Preclinical Development

TAK-960, a Novel, Orally Available, Selective Inhibitor of Polo-Like Kinase 1, Shows Broad-spectrum Preclinical Antitumor Activity in Multiple Dosing Regimens

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Abstract

Polo-like kinase 1 (PLK1) is a serine/threonine protein kinase involved in key processes during mitosis. Human PLK1 has been shown to be overexpressed in various human cancers, and elevated levels of PLK1 have been associated with poor prognosis, making it an attractive target for anticancer therapy. TAK-960 [4-[(9-cyclopentyl-7,7-difluoro-5-methyl-6-oxo-6,7,8,9-tetrahydro-5H-pyrimido[4,5-b][1,4]diazepin-2-yl)amino]-2-fluoro-5-methoxy-N-(1-methylpiperidin-4-yl) benzamide] is a novel, investigational, orally bioavailable, potent, and selective PLK1 inhibitor that has shown activity in several tumor cell lines, including those that express multidrug-resistant protein 1 (MDR1). Consistent with PLK1 inhibition, TAK-960 treatment caused accumulation of G2–M cells, aberrant polo mitosis morphology, and increased phosphorylation of histone H3 (pHH3) in vitro and in vivo. TAK-960 inhibited proliferation of multiple cancer cell lines, with mean EC50 values ranging from 8.4 to 46.9 nmol/L, but not in nondividing normal cells (EC50 > 1,000 nmol/L). The mutation status of TP53 or KRAS and MDR1 expression did not correlate with the potency of TAK-960 in the cell lines tested. In animal models, oral administration of TAK-960 increased pHH3 in a dose-dependent manner and significantly inhibited the growth of HT-29 colorectal cancer xenografts. Treatment with once daily TAK-960 exhibited significant efficacy against multiple tumor xenografts, including an adriamycin/paclitaxel-resistant xenograft model and a disseminated leukemia model. TAK-960 has entered clinical evaluation in patients with advanced cancers.

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Introduction

The polo-like kinases (PLK) are evolutionarily conserved serine/threonine kinases that were first described in the so-called polo mutants of Drosophila melanogaster that failed to undergo normal mitotic progression (1). Since then, many important functions of PLK1 have been discovered, such as activation of cell division cycle 2, chromosome segregation, centrosome maturation, bipolar spindle formation, regulation of the anaphase-promoting complex, and execution of cytokinesis (2–4). In addition to these general cellular functions, human PLK1 is overexpressed in various human cancers, including head and neck squamous cell carcinoma, non-small cell lung cancer, oropharyngeal carcinoma, melanoma, ovarian and endometrial carcinoma, and prostate cancer; elevated PLK1 has been associated with poor prognosis (2, 5, 6). Several reports have shown that PLK1 depletion by short interfering RNA or PLK1 inhibition by small-molecule inhibitors resulted in cell-cycle arrest at mitosis and induced apoptosis in cancer cell lines, but not in normal diploid cells or nondividing cells (7–10). Currently PLK1 inhibitors are being evaluated in registered clinical trials (4). To date, clinical responses have been reported with intravenous volasertib (11). Antimitotic agents that target tubulin, such as taxanes, have been clinically validated; however, these drugs have several drawbacks including development of peripheral neuropathy (12), the need for pretreatment to reduce anaphylaxis (13), and the potential for tumors to become refractory or resistant due to multidrug resistance (6). Consequently, there is a need for novel antimitotic drugs that target nonmicrotubule proteins such as the mitotic kinases (10). Here we report the preclinical evaluation of TAK-960 [4-[(9-cyclopentyl-7,7-difluoro-5-methyl-6-oxo-6,7,8,9-tetrahydro-5H-pyrimido[4,5-b][1,4]diazepin-2-yl)amino]-2-fluoro-5-methoxy-N-(1-methylpiperidin-4-yl) benzamide].
amino]-2-fluoro-5-methoxy-N-(1-methylpiperidin-4-yl) benzamide], a potent and selective PLK1 inhibitor that has oral bioavailability and shows single-agent antitumor activity in a variety of tumor cell lines and xenograft models of solid tumors and hematologic malignancies.

