Small Molecule Therapeutics

Activity of a Novel Hec1-Targeted Anticancer Compound against Breast Cancer Cell Lines In Vitro and In Vivo

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Abstract

Current cytotoxic chemotherapy produces clinical benefit in patients with breast cancer but the survival impact is modest. To explore novel cytotoxic agents for the treatment of advanced disease, we have characterized a new and pharmacokinetically improved Hec1-targeted compound, TAI-95. Nine of 11 breast cancer cell lines tested were sensitive to nanomolar levels of TAI-95 (GI₅₀ = 14.29–73.65 nmol/L), and more importantly, TAI-95 was active on a number of cell lines that were resistant (GI₅₀ > 10 μmol/L) to other established cytotoxic agents. TAI-95 demonstrates strong inhibition of in vivo tumor growth of breast cancer model when administered orally, without inducing weight loss or other obvious toxicity. Mechanistically, TAI-95 acts by disrupting the interaction between Hec1 and Nek2, leading to apoptotic cell death in breast cancer cells. Furthermore, TAI-95 is active on multidrug-resistant (MDR) cell lines and led to downregulation of the expression of P-glycoprotein (Pgp), an MDR gene. In addition, TAI-95 increased the potency of cytotoxic Pgp substrates, including doxorubicin and topotecan. Certain clinical subtypes of breast cancer more likely to respond to Hec1-targeted therapy were identified and these subtypes are the ones associated with poor prognosis. This study highlights the potential of the novel anticancer compound TAI-95 in difficult-to-treat breast cancers. Mol Cancer Ther; 13(6); 1–12. ©2014 AACR.

Introduction

Despite advances in treatment, 40,000 of the 200,000 newly diagnosed patients with breast cancer die each year (1). Approximately 15% of globally diagnosed breast cancers are triple-negative breast cancer (TNBC), which lack the presence of targets of hormone-based therapies [estrogen receptor (ER)−, progesterone receptor (PR)−, and Her2/neu] and are aggressive, highly metastatic cancers carrying poor prognoses (2). For these hormone receptor-negative patients, cytotoxic chemotherapy is the first-line treatment and yields clinical benefit (response and disease stabilization) in 60% to 80% of patients, but survival benefit is modest (3). The proportion of chemoresistant disease represents more than 50% of TNBC (4). In addition to intrinsic resistance, many resistant tumor cells in human acquire resistance during chemotherapy.

Sequential pulses of chemotherapeutic drugs, including docetaxel and cisplatin, lead to the development of resistance to these drugs and in some cases even cross-resistance to other drugs (5). In in vitro experiments, prolonged exposure to cytotoxic drugs also leads to chemoresistance in breast cancer cells (6). Multidrug-resistant (MDR) gene expression levels, including those of ABCB1 (Pgp), ABCC1 (MRP1), and ABCG2 (BCRP), were found to be elevated in these cells with acquired resistance (5, 6).

Because of the heterogeneous nature of breast cancer, microarray platforms have been developed to help classify patients into manageable treatment types (7). The 70-gene prognosis profile showed high correlation with prognosis prediction (8). Such personalized medicine approach help improve clinical outcomes by providing suitable drug in a timely manner, minimizing the possibility of acquiring resistance.

NDC80/Hec1 is a key component of the mitotic kinetochore complex and an attractive molecular target for cancer (9). As a conserved mitotic regulator, Hec1 is critical for spindle checkpoint control, kinetochore functionality, and cell survival (9). Hec1 is expressed in rapidly dividing cells, and its levels are increased in cell lines upon transformation (10). Hec1 is overexpressed in human cancers, including breast, lung, cervical, brain, liver, colorectal, and gastric cancers (9, 11, 12). Hec1 expression is low in slow dividing and differentiated organs such as the heart, lung, liver, kidney, and brain.

