Preclinical characterization of SHR6390, a novel CDK 4/6 inhibitor, in vitro and in human tumor xenograft models

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Inhibition of the cyclin-dependent kinase (CDK) 4/6-retinoblastoma (RB) pathway is an effective therapeutic strategy against cancer. Here, we performed a preclinical investigation of the antitumor activity of SHR6390, a novel CDK4/6 inhibitor. SHR6390 exhibited potent antiproliferative activity against a wide range of human RB-positive tumor cells in vitro, and exclusively induced G1 arrest as well as cellular senescence, with a concomitant reduction in the levels of Ser780-phosphorylated RB protein. Compared with the well-known CDK4/6 inhibitor palbociclib, orally administered SHR6390 led to equivalent or improved tumor efficacy against a panel of carcinoma xenografts, and produced marked tumor regression in some models, in association with sustained target inhibition in tumor tissues. Furthermore, SHR6390 overcame resistance to endocrine therapy and HER2-targeting antibody in ER-positive and HER2-positive breast cancer, respectively. Moreover, SHR6390 combined with endocrine therapy exerted remarkable synergistic antitumor activity in ER-positive breast cancer. Taken together, our findings indicate that SHR6390 is a novel CDK4/6 inhibitor with favorable pharmaceutical properties for use as an anticancer agent.

KEYWORDS
breast cancer, CDK 4/6, drug resistance, palbociclib, SHR6390

1 | INTRODUCTION

Cell cycle, the process that governs cell division, consists of several distinct phases.1 Growth factors, estrogens and other mitogenic stimuli initiate cell cycle progression by inducing the expression of cyclins and cyclin-dependent kinases (CDK).2 Among them, CDK4/6 manages cell cycle progression by reversibly combining with cyclin D1. In early G1-phase, active CDK4 and CDK6 phosphorylate retinoblastoma (RB) protein, a tumor suppressor, causes partial release of E2F transcription factors and subsequently promotes the transcription of downstream genes required for passing through the G1 restriction point into S-phase.3,4 P16 is an endogenous CDK4 inhibitor and negatively regulates the cell cycle, which is lost in a wide range of malignancies.5 Disordered cell cycle regulation results in uncontrolled cell proliferation: a key feature of tumorigenesis. Given its significance in cell cycle control, the cyclin D1-CDK4/6-RB pathway is a well-substantiated target for anticancer drugs.

Breast cancer, one of the most commonly diagnosed cancers among women, is expected to account for 15% of all new cancers in China.6 Although considerable progress in breast cancer treatment has been made in recent decades, metastatic breast cancer remains incurable. Breast cancer is a highly heterogeneous malignant disease
that is classified into 5 distinct molecular subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, basal-like and claudin-low. Of these, the luminal type accounts for most breast tumors that express the estrogen receptor (ER) and/or progesterone receptor. Approximately 70% of human breast cancers present as ER-positive, a cancer cell subtype that requires estrogen and progesterone receptor. Approximately 70% of human breast cancers present as ER-positive, a cancer cell subtype that requires estrogen for survival and growth. Endocrine therapy including tamoxifen, fulvestrant and letrozole is currently recognized as the most effective for ER-positive, a cancer cell subtype that requires estrogen for survival and growth. Endocrine therapy including tamoxifen, fulvestrant and letrozole is currently recognized as the most effective agent.

However, the prevalence of primary and acquired resistance severely restricts the efficacy of these agents in the clinic, and the development of new treatments for breast cancer is a research priority.

First-generation CDK inhibitors were nonselective, pan-CDK blocking agents that showed limited antitumor activity and excessive toxicity. More recently, increasingly potent, selective, small-molecule CDK4/6 inhibitors, such as palbociclib, ribociclib and abemaciclib, have been developed in the setting of metastatic luminal breast cancer. Clinical studies have demonstrated encouraging efficacy of combined treatment with palbociclib and fulvestrant or letrozole. Although evidence for the efficacy of palbociclib is compelling, daily treatment causes severe neutropenia, which necessitates a 7-day treatment “holiday.” Overall survival is not extended by implementation of this strategy, however, indicating that a better understanding of resistance mechanisms and further evaluation of combination strategies are needed. These drugs are also usually expensive or unavailable to patients in China. Collectively, these observations highlight the desirability of developing new CDK4/6 inhibitors with improved efficacy and reduced toxicity.

SHR6390, a novel CDK4/6 inhibitor developed by the Chinese pharmaceutical company Jiangsu Hengrui Medicine (Jiangsu, China), has been shown to exhibit potent antitumor activity in esophageal squamous cell carcinoma. In the current study, we performed a pharmacological characterization of SHR6390, testing it against a panel of tumor models. SHR6390 showed potent and broad-spectrum antitumor activity in RB-positive cancer cell lines. In vivo xenografts, SHR6390 exhibited favorable pharmacokinetic and pharmacodynamic properties without causing noticeable toxicity. Importantly, SHR6390 overcame acquired resistance to tamoxifen and trastuzumab, and exerted synergistic antitumor activity in combination with endocrine therapy in breast cancers. Thus, these results provide a rationale for the ongoing clinical trials of SHR6390.