Materials and Methods

Compounds

TAK-960 was synthesized at Takeda California, Inc., San Diego, CA. Paclitaxel was purchased from Bristol-Myers Squibb. AraC was purchased from Sigma Aldrich.

Cell culture

Human tumor cell lines were obtained from the American Type Culture Collection with the exception of A2780 (ovarian cancer), SW620 (colorectal cancer), and MRC5 (normal lung fibroblast; all 3 from Dainippon Sumitomo Pharma Co., Ltd.); KARPAS299 (lymphoma; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). K562ADR was generated at Takeda Pharmaceutical Company Ltd., (TPC) by exposing the K562 parent cell line to doxorubicin at 10 nmol/L for 8 days, 15 nmol/L for 11 days, 20 nmol/L for 4 days, 30 nmol/L for 9 days, and 50 nmol/L for 10 days. MV4-11uc cells were established at TPC by transfecting a firefly luciferase expression vector (Promega Corp.) into MV4-11 cells. The tissue culture medium recommended by suppliers was used for cultivation of each cell line. Commercially obtained cells were not authenticated by the authors.

Biochemical kinase inhibition assays

The inhibitory activity of TAK-960 was assessed by the TR-FRET (fluorescence resonance energy transfer) assay, which measured the ATP-dependent phosphorylation of a biotinylated substrate peptide corresponding to residues 2,470 through 2,488 of the mTOR protein (Biotin-AGAGTVPSIIHSFIGDGLV). A total of 288 kinases were screened for TAK-960 inhibition (1 μmol/L) using HotSpot technology (Reaction Biology Corporation) and IC50 values for the selected kinases were determined.

Cell-cycle DNA analysis

HT-29 colorectal cancer cells were seeded in 12-well tissue culture plates, cultured overnight at 37°C, and incubated for 48 hours with dilutions of TAK-960. Cells were harvested by trypsin treatment and fixed in 70% ethanol at 4°C. After treatment with RNAse, cells were stained with propidium iodide and DNA content was measured using a FACSCalibur System (BD Biosciences).

Immunocytochemistry

HT-29 cells were treated with serial dilutions of TAK-960 for 24 hours and fixed with 4% paraformaldehyde for 10 minutes, blocked in StartingBlock TBS-T (Thermo Scientific) for 30 minutes, followed by incubation with antibodies against α-tubulin and γ-tubulin (Abcam) for 2 hours. Alexa fluorophore–conjugated antibodies (Invitrogen Corporation) were used as secondary antibodies for detection, and 4',6-diamidino-2-phenylindole (DAPI) was used for nuclei detection.

Analysis of cell proliferation

Cells were seeded into 96-well plates at 3,000 to 30,000 cells per well in appropriate medium plus 10% fetal calf serum. After 24 hours, cells were treated with serial dilutions of TAK-960, and 72 hours later, the number of viable cells was assessed using the CellTiter-Glo Assay (Promega). Calculation of EC50 values and statistical analysis were done using GraphPad Prism software (Version 5.01; GraphPad Software).