References:

1. For references, please see the full text of this article.
(10). In vivo overexpression of Hec1 leads to tumor formation (13). Analysis of clinical tumor samples showed high levels of this protein and the levels correlates with tumor grade and prognosis in primary breast cancers (9). The role of Hec1 in cell cycle and its differential expression in cancer relative to normal tissues highlights its suitability as a cancer killing target. From a mechanistic standpoint, targeted inhibition of Hec1 by RNA interference or small molecules has been shown to effectively block tumor growth in animal models (9, 14). Therefore, Hec1 is a good potential molecular target for treating cancer clinically.

Small molecules targeting the Hec1/Nek2 pathway were discovered in the laboratory of Dr. WH Lee (University of California, Irvine, CA) by a yeast two-hybrid screening of a library of about 24,000 compounds (9). The original molecular hit, INH1, binds to and disrupts the functions of Hec1, leading to abnormal mitotic processes, activation of apoptosis, and cell death (9, 15). With INH1 as the starting template, structural modeling was applied to rationally design a series of compounds to generate a lead compound with significantly improved potency [MDA-MB-468 IC50: INH1 = 10.5 μmol/L (ref. 15); TAI-1 = 33.64 nmol/L (ref. 16)]. C-4′ methyl, C-4′-4-methoxyphenoxo, and 4-pyridyl groups were introduced to the core structure of INH1 to generate TAI-1. To optimize the oral bioavailability, solubility, and pharmacokinetic parameters, we introduced various heterocycles at the C-4′ position. The final compound selected for further development, TAI-95, has a C-4′-2-methoxyethoxy thiopyrazine and maintains excellent potency (MDA-MB-468 IC50 = 22.21 nmol/L). A summarized structural modification scheme is provided in Supplementary Fig. S1.

In this study, we report the characteristics of a novel and pharmacokinetically improved Hec1 molecularly targeted anticancer compound TAI-95 for the treatment of drug-resistant breast cancer. We showed the potent anti-proliferative activity of TAI-95 in a series of breast cancer cell lines, which compare favorably to currently available cytotoxic agents. TAI-95 was also demonstrated to be effective orally for the inhibition of breast tumor in animal xenograft models. TAI-95 downregulated several MDR genes and could enhance the potency of other cytotoxic agents. Finally, molecular subtypes of patients with breast cancer who may be sensitive to Hec1-targeted therapy were identified.

Materials and Methods

Cell lines

MDA-MB-231, MDA-MB-468, MCF7, HCC1954, BT474 [Development Center for Biotechnology (DCB), New Taipei City, Taiwan], T47D, Hs578T, ZR-75-1, ZR-75-30, MDA-MB-453, MDA-MB-361 [Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan] cell lines were maintained in complete 10% FBS (Biowest) and physiologic levels of glucose (1 g/L) Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma) at 37°C under 5% CO2. BCRC uses STR-PCR profile to authenticate their cell lines. Purchase dates from BCRC were within 2 years at the time of testing. Drug-resistant cell line study (MES-SA/Dx5, NCI/ADR-RES, and K562R) was conducted by Xenobiotic Laboratories.

In vitro drug potency assay

The preparation of TAI-95 is provided in Supplementary Materials and Methods. Cells were seeded in 96-well plates, incubated for 24 hours, drugs added, and again incubated for 96 hours. All testing points were done in triplicate. Cell viability was determined by MTS assay using CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay system (Promega) and PMS (Sigma). Data retrieved from spectrophotometer (BIO-TEK 340, BIO-TEK) were processed in Excel and GraphPad Prism 5 (GraphPad Software) to determine the concentration–response curves for calculating the relative concentration that exhibit 50% growth inhibition (GI50). All data represent the results of triplicate experiments.

To test downregulation of MDR genes, cells were seeded in 96-well plates and incubated for 24 hours. Cells were pretreated with TAI-95 for 6 hours and cytotoxic agent was added and incubated for 48 hours. Cell viability was determined as described above.