2 | MATERIALS AND METHODS

2.1 | Materials

SHR6390 and palbociclib were provided by Jiangsu Hengrui Medicine. 4-OH tamoxifen, tamoxifen, trastuzumab, fulvestrant and letrozole were purchased from Selleckchem (Shanghai, China). Antibodies against Ser780-phosphorylated RB (p-RB), RB, cyclin D1, p16 and tubulin were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2 | Cell lines and culture

The cell lines MCF7, ZR-75-1, T-47D, A549, SK-OV-3, NCI-H292, U-87 MG, PC-3, SNU-398, SNU-475, SNU-182, OVCAR-3, LN-18, HS-578T, MDA-MB-231 and MV-4-11 were purchased from the American Type Culture Collection (Manassas, VA, USA), MDA-MB-468, ES-2, SW620, COLO 205, A2780, Calu-3 and Hep G2 cell lines were purchased from Shanghai Cell Bank (Shanghai, China). The MCF7/ARO cell line was established by stably transfecting the MCF7 with human aromatase. The tamoxifen-resistant cell line MCF7/TR was established by culturing MCF7 cells with gradually increasing concentrations of tamoxifen. The trastuzumab-resistant cell line BT-474/T was established from the BT474 cell line as described previously.

2.3 | Cell proliferation assay

Cells were seeded into 96-well plates and then treated with different concentrations of drugs for 6 days. Cell proliferation was evaluated using the SRB (Sigma-Aldrich, St. Louis, MO, USA) or MTT (Sigma-Aldrich) assay. Drug combination studies were performed by assessing the combination index (CI) value, which defines the interaction between 2 drugs as synergistic (CI < 1), additive (CI = 1) or antagonistic (CI > 1), on the basis of the median-effect principle, calculated using CalcuSyn software (Biosoft, Cambridge, UK).

2.4 | Western blotting

Cells were lysed after treatment with different concentrations of drugs. Proteins were separated and transferred as well as incubated with antibodies. Proteins were visualized using a Western blot imaging System (Clinx Science Instruments, Shanghai, China).

2.5 | Cell cycle analysis

Cells were treated with drugs for 24 hours and harvested, then stained with propidium iodide (BD Biosciences, San Jose, CA, USA) and incubated. DNA content was determined using a FACS Calibur flow cytometry system (BD Biosciences). Data were analyzed using ModFit 3.0 software (BD Biosciences).

2.6 | Senescence assay (β-galactosidase staining)

According to the manufacturer’s instructions of the Senescence Associated β-Galactosidase Staining Kit (Cell Signaling), Cells were treated with indicated drugs or vehicle for 6 days and then stained by β-Galactosidase Staining Solution. The number of SA-β-gal positive cells (blue stained) was evaluated by cell counting in 5 randomly chosen microscope fields (200× magnification).

2.7 | In vivo study

Animal experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of
the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Tumor xenografts were established by subcutaneously inoculating tumor cells into 5-week-old female Balb/cA-nude mice (Shanghai Laboratory Animal Center, Shanghai, China). Mice were randomized into control or treatment groups when tumors reached a volume of approximately 100-200 mm³.

Control groups were given vehicle alone, and treatment groups received oral SHR6390, palbociclib, or SHR6390 plus tamoxifen or letrozole. Tumor volume was calculated as (length × width²)/2. Pharmacokinetic/pharmacodynamic studies were carried out as described previously. Concentrations of SHR6390 in plasma and tumor tissue were determined by high-pressure liquid

FIGURE 1 SHR6390 predominantly inhibits the proliferation of retinoblastoma (RB)-positive tumor cell lines. A, Antiproliferative activity of SHR6390 against a panel of human cancer cell lines derived from different tissues of origin and with varying RB status. Cancer cells were treated with different concentrations of SHR6390 for 6 d. B, Whole-cell lysates from a panel of human cancer cell lines was analyzed by western blotting. C, Cells were treated with 4-OH tamoxifen, tamoxifen or SHR6390 for 6 d. D, Cells were treated with trastuzumab or SHR6390 for 6 d. Cell viability was determined by SRB assay (n = 3; error bars denote SD; *P < .05 vs parent cells)
chromatography tandem mass spectrometry. Tumor samples were analyzed by western blotting.