Animal model studies

Mice were housed and maintained within the facility at TPC in accordance with the Takeda Experimental Animal Care and Use Committee approved protocol. Athymic nude mice (BALB/cAJcl-nu/nu) or severe combined immunodeficiency (SCID) mice (C.B17-Scid/Scid) of approximately 5 weeks of age were obtained from CLEA Japan, Inc. Nonobese diabetic (NOD)-SCID mice (NOD.CB17-Prkdcscid/j) were obtained from Charles River Laboratories Japan, Inc. For subcutaneous-implanted tumor xenograft models, nude mice or SCID mice (for K562ADR leukemia xenografts) were injected with 5 × 106 cells per mouse. When tumor volumes reached approximately 200 mm3, mice were randomly assigned to treatment groups. Tumor volumes and body weights were measured approximately twice weekly throughout the study. Tumor volume was calculated using calipers and the equation (L × W2)/2, where L and W refer to the larger and smaller dimensions collected at each measurement, respectively. Complete regression (CR) was defined as tumors reduced to 13.5 mm3 or less and partial regression (PR) was defined as tumors reduced by more than 50% of their initial size but that were more than 13.5 mm3. For the disseminated leukemia study, NOD/SCID mice were pretreated intraperitoneally with TM-B1, an anti-CD122 antibody (0.05 mg/mouse; BD Biosciences), to deplete natural killer cells. The next day, mice were sublethally irradiated with 3 Gray of X-ray, after which human AML cells (MV4-11uc) were injected via tail vein. In vivo optical images were obtained with an IVIS 200 optical imaging system (Caliper Life Sciences) twice weekly. One hundred fifty mg/kg of α-luciferin (Promega) dissolved in PBS were administered to animals by intraperitoneal injection 10 minutes before each image acquisition. Significant luminescence was detected around hind limb bone joints on day 22 after implantation and vehicle, AraC, or TAK-960 was administered. AraC and paclitaxel were diluted in saline for intraperitoneal dosing and TAK-960 was suspended in 0.5% methyl cellulose for oral dosing.
pHH3 analysis
The PathScan Phospho-Histone H3 (serine 10; pHH3) sandwich ELISA was carried out as per the manufacturer’s instructions (Cell Signaling Technology) using in vitro cell lysates or in vivo tumor tissue lysates. The increase in pHH3 was shown as the fold increase in pHH3 normalized to the value in a vehicle control. Tissue samples from tumor xenografts were fixed in 10% formalin before paraffin embedding. Sections were rehydrated and incubated with anti-pHH3 antibody. Peroxidase-conjugated antibody was used as a second antibody and detected by diaminobenzidine staining. Two representative pictures were taken from each section, and pHH3-positive cells were counted using an AxioVision system (Carl Zeiss).

Pharmacokinetic/pharmacodynamic studies
Female athymic nude mice (Hsd:Athymic Nude-Foxn1tnnu) from Harlan Labs, Inc. were housed and maintained within the Comparative Medicine department at the Sidney Kimmel Cancer Center (SKCC, San Diego, CA) in accordance with SKCC Institutional Animal Care and Use Committee, and state and federal guidelines for the humane treatment and care of laboratory mice. Tumors were collected at multiple time points after oral administration of TAK-960 and stored on dry ice or at −80°C. Homogenization was done using Beadbeater (Biospec Products, Inc.) with sample buffer containing 2% SDS. TAK-960 concentrations were determined by extraction from mouse plasma and tumor homogenates by protein precipitation, followed by liquid chromatography–tandem mass spectrometry. pHH3 was determined as a pharmacodynamic (PD) marker using the tumor lysates by the pHH3 ELISA as described above.

Results
Kinase inhibition and selectivity of TAK-960
TAK-960 was designed and synthesized as a novel PLK1 inhibitor that binds to the ATP-binding pocket of PLK1 (Fig. 1A). Data reported herein using the TR-FRET assay confirmed that TAK-960 is a potent PLK1 inhibitor with a mean IC50 of 1.5 nmol/L against the kinase domain of PLK1 at 3 μmol/L ATP (Fig. 1B). The ATP concentration in living cells is much higher, in the millimolar range, which significantly reduces the inhibitory activity of pure ATP-competitive inhibitors. In the presence of a high ATP concentration (1,000 μmol/L), the inhibitory activity of TAK-960 was reduced less than 5-fold (IC50 = 6.5 nmol/L; Fig. 1B).

To determine the specificity of TAK-960 across the kinome, a panel of 288 kinases was screened by the HotSpot technology. Of the kinases tested, 243 (84%) were inhibited by less than 20% and 6 (PLK1, PLK2, PLK3, FAK, MLCK, and FES) were inhibited by more than 80% at 1,000 nmol/L TAK-960 (Fig. 1C); PLK1 showed the greatest inhibition (101.6%). To further examine the selectivity of TAK-960 for inhibition of PLK1, IC50 values for TAK-960 inhibition of the 6 kinases that were inhibited by more than 80% were determined in the presence of 10 μmol/L ATP (Table 1). The IC50 value for inhibition of full-length PLK1 protein by TAK-960 was 0.8 nmol/L in this assay, which was more than 20-fold lower than the next lowest IC50 value (PLK2: 16.9 nmol/L).
Mitotic arrest has been implicated as an outcome of PLK1 inhibition (14). Flow cytometry analysis showed that treatment of HT-29 colorectal cancer cells with TAK-960 resulted in concentration-dependent accumulation of cells in G2–M phase (Fig. 2A). A correlation between the accumulation of G2–M-phase cells and the increase in pHH3 was observed. In addition, the pattern of increase of pHH3 at 24 hours after TAK-960 treatment clearly indicated a decrease in viability at 72 hours after TAK-960 treatment (Fig. 2B). EC₅₀ values for the increase of pHH3 and the loss of viability were 9.8 and 8.4 nmol/L, respectively, similar to the IC₅₀ value of 6.5 nmol/L for PLK1 kinase enzyme inhibition at 1,000 μmol/L ATP.

The effects of TAK-960 on chromosome dynamics were examined by immunofluorescent microscopy. The far-left panel of Fig. 2C shows a representative control dimethyl sulfoxide (DMSO)-treated HT-29 cell in G₂–M phase. In contrast, accumulation of aberrant spindles was observed following treatment with TAK-960 at 10 or 30 nmol/L. At 100 nmol/L or more concentration, the appearance and distribution of phenotypes consisted of monopolar spindles with characteristic polo spindle morphology (15).

**Effect of TAK-960 on proliferation in human tumor cell lines, including those with TP53 or KRAS mutations or MDR1 overexpression**

TAK-960 was tested for its ability to inhibit the proliferation of selected human cancer cells. The mean EC₅₀ values of proliferation inhibition in the 18 cell lines tested are shown in Fig. 3A, along with the mutation status of PLK1.

### TAK-960 mechanism of action

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### Table 1. Enzyme selectivity profile of TAK-960 on selected kinases

<table>
<thead>
<tr>
<th>Kinase</th>
<th>% inhibition at 1,000 nmol/L</th>
<th>IC₅₀ (nmol/L)</th>
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<td>PLK1</td>
<td>101.6</td>
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<tr>
<td>PLK2</td>
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<td>16.9</td>
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<td>FES/FPS</td>
<td>81.5</td>
<td>58.2</td>
</tr>
<tr>
<td>282 kinases</td>
<td>&lt;80</td>
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Figure 2. TAK-960 induces accumulation of aberrant mitotic cells in HT-29 cells. A, DNA profiles of asynchronously cultured HT-29 cells treated with a variety of concentrations indicated in the figure for 48 hours were evaluated by flow cytometry. B, accumulation of pHH3 in HT-29 cells was determined by pHH3 ELISA at 24 hours (●). Growth inhibition at 72 hours (○) was measured by CellTiter-Glo as described in Materials and Methods. Data are shown as mean ± SD of 3 samples. C, HT-29 cells were treated for 24 hours with different concentrations of TAK-960 and analyzed by immunofluorescence for α-tubulin (green), γ-tubulin (red), and chromatin (blue).
TP53 and KRAS. TAK-960 inhibited the proliferation of these human cancer cell lines in a concentration-dependent manner, with mean EC_{50} values ranging from 8.4 to 46.9 nmol/L (Fig. 3A). Recent studies have suggested a correlation between sensitivity to PLK1 inhibition and TP53 or KRAS mutation status (9, 16, 17). However, no statistical differences were observed when EC_{50} values were compared between cell lines with wild-type alleles and those with mutated alleles of TP53 or KRAS (P > 0.05; 2-tailed Mann–Whitney test; Supplementary Fig. S1). Because many chemotherapeutic agents, such as paclitaxel and doxorubicin, are susceptible to the activity of multidrug-resistant protein 1 (MDR1), the effect of MDR1 expression on the potency of TAK-960 was evaluated. Western blot analysis was carried out on selected cell lines to confirm expression levels of MDR1 (Fig. 3C). Although expression levels of MDR1 were high in HCT-15 and COLO320DM colorectal cancer cells, EC_{50} values were similar to those in cell lines that did not express MDR1 (Fig. 3A). The lack of effect of MDR1 expression was further shown with the doxorubicin-resistant K562ADR leukemia cell line. Interestingly, although this cell line was established as a doxorubicin-resistant cells, it showed resistance not only to doxorubicin but also to paclitaxel when compared with its parent cell line K562 (Fig. 3D). Indeed, Western blot data showed that K562ADR expressed MDR1 at a level similar to that of K562ADR. 