Mouse xenograft model

The procedure was adapted from published protocol (9) and were in accordance to the Institutional Animal Care and Use Committee of DCB. Male C.B-17 SCID mice (6–7 weeks, 21–24 g) were used for BT474 and MCF7 and nude/nude mice (6–7 weeks, 27–32 g) was used for MDA-MB-231 (Biosasco). Cells were injected subcutaneously into the flank in 50% Matrigel solution (BD Biosciences). A total of 6 × 106, 1.5 × 107, and 1 × 107 implanted cells per mouse was used for MDA-MB-231, BT474, and MCF7, respectively. When tumor volume reached 150 mm3 for MDA-MB-231 and 200 mm3 for BT474 and MCF7, mice were administered vehicle [10% dimethyl sulfoxide (DMSO), 25% PEG200] or TAI-95 twice a day for 28 days. Mice were dosed orally at 10, 25, 50 mg/kg (mpk) for BT474, orally at 10 and 25 mpk for MDA-MB-231, and intravenously at 10, 25, 50 mpk for MCF7 (for early screenings, intravenous administration were done). Tumor size were measured with digital calipers and volume calculated using the formula (L × W × H)/2, of which L and W represented the length and the width in diameter (mm) of the tumor, respectively. Body weights and tumor growth were measured twice a week. Mean tumor growth inhibition of each treated group was compared with vehicle control and a tumor growth inhibition value calculated using the formula: 1 – (T/C) × 100% (T, treatment group; C, control group tumor volume).

For re-dosing, the BT474 model was withheld from dosing 2 weeks before re-administration with the same regimen. Slopes of the tumor growth curves were calculated to compare growth rates.
**Immunoblot and co-immunoprecipitation analysis**

For immunoblotting, lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP40, 0.25% DOC, 0.1% SDS, 1 mmol/L NaVO₄, 1 mmol/L EDTA, 1 μg/mL leupeptin, 1 μg/mL pepstatin), separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Antibodies used for immunoblotting were mouse anti-Nek2, mouse anti-Mcl-1 (BD Biosciences); rabbit anti-Hec1 (GeneTex, Inc); mouse anti-actin (Millipore); rabbit anti-cleaved caspase-3, rabbit anti-PARP, rabbit anti-XIAP (Cell Signaling Technology); mouse anti-Bcl-2 (Santa Cruz); and mouse anti-α-Tubulin (FITC Conjugate; Sigma).

For co-immunoprecipitation, cells were lysed in buffer [50 mmol/L Tris (pH 7.5), 250 mmol/L NaCl, 5 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 50 mmol/L NaF, and protease inhibitor cocktail (Sigma P8340)] for 1 hour and then incubated with anti-Nek2 antibody (rabbit; Rockland) or anti-Hec1 antibody (mouse; Abcam) or rabbit or mouse IgG as control (Sigma-Aldrich) for 4 hours at 4°C, collected by protein G agarose beads (Amersham) and processed for immunoblotting.

**Immunofluorescent staining and microscopy**

For quantification of mitotic abnormalities, cells were grown on Lab-Tek II Chamber Slides and washed with PBS buffer (pH 7.4) before fixation with 4% paraformaldehyde. Following permeabilization with 0.3% Triton X-100, cells were blocked with 5% BSA/TBST and incubated with anti-α-tubulin antibodies. Then, 4',6-diamidino-2-phenylindole (DAPI) staining was applied and cells were mounted with ProLong gold antifade (Life Technologies). Images were examined with ZEISS LSM700 microscope at 400× magnification and captured with Spot Digital Camera and Spot Advanced Software Package (Diagnostic Instruments). Merged image was produced with ImageJ (U.S. NIH, Bethesda, MD). The percentage of cells with mitotic abnormalities was calculated by the number of the cells showing the abnormal mitotic figures (including chromosomal misalignment and formation of multipolar spindles) divided by the total number of mitotic cells counted. A minimum of 500 cells from randomly selected fields were scored per condition per experiment.

For Hec1 localization, cells were fixed, permeabilized, and incubated with Texas red–labeled anti-centromere antibody (Antibodies Inc.) overnight, followed by Hec1 antibody (GeneTex) at 37°C for 4 hours, followed by Alexa Fluor 488 Goat Anti-Mouse IgG (Life Technologies), and finally DAPI staining. Images were examined with ZEISS LSM700 microscope (Zeiss) at 1,000× magnification.

**Flow cytometric analysis**

For DNA content analysis, cells were cultured in 10-cm culture plate overnight, incubated with compound, and collected. Cells were fixed with 70% ethanol overnight, stained with propidium iodide (PI) solution (Sigma), and subjected to fluorescence-activated cell-sorting (FACS) analysis (FACScalibur, BD Biosciences). The results were analyzed by CellQuest software (BD Biosciences).

For Annexin V detection, FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) was used. FACS was used to analyze cells.

**Quantitative real-time reverse transcription PCR**

Cells were cultured in 6-cm culture plates overnight, incubated with compound for 48 hours, and collected. TAI-95 concentration 3 times the corresponding GI₅₀ was used. Total RNA was isolated with Quick-RNA miniPrep (Zymo Research). Reverse transcription and quantitative real-time PCR were performed on ABI Prism 7500 (PE Applied Biosystems) using the One-Step SYBR ExTaq qRT-PCR Kit (Takara). Primers from QuantiTect Primer Assay (Qiagen) were used and include GAPDH (QT01192646), ABCB1 (P-glycoprotein, Pgp) (QT00081928), ABCCI (MRP1; QT00061159), ABCC2 (MRP2; QT0056294), ABCC3 (MRP3; QT00076002), and ABCG2 (BCRP; QT00073206). All data represent the results of triplicate experiments.

**Gene expression in clinical samples—data from databases**

NDC80 (Hec1) gene expression data were retrieved from public dataset [GEO-GSE20685 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20685)]. Gene expression intensities were normalized with quantile normalization. Breast cancer samples in the dataset were subtyped on the basis of expression of 783 probe sets as reported (8). The sample number for normal, subtypes I, II, III, IV, V, and VI were 40, 37, 34, 41, 81, 41, and 93, respectively. P value was determined using ANOVA.

In addition, NDC80 and 8 other genes known to associate with NDC80 were identified (17, 18). One-way hierarchical clustering analysis was performed for 6 breast cancer subtypes using R package software (http://www.r-project.org/).

**Results**

**TAI-95 targets the Hec1/Nek2 pathway and leads to apoptosis**

To confirm the mechanism of action of the improved Hec1 compound TAI-95, co-immunoprecipitation and Western blot experiments were performed in breast cancer cell lines. In both Nek2 and Hec1 antibody co-immunoprecipitates, Hec1 failed to co-immunoprecipitate with Nek2, indicating that the Hec1/Nek2 complex was disrupted by treatment with TAI-95 (Fig. 1A). In addition, Nek2 was significantly reduced in a time-dependent manner with TAI-95 in ZR-75-1, with little change in the level of Hec1 expression, consistent with characteristics of Hec1/Nek2 disruption (9). This was also observed in TAI-95–treated MDA-MB-468, MDA-MB-231, and Hs578T breast cancer cell lines (Fig. 1B).
As cells defective in Hec1 or Nek2 show chromosomal and spindle abnormalities (10, 19–23), we tested the effects of TAI-95. Mitotic cells were counted for the presence of abnormal mitotic figures, including chromosomal misalignment and multipolar spindles (Fig. 1C). A dose-dependent increase in the mitotic population with multipolar spindles was seen with TAI-95 (Fig. 1C). Furthermore, no significant change in the localization of Hec1 with the centromere was observed by immunofluorescent staining; however, a decrease in the fluorescence intensities of both Hec1 and centromere proteins was observed at the chromosome (Supplementary Fig. S2). TAI-95 targets the Hec1/Nek2 pathway leading to the disruption of Hec1/Nek2 interaction, induction of Nek2 degradation, and deregulation of spindle formation.