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 7 software (GraphPad Software, San Diego, CA, USA). Half-maximal inhibitory concentration (IC₅₀) values were calculated by nonlinear regression analysis of dose-response curves. CI values were calculated using CalcuSyn software. Means ± SD (in vitro) and means ± SEM (in vivo) represent the results of repeated experiments. Student's t test was used to test differences between groups. P-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | SHR6390 inhibits the proliferation of retinoblastoma-positive tumor cell lines

On the basis of biochemical kinase assay (Table S1) and previously demonstrated CDK4/6 inhibitory activity of SHR6390, we tested the effects of SHR6390 against a panel of human cancer cell lines derived from different tissues of origin and with varying RB status. As expected, SHR6390 potently inhibited the proliferation of most RB-positive cell lines (IC₅₀ < 800 nmol/L), with the exception of Calu-3 cells. SHR6390 exerted little cytotoxicity against the RB-negative MDA-MB-468 cell line (IC₅₀ > 10 000 nmol/L), and showed limited efficiency against tumor cell lines with low expression of RB, including SNU-182 and OVCAR-3 cells (Figure 1A, B). Taken together, these findings indicate that SHR6390 exerts wide-spectrum cytotoxic effects against RB-positive tumor cell lines, without exhibiting significant tissue specificity.

A previous study reported that dysregulation of the CDK4-RB pathway is an important contributor to endocrine therapy resistance. To test this, we established the tamoxifen-resistant MCF7/TR cell line through long-term culture of ER-positive MCF7 cells with increasing concentrations of tamoxifen. The IC₅₀ values for 4-OH tamoxifen and tamoxifen in parental MCF7 cells were 368 and 1533.7 nmol/L, respectively, whereas the IC₅₀ values for these 2 drugs were greater than 10 000 nmol/L in MCF7/TR cells. Strikingly, SHR6390 demonstrated similar potency in MCF7/TR cells and parental MCF7 cells, with an IC₅₀ value of 229.5 and 115.4 nmol/L, respectively (Figure 1C). Furthermore, the BT-474/T cell line, which has been demonstrated to possess resistance to the HER2-targeted antibody trastuzumab, was even more sensitive to SHR6390 than the parental BT-474 cell line, exhibiting IC₅₀ values of 626.8 and 210.7 nmol/L in parental and BT-474/T resistant cell lines, respectively (Figure 1D).

3.2 | SHR6390 induces G₁-phase cell cycle arrest and cellular senescence through inhibition of the CDK4/6-RB pathway

CDK4/6 complexes with cyclin D1 to phosphorylate and inactivate RB, thereby allowing cell cycle progression. SHR6390 induced a clear decrease of RB phosphorylation in these sensitive tumor cells, with either a response or no response in other cell cycle-related proteins, such as cyclin D1 and p16. SHR6390, similar to the well-known selective CDK4/6 inhibitor palbociclib, substantially reduced the expression of RB and phosphorylated RB (p-RB) in COLO 205, U-87 MG and ES-2 cell lines, derived from colon, brain and ovarian cancers, respectively. Moreover, SHR6390 treatment increased the expression of cyclin D1 in all 3 of these cell lines and reduced the expression of p16 in COLO 205 and U-87 MG cell lines (Figure 2A).

Next, we investigated the effects of SHR6390 on breast cancer cell lines with different genetic backgrounds. As shown in Figure 2B, SHR6390 prominently reduced RB expression and phosphorylation at Ser780 in a concentration-dependent manner in RB-positive MCF7 and ZR-75-1 cell lines, and caused a corresponding increase in the expression of cyclin D1 as well as a reduction in expression of p16. Consistent with the results of cytotoxicity experiments, SHR6390 also inhibited RB phosphorylation in the MCF7/TR cell line, implying that RB protein-related changes are a universal phenomenon associated with CDK4/6 inhibitor treatment and may be a determinant of therapeutic response. Notably, SHR6390 treatment did not have a similar significant effect on the expression of cyclin D1 and p16 in RB-negative MDA-MB-468 cells. Time-course experiments further showed that SHR6390 rapidly inhibited RB expression and phosphorylation, increased the expression of cyclin D1 and reduced the expression of p16 for at least 72 hours in MCF7. Similar effects were observed in ZR-75-1 but not in MDA-MB-468 cells (Figure 2C).

Accordingly, SHR6390 induced a concentration-dependent G₁-phase cell cycle arrest in RB-positive MCF7 and ZR-75-1 breast cell lines but had minimal effects in the RB-negative MDA-MB-468 cell line, even at a concentration of 1000 nmol/L (Figure 2D). There was no evidence that SHR6390 induced apoptosis in either sensitive or resistant cell lines (data not shown).

Irreversible cell cycle arrest can result in cellular senescence. Next, senescence β-galactosidase staining was used to investigate whether SHR6390 could induce senescence. As show in Figure 2E, the proportion of senescence SA-β-gal positive cells increased from control levels of 10.6%-74.4% and 77.0% by 1 μmol/L SHR6390 and 1 μmol/L palbociclib in MCF7 cells, respectively. SA-β-gal positive cells increased from 18.0% to 85.8% and 88.6% after SHR6390 and palbociclib treatment in ZR-75-1 cells, respectively. