally supported by many preclinical studies. We also proposed experimentally predicted target cancer types for the combination based on sensitivity-modulating molecules. These findings indicate the potential of TAK-931 combination strategies in next-generation anticancer therapeutics.

**MATERIALS AND METHODS**

**Compounds**

TAK-931 was synthesized by Takeda Pharmaceutical Company Ltd. (23). Niraparib was purchased from Selleck China (Shanghai, China). Gemcitabine was purchased from Eli Lilly and Company (Indianapolis, IN, USA). CPT-11 (irinotecan hydrochloride injection) was purchased from Pfizer (Perth) Pty Limited (Bentley, WA, Australia). 5-FU (fluorouracil injection; 25 mg/ml) was purchased from Shanghai Xudong Haipu Pharmaceutical Inc. (Shanghai, China).

**Double-agent studies**

The indicated compounds were added to the cell test plates 16 hours after cell plating, and then the viability was assessed using the adenosine triphosphate (ATP)-based assay 72 hours later. An inward curving isobologram indicates Loewe synergy, whereas an outward curving isobologram indicates Loewe antagonism. When a drug is mixed with itself (sham experiment), the isobologram should have straight lines. Thus, linearity indicates Loewe additivity. Horizontal slices were obtained from the response surface at various levels of survival to generate a fitted isobologram.

**Cell growth assay**

Cell growth assays were performed as described previously (44). Cell growth was evaluated by measuring intracellular ATP concentrations by using the CellTiter-Glo luminescent cell viability assay (Promega Corp., Madison, WI, USA). Absorbance and luminescence were measured using a microplate reader. Relative cell growth activities were calculated by comparing with the absorbance or chemiluminescence value of the control cells [means ± SD (n = 3)].

**DSB recovery assay**

HeLa cells were treated with DMSO or TAK-931 at 300 nM for 1 hour and then irradiated at 0 or 4 Gy. The cells were collected after 8 or 48 hours after IR treatment for the immunofluorescence of 53BP1 foci formation, which was quantified as the percentage of the cells with >10 53BP1 foci in each treatment. Statistical analysis was performed using Student’s t test (n = 3). Differences were considered significant at P < 0.05.

**Immunofluorescence assay**

The immunofluorescence assay was performed as described previously (44). HeLa cells were fixed for 15 min with 4% paraformaldehyde in

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phosphate-buffered saline, followed by permeabilization for 5 min with Triton X-100–containing buffer. The anti-53BP1 (sc-515841; Santa Cruz Biotechnology) and anti-BRCA1 (sc-6954; Santa Cruz Biotechnology) antibodies were used at 1 to 2 μg/ml for immunofluorescence assays. Images were captured using an Axiovert 200M microscope (Carl Zeiss) or InCell Analyzer 2000 (GE Healthcare).

**Immunoblotting**

Immunoblotting was performed as described previously (44). The following antibodies were used at 0.1 to 0.5 μg/ml: anti-pMCM2 (3378-1; Epitomics Inc.), anti-MCM2 (sc-9839; Santa Cruz Biotechnology), pChk1 (#2348; Cell Signaling Technology), anti-Chk1 (#2345; Cell Signaling Technology), anti-pCDK1 (#9111; Cell Signaling Technology), anti-CDK1 (sc-954; Santa Cruz Biotechnology), anti-pBRCA1 (#9009; Cell Signaling Technology), anti-pp53 (#9286; Cell Signaling Technology), anti-pATM (2152-1; Epitomics Inc.), anti-p21 (#2947; Cell Signaling Technology), anti-gH2AX (#2577; Cell Signaling Technology), anti-p53 (sc-126; Santa Cruz Biotechnology), anti-PARP1 (#9542; Cell Signaling Technology), anti-BRCA1 (sc-6954; Santa Cruz Biotechnology), anti-CDC7 (sc-56274; Santa Cruz Biotechnology), anti-DBF4 (ab124707; Abcam), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (MAB374; Chemicon). Immunoblotted proteins were visualized using chemiluminescence.

**HRR assay**

HRR assay was performed as described previously (27). In brief, the efficiency of HRR was assessed using an I–Sce I expression plasmid (pCBASce) and I–Sce I repair reporter plasmid (DR-GFP) composed of two differentially mutated GFP genes, one of which contained a unique I–Sce I restriction site. The assay involves gene conversion repair of a DSB caused by I–Sce I digestion. DR-GFP plasmids were repaired by HRR. The 293T cells were transfected with 2 μg of DR-GFP and 8 μg of pCBASce in the presence or absence of TAK-931. At 72 hours after transfection, the cells were harvested, and the number of GFP-expressing cells was assessed using the FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

**Antitumor efficacy studies in PDX models**

The antitumor efficacy studies in PDX models were performed at Shanghai Medicilon Inc. (Shanghai, China). The PDX tumor fragments at 2 to 4 mm in diameter were subcutaneously xenografted into BALB/c nude mice. The PDX-inoculated mice were selected and randomly categorized into the study groups (eight mice per group) when the mean tumor size reached approximately 180 to 250 mm³. Kaplan-Meier survival analysis was performed using an endpoint tumor volume of 600 mm³ as a surrogate for mortality. Log-rank test was used for comparison of survival between the combination and other treatment groups.

The protocol and any amendments or procedures involving the care and use of animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Medicilon Inc. before study initiation. The care and use of animals were conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (experimental protocol number: 00004407).

**Statistical analysis**

Parametric statistical analysis by Student’s t test was used for the comparison, if both normal distribution and variance equality of the sample data were verified by Shapiro-Wilk normality and Gaussian distribution tests, respectively. If either normal distribution or variance equality was not determined, then nonparametric statistical analysis by Wilcoxon-Mann-Whitney test was used. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) or R version 3.3.0, and differences were considered significant at P < 0.05.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/21/eabf0197/DC1

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Competing interests: K.I., T.N., J.Y., K.E., K.M., K.Kag., M.G., T.T., A.K.J., S.S., S.M., O.K., H.N., K.Kan., and A.O. are/were employees of Takeda Pharmaceutical Company Ltd. A.O. reported paid consulting or advisory roles for Ono Pharmaceutical Company Ltd. and Craif Inc. out of this study. K.I., T.N., H.N., and A.O. are inventors on a patent related to this work (WO2019/245061A1). K.I., T.N., and A.O. are inventors on a patent related to this work (WO2018/158988A1). O.K. is an inventor on a patent related to this work (WO2011/103299). The authors declare that they have no other competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. Data and materials will be provided by Takeda pending scientific review and a completed material transfer agreement. Request for the data and materials should be submitted to A.O. as point of contact.

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