**TAI-95 is highly potent in breast cancer cell lines**

To examine in vitro antitumor efficacy of TAI-95, 11 breast cancer cell lines were screened and 9 of 11 tested cell lines were found to be highly sensitive to TAI-95, including MDA-MB-231, MDA-MB-468, T47D, Hs578T, ZR-75-1, MCF7, HCC1954, BT474, and ZR-75-30, with GI50...
between 14.29 and 73.65 nmol/L. Notably, some of these cell lines which were resistant (GI50 > 10 µmol/L) to other tested cytotoxic agents, including approved breast cancer drugs paclitaxel, doxorubicin, gemcitabine, and sorafenib, were very sensitive to TAI-95 (Table 1). In addition, we investigated the combinatorial effects of TAI-95 with paclitaxel, doxorubicin, topotecan, and sorafenib in these cell lines but found no consistent substantial synergistic or antagonistic effects (Supplementary Table S1). These data suggest that TAI-95 has a wide spectrum of potency in breast cancer.

TAI-95 leads to apoptotic cell death
To characterize the cell death pathway induced by TAI-95 in breast cancer cell lines, we treated various cellular models HeLa, MDA-MB468, MCF7, and MDA-MB-231, and detected representative features of apoptotic cell death. Cleavage of PARP (Fig. 2A) indicated the activation of apoptosis. Topotecan was included as a positive control drug to show that that the different cell lines were competent of undergoing PARP cleavage. MCF7, which is caspase deficient (24) and resistant to various drugs (Table 1), was also susceptible to cell death and PARP1 (PARP) cleavage by TAI-95 (Figure 2A). Cleavage of caspase-3 and degradation of anti-apoptotic proteins MCL1 (Mcl-1) and BIRC5 (survivin; Supplementary Fig. S3) were also observed.

To further characterize TAI-95–induced cell death, the appearance markers for early and late apoptosis, Annexin V (ANXA5) and PI, respectively, were detected with flow cytometry. Results showed a significant increase in the Annexin V–positive PI–positive population, indicating the induction of apoptotic cell death by TAI-95 (Fig. 2B). The presence of DNA fragmentation, which is a hallmark of apoptosis, was determined by the sub-G1 assay. Results showed that TAI-95 led to an increase in the percentage of sub-G1 population, suggestive of apoptosis (Fig. 2C). The observation of cleavage of anti-apoptotic proteins PARP and caspase-3, the shrinkage of cells, the increase in Annexin V membrane protein, and the appearance of fragmented DNA indicated that TAI-95 induced cell death through the apoptotic pathway.

TAI-95 inhibits tumor growth of breast cancer model
To examine the in vivo antitumor efficacy of TAI-95, mouse harboring triple-positive breast cancer cell line–derived tumor xenografts were used. Remarkably, oral dosing of TAI-95 at 10, 25, and 50 mg/kg significantly inhibited tumor growth in the breast cancer model in a dose-dependent manner (Fig. 3A) but did not lead to any loss of body weight (Fig. 3B), nor liver and kidney toxicity (Supplementary Figs. S4 and S5). In addition, a preliminary screening shows growth-inhibitory trends in MDA-MB-231 and MCF7 xenograft models (Supplementary Fig. S6).

TAI-95 is active on large tumors and does not lead to drug resistance
Tumor size is a major prognostic indicator in breast cancer (25), whereas acquired resistance with chemotherapy