Figure 3. TAK-960 inhibits proliferation of human cancer cell lines regardless of TP53 and KRAS mutation and MDR1 expression status. A, B, and D, CellTiter-Glo assay was carried out on a panel of human cell lines treated with TAK-960 (concentration range = 2-1,000 nmol/L) for 72 hours. A, the list of EC_{50} in cell lines with various TP53 and KRAS status, M, mutant, WT, wild-type, NA, not available. Shading indicates tumor origin. B, representative concentration–response curve of MRC5. MRC5 human normal lung fibroblasts were seeded at 3 \times 10^4 (proliferating, *) and 3 \times 10^5 (quiescent, *) cells per well in 100 μL of media) at 24 hours before TAK-960 treatment. Data represent the mean of 3 samples. C, MDR1 expression in selected cancer cell lines was determined by Western blotting. D, concentration–response curve of K562 leukemia cells (□) and K562ADR (■) for doxorubicin, paclitaxel, and TAK-960. Data are shown as mean ± SD of 3 samples.
of HCT-15 and COLO320DM (Fig. 3C). TAK-960 inhibited proliferation of K562ADR cells with an EC50 value similar to that of the parent cell line, K562 (Fig. 3A and D).

Pharmacokinetic/pharmacodynamic and antitumor activity relationships in xenograft models

To confirm that TAK-960 inhibited PLK1 activity in vivo, mice bearing established HT-29 colorectal tumors were administered a single oral dose (5, 10, or 30 mg/kg, Fig. 4A) of TAK-960. Plasma and tumor concentrations of TAK-960 were measured at various time points using a high-performance liquid chromatography/tandem mass spectrometry assay. Plasma exposure to TAK-960 increased in an approximately dose-proportional manner and was partitioned preferentially into the tumor (Fig. 4A, ●) compared with the circulating levels in plasma (Fig. 4A, ○). As shown previously, increased pH3 is a marker of PLK1 inhibition/mitotic arrest. Levels of pH3 were measured by ELISA in HT-29 colorectal tumor samples. The maximum increase in tumor pH3 over baseline (Fig. 4A, ■) was observed at 24 hours post dose. Thus, the peak in pHH3 levels occurred after the tumor concentration of TAK-960 had peaked, which occurred between 4 to 8 hours post dose. This result is consistent with the fact that increases in pH3 are a consequence of mitotic arrest induced by PLK1 inhibition by TAK-960 treatment. Exposure to TAK-960 (AUC) in plasma and tumor was linearly correlated with dose. In addition, the peak of pH3 response (Emax) at 10 and 30 mg/kg was similar, whereas total PD response (AUE) over the time was relatively dose dependent (Supplementary Table S1).

To further evaluate relationship between PD response and antitumor activity, TAK-960 was evaluated in mice bearing established HT-29 colorectal tumor xenografts using various regimens (Fig. 4B and C). Doses of 6.25 or 10 mg/kg were administered orally once daily for 21 days (●, QD×21) or once daily for 3 days per week for 3 weeks (▲, QD×3/w×3w) or twice weekly 2 days apart for 3 weeks (▲, Q2D×2/w×3w) at the dosage indicated in figure. Arrows in the figure indicate the days of administration for each schedule. No regrowth was observed in 3 of 5 tumors in 10 mg/kg administered groups on once daily for 21 days schedule until the measurement on day 86. Data represent the mean ± SD of 5 samples.
once daily for 3 consecutive days per week for 3 weeks (QD×3/w×3w). All TAK-960 treatment schedules inhibited tumor growth in a dose-dependent manner (Fig. 4B) without obvious body weight loss (Fig. 4C); however, the degree of inhibition was schedule dependent. Tumor regression was greater in the continuous dosing group (10 mg/kg, QD×3; CR: 3 of 5 mice at day 86, Fig. 4B). In the intermittent dose group, 1 CR and 1 PR were observed at the 20 mg/kg TAK-960 dose (QD×3/w×3w, Fig. 4B). An additional xenograft study was carried out to evaluate another intermittent dosing schedule (Q2D×2/w×3w), in which the total amount of TAK-960 administrated was similar to that achieved with daily dosing; however, in all intermittent schedules, tumor recurrence was observed by 48 days after the first dosing day (Supplementary Fig. S2).

**TAK-960 antitumor activity in various models of human cancer**

Nine additional tumor xenograft models, HCT116 (colorectal cancer), PC-3 (prostate cancer), BT474 (breast cancer), A549 (lung cancer), NCI-H1299 (lung cancer), NCI-H1975 (lung cancer), A2780 (ovary cancer), and MV4-11 (myeloid leukemia) were tested using the optimal dose of 10 mg/kg once daily for 2 weeks selected on the basis of experiments with HT-29 colorectal cancer xenografts. In each of these models (Fig. 5A–H), substantial antitumor activity and good tolerability was observed. TAK-960, administered orally once daily for 9 consecutive days (QD×9), was effective in inhibiting tumor growth in all models tested.

Figure 5. Single-agent efficacy of TAK-960 in various tumor xenograft models using human cancer cell lines. A, HCT116 colorectal cancer; B, PC-3 prostate cancer; C, BT474 breast cancer; D, A549 lung cancer; E, NCI-H1299 lung cancer; F, NCI-H1975 lung cancer; G, A2780 ovary cancer; and H, MV4-11 leukemia cells were inoculated subcutaneously in nude mice or SCID mice. Vehicle (○, 0.5% MC) or TAK-960 (●, 10 mg/kg) administered orally once daily for 14 days. Data are shown as mean ± SD of 5 samples. I, survival of mice inoculated with MV4-11Luc was assessed following treatment with vehicle, AraC, and TAK-960. AraC 5 mg/kg was administered intraperitoneally once daily for 5 days per week for 2 weeks (QD×5/w×2w). TAK-960 (7.5 mg/kg) was administered orally once daily for 9 consecutive days (QD×9).
observed (no treatment-related deaths occurred in this regimen).

On the basis of the significant tumor growth inhibition observed in the subcutaneously implanted MV4-11 human leukemia model (Fig. 5H), TAK-960 efficacy was also explored in a model of disseminated leukemia using MV4-11Luc cells, which may more closely reflect the pathogenesis of the human disease. In this model, treatment with TAK-960 at 7.5 mg/kg orally once daily for 9 days showed a significant increase in median survival compared with vehicle (39.5 vs. 25 days, respectively; \( P = 0.0055 \), Log-rank (Mantel–Cox) test; Fig. 5I). Disseminated tumor growth was also evaluated by in vivo whole body fluorescence imaging 14 days after the first day of dosing; inhibition of tumor growth was observed in animals treated with TAK-960 but not in animals treated with AraC (Supplementary Fig. S3A). Animals treated with vehicle or AraC showed increasing total body flux (i.e., tumor growth) during 2 weeks of treatment; flux was lower in animals treated with TAK-960 and reached statistical significance on day 14 (\( P = 0.0017 \); Supplementary Fig. S3B).

K562ADR was established as an adriamycin-resistant leukemia cell line and also showed resistance to paclitaxel (Fig. 3D). Paclitaxel induces G2–M arrest and increases pH3, as observed with PLK1 inhibition. Therefore, we compared changes in PD markers between TAK-960 and paclitaxel in K562 or K562ADR cells that were inoculated subcutaneously in SCID mice. After a single oral administration of TAK-960 or intraperitoneally administered paclitaxel, pH3-positive cells in tumor tissues were detected and counted immunohistochemically. In K562 tumors, the maximum increase in pH3 was observed at 24 hours post dose at 30 mg/kg TAK-960, consistent with HT-29 colorectal cancer cells (Fig. 6A) and 20 mg/kg of intraperitoneal paclitaxel. TAK-960 also showed a similar pattern of pH3 response in K562ADR tumors, but paclitaxel did not induce pH3 in MDR1-expressing tumors (Fig. 6A–C). Indeed, TAK-960 at 10 mg/kg (once daily for 6 days per week for 2 weeks, orally) inhibited tumor growth in the MDR1-expressing K562ADR-bearing leukemia xenograft model, whereas paclitaxel at 10 mg/kg

TAK-960 in vivo efficacy in MDR1-expressing tumors

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Figure 6. TAK-960 shows PD activity in paclitaxel-resistant model.
A, pH3, as a PD marker for TAK-960 and paclitaxel in mice bearing K562 or K562ADR leukemia cells, was analyzed by immunohistochemistry. TAK-960 30 mg/kg was administered orally and 20 mg/kg of paclitaxel was administered intraperitoneally. The time course of the PD response was assessed up to 48 hours after the single dose of TAK-960 (B) or paclitaxel (C) in mice bearing K562 (○ and K562ADR (●) tumors. Data are shown as the mean of 3 samples. D, the single-agent efficacy study was carried out using the K562ADR-bearing xenograft model. Vehicle (○, 0.5% methyl cellulose) or TAK-960 (●, 10 mg/kg) was administered orally, once daily for 6 days per week for 2 weeks (OD×6/w×2w). Paclitaxel (○, 10 mg/kg) was administered intraperitoneally once daily for 5 days per week for 2 weeks (ID×5/w×2w). Data are shown as the mean ± SD of 3 samples.
found that mutation of Arg136 to Gly in the PLK1 ATP-binding pocket could alter the sensitivity to PLK1 inhibitors in a compound-specific manner. Further efforts will be required to define potential patient stratification factors to help identify the tumors most vulnerable to TAK-960 and to determine whether combinatorial approaches may lead to durable tumor regression.

Our results showed that TAK-960 inhibited intracellular PLK1 and led to dysregulation of the cell cycle. The mechanism of action of TAK-960 was indicated in HT-29 colorectal cancer cells, in which concentrations that inhibited growth at 72 hours were able to induce pH3 at 24 hours and cause aberrant mitosis in a dose-dependent manner, suggesting that pH3 would be a predictive marker of antitumor activity. Consistent with in vitro observations, TAK-960 treatment in mice bearing HT-29 colorectal cancer or K562ADR leukemia xenografts induced pH3 in tumors and showed significant antitumor efficacy in vivo. Recently, a number of PLK1 substrates have been reported (25, 26). Although the availability of an appropriate antibody against PLK1 substrates for immunohistochemistry or ELISA is still limited, the reliability of PLK1 substrates as potential predictive or diagnostic biomarkers should be compared with pH3 in future studies.

Early preclinical studies of taxanes showed poor correlations between efficacy and peak mitotic index (27, 28). Although there seemed to be a poor correlation between doses and peak pH3 levels ($E_{\text{max}}$), we found a good correlation between doses of TAK-960 and total PD responses over the time for pH3 (AUE) in the HT-29 xenograft model. Given that the peak level of PD response was the same for 10 and 30 mg/kg of TAK-960 at 24 hours post dose (Supplementary Table S1), the $E_{\text{max}}$ PD response could be saturated at 10 mg/kg. Considering the relationship between pharmacokinetic (PK), PD, and TAK-960 dose, it is likely that consecutive daily 10 mg/kg administration would result in an effective PD response without toxic drug accumulation. QD dosing at 10 mg/kg showed superior antitumor activity compared with intermittent dosing in the HT-29 colorectal cancer xenograft model without obvious body weight loss. Using the QD schedule, TAK-960 exhibited significant antitumor activity as a single agent in several solid tumor xenograft and hematologic malignancy models, including MDR1-expressing tumors. In the disseminated leukemia model using MV4-11uc cells, QD treatment with TAK-960 significantly extended survival, whereas treatment with AraC did not. In K562ADR-bearing tumors, which overexpress MDR1, the lack of PD response or efficacy was likely due to drug efflux. As mentioned above, the ability to exit from mitosis may be another influencing factor for drug sensitivity. However, such resistance could also be observed by a decrease in pH3. Taken together, PD response for pH3, especially AUE, could be a valuable biomarker not only for prognosis but also for determining a suitable dose and treatment schedule.
Through PK/PD and efficacy studies, we established the optimal dosing regimen for TAK-960 in the mouse xenograft model; however, an optimal human dose and schedule will need to be determined in clinical studies. In summary, TAK-960 has high oral bioavailability, thus providing the potential for convenient, controlled, and flexible dosing schedules compared with intravenously administered drugs. TAK-960 is also a potent, selective PLK1 inhibitor with activity in various tumor cell lines in vitro and in vivo, including cells that overexpress MDR1. TAK-960 is currently undergoing phase I evaluation in adult patients with advanced cancers.

Disclosure of Potential Conflicts of Interest
All authors are employees of Takeda Pharmaceutical Company, Ltd., or California, Inc., or Millennium Pharmaceuticals, Inc.

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