### Table 1. GI50 values of TAI-95 in various breast cancer lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>GI50 (nmol/L)</th>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td>16.48 nmol/L</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>22.21 nmol/L</td>
</tr>
<tr>
<td>MCF7</td>
<td>14.29 nmol/L</td>
</tr>
<tr>
<td>HCC1954</td>
<td>43.39 nmol/L</td>
</tr>
<tr>
<td>BT474</td>
<td>52.35 nmol/L</td>
</tr>
<tr>
<td>ZR-75-30</td>
<td>39.17 nmol/L</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>25.46 nmol/L</td>
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Abbreviation: ND, not determined.
leads to poor clinical outcome (26). To study whether TAI-95 will be useful for treating large tumors, a modified version of the BT474 model was used. After the standard drug testing in Fig. 3A, animals were put off the compound for 2 weeks and then the treatment was re-initiated (Fig. 4A). Oral dosing of 10, 25, and 50 mpk of TAI-95 to mice did not lead to any loss of body weight during this period (data not shown). Figure 4B shows the relationship of starting tumor size, treatment dose, and growth rate. The pattern in Fig. 4B suggests that TAI-95 remains active against large tumors (up to 920 mm³) during re-dosing and shows that 50 mg/kg TAI-95 maintains growth rates at similar rates (<10 mm³/d) in naive and previously treated tumors, indicating that the first treatment did not lead to acquired resistance in the second cycle despite a break window of 2 weeks. The current study shows that TAI-95 is able to target large breast tumors and remain active on previously treated animals.

TAI-95 downregulates Pgp and improves the potency of doxorubicin and topotecan

TAI-95 was tested for its potency against known MDR cell lines. MDR cell lines MES-SA/Dx5 and NCI/ADR-RES and Gleevec-resistant K562R were inhibited by TAI-95 in the GI50 range 7.278 to 19.46 nmol/L (Fig. 5A). For the 2 paclitaxel-resistant cell lines, the effect is 200 times more potent than paclitaxel. In addition, paclitaxel-resistant (GI50 > 10 nmol/L) breast cancer cell lines in Table 1, including MCF7 and HCC1954, were also sensitive to TAI-95. These data suggest that TAI-95 is not a Pgp (ABCB1/Pgp) substrate, consistent with our Caco-2 study (model for efflux transporter, especially Pgp; data not shown).

To elucidate whether TAI-95 affects MDR genes, the gene expression of MDR genes, including Pgp (ABCB1), MRP-1 (ABCC1), MRP-2 (ABCC2), and BCRP (ABCG2), was assessed with quantitative real-time PCR.
Interestingly, TAI-95 led to a time-dependent downregulation of the mRNA expression of Pgp in HeLa cells (Fig. 5B). In MDA-MB-231, the expression level of Pgp was significantly downregulated by TAI-95, whereas the expression levels of MRP-1, MRP-2, and BCRP were moderately affected (Fig. 6A). In addition, 3 other breast cancer cell lines were screened and showed downregulation of Pgp by TAI-95 (Fig. 6B). TAI-95 also inhibits Pgp expression more than 70% in MES-SA/Dx-5 cells (Fig. 6B), which express Pgp at levels more than 4 logs that of MDA-MB-231 cells (data not shown). To determine whether the downregulation of Pgp by TAI-95 could affect the potency of known Pgp substrates, well-known Pgp substrates doxorubicin and topotecan (27) were used for testing. Cells were pretreated for 6 hours with TAI-95 before being treated with the indicated Pgp substrate. As TAI-95-induced cell death peaked at 96 hours after treatment, we detected the cytotoxic effect of the added Pgp substrate at an earlier time point, 48 hours. Results showed that TAI-95 significantly increased the inhibitory effect of the Pgp substrates doxorubicin and topotecan (Fig. 5C, left and middle), with corresponding downregulation of Pgp at 6 hours (Fig. 5C, right). These results suggest that the synergistic effect of TAI-95 with doxorubicin and topotecan could, at least in part, be due to the effect of TAI-95 on Pgp expression.

**Differential expression of HEC1/NDC80 and associated genes among breast cancer subtypes**

To evaluate the characteristics of breast cancer subtypes relevant to Hec1 inhibitors, we used Western blotting and publicly available database to analyze HEC1 gene expression. Western blotting showed that the cells less sensitive to Hec1 inhibition had lower Hec1

Figure 3. TAI-95 inhibits tumor growth in breast cancer xenograft mouse models. BT474 breast cancer xenograft model in nude mice were used to test the in vivo efficacy of TAI-95. When tumor size reached 200 mm³, a twice daily, 28-day oral dosing treatment of TAI-95 was initiated. Tumor size (A) and body weights (B) were measured. **, P < 0.01; ***, P < 0.001, by 2-tailed t test.

Figure 4. TAI-95 inhibits large tumor growth and does not lead to resistance. BT474 (triple-positive) breast cancer xenograft model in nude mice was used to test the in vivo re-dosing efficacy of TAI-95. TAI-95 dosing was re-initiated after 2 weeks of non-dosing period and restarted on day 42 lasting for another 28 days. Vehicle group mice were sacrificed according to animal protocols and tumor volume postulated with linear assumption. A, tumor volume plotted against time. B, starting tumor size was plotted against treatment dose with circles with size and numbers indicating growth rates obtained at the end of the 28-day treatment period. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, by 2-tailed t test.
expression levels (Fig. 7A and B). The finding suggests that breast cancer with higher expression of Hec1 could be more sensitive to Hec1 inhibitors. We therefore investigated HEC1 expression in different molecular subtypes of breast cancer. As shown in Fig. 7C, HEC1 expression varied significantly among breast cancer subtypes. Subtypes I, II, and IV have a higher expression of HEC1. These 3 subtypes are also known to have high expression of genes for cell cycling/proliferation (8). These are types known to have worse recurrence-free and overall survival. As reported previously (8), subtype I corresponds to basal-like breast cancer, subtype II corresponds to ERBB2/HER2 overexpression breast cancer, subtype III has weak ESR (ER) expression and variable HER2 expression, subtype IV is a subset of luminal B breast cancer, subtype V is a subset of luminal A breast cancer with low risk of recurrence and excellent long-term survival, and subtype VI is a subset of luminal A with higher risk of

<table>
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<tr>
<th>GI50 (nmol/L)</th>
<th>TAI-95</th>
<th>Paclitaxel</th>
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<tbody>
<tr>
<td>MES-SA/Dx5</td>
<td>19.46</td>
<td>6247</td>
</tr>
<tr>
<td>NCI/ADR-RES</td>
<td>10.44</td>
<td>2329</td>
</tr>
<tr>
<td>K562R</td>
<td>7.28</td>
<td>34.45</td>
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Figure 5. TAI-95 potency in MDR lines. A, MDR cells treated with TAI-95 for 96 hours and quantitated with MTS assay to generate GI50. MES-SA/Dx5 (uterine sarcoma), NCI/ADR-RES (ovarian carcinoma), and K562R (leukemia) were tested and 2 paclitaxel-resistant breast cancer cell lines are included as reference. B, cells were treated with 50 nmol/L TAI-95 for the indicated time period, collected, RNA extracted, and analyzed with quantitative PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control and mRNA expression is presented as percentage of DMSO (vehicle control). * P < 0.05; ** P < 0.01; *** P < 0.001.
recurrence than subtype V. Subtypes II and IV had worst recurrence-free and overall survival. In addition, gene cluster analysis using \textit{HEC1} and genes associated with \textit{HEC1} (\textit{SPC24}, \textit{SPC25}, \textit{NUF2}, and SMC proteins; refs. 9, 18) revealed that the expression of these associated genes was proportional to \textit{HEC1} expression according to breast cancer molecular subtypes (Fig. 7D). This finding suggests that high \textit{HEC1} gene expression is associated with high expression of its associated genes but not with genes less associated with the kinetochore, such as \textit{RB1}. Subtype I, II, and IV breast cancer therefore may be more sensitive and responsive to treatment of Hec1 inhibitor. Breast cancer subtyping might serve as an effective predictive and triage tool to identify patients who are sensitive to Hec1-targeted therapy.

Discussion

This study demonstrates the potential utility of a novel Hec1-targeted small-molecule, TAI-95, in the treatment of
resistant breast cancers. TAI-95 is shown to be active on a number of breast cancer cell lines, notably those that were resistant to conventional cytotoxic agents. TAI-95 showed excellent oral efficacy in an in vivo breast cancer model. In addition to the activity in MDR cell lines, TAI-95 was shown to downregulate Pgp expression.

Hec1 is an oncogene that was discovered through a yeast two-hybrid screen to screen for the interacting partner of RB. Soon after its discovery, the functional aspects of the novel protein were established and were found to be closely associated with cancer. Hec1 is a member of the kinetochore that attaches the chromosomes to the centromere through microtubules for proper alignment during mitosis. The protein was found to be highly expressed in various cancers screened and little expressed in slow dividing cells (9), which suggests that it is an excellent candidate to target cancer. Further screening of a library of 20,000 compounds revealed a small molecule structure capable of the disruption of interaction between Hec1 and Nek2 and the inhibition of tumor growth in vivo (intraperitoneal). We started out aiming to optimize the drug attributes of the Hec1-targeted small-molecule INH1 and to develop it into an orally efficacious anticancer drug. To achieve the druggability of the targeted drug, the potency must reach nanomolar levels, which we have achieved (Supplementary Fig. S1). In addition, the
pharmacokinetic properties were optimized to promote oral efficacy, which is demonstrated in this study in the in vivo model. These new druggable attributes of TAI-95 have driven TAI-95 into preclinical drug development stage.

TAI-95 showed good activity against breast cancer cells derived from various origins. The potency profile is remarkable and demonstrates the potential utility of this drug in unmet medical needs in breast cancer. Even though the site of action is very closely associated with microtubules, the potency profile shows that the mechanism of action of TAI-95 has interesting features that may give it an advantage over conventional cytotoxic drugs. A study has shown that Hec1 overexpression was associated with paclitaxel resistance and poor prognosis in ovarian cancer (28), further illustrating the role of the target, Hec1, in drug-resistant cancers. Even though more studies will be necessary to elucidate the underlying mechanisms, the potential of this drug draws immense interest and we hold great expectations on its impact on the clinical treatment of drug-resistant breast cancers.

The in vivo xenograft studies showed that TAI-95 significantly inhibited the growth of BT474 xenografts. In addition to the triple-positive model (BT474), we have also performed xenograft studies on triple-negative (MDA-MB-231) and caspase-deficient (MCF7) models. Preliminarily, TAI-95 shows an inhibitory trend on the average size of the xenograft; however, the data did not show statistical significance within our standard observation period of 28 days (Supplementary Fig. S6). We believe that the inhibitory trend will be maintained to demonstrate statistical significance when the observation period of these models were extended to more than 10 weeks or if the models were optimized to allow for more stable growth and smaller variation of the size of tumors.

The development of refractory disease in breast cancer is frequently associated with the development of MDR. Overexpression of the multidrug transporter Pgp and MDR protein (MRP1) has been extensively studied for its role in MDR (29). Pgp may be induced in cancer cells through exposure to irradiation (30) or cytotoxic agents such as paclitaxel, doxorubicin, or cisplatin (31). On average 40% and 50% of untreated breast cancers show expression of Pgp and MRP-1, respectively, and exposure to chemotherapy increases the expression of both proteins (29). We show that TAI-95 downregulation of Pgp levels, TAI-95 does not lead to weight loss. TAI-95 down-regulates anti-apoptotic proteins and Pgp and enhances the effect of cytotoxic agents, demonstrating its potential to be incorporated into combination treatments. The current study shows that TAI-95 is a potent and safe anticancer drug candidate with excellent therapeutic potential for difficult-to-treat breast cancers.

**Disclosure of Potential Conflicts of Interest**

J.Y.N. Lau has ownership interest (including patents) in Taives and has received other commercial research support from Kinex Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: L.Y.L. Huang, Y-S. Lee, J-J. Huang, J.Y.N. Lau

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Study supervision: L.Y.L. Huang, Y-S. Lee, J.Y.N. Lau

Other: Drug design, J-J. Huang

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**References**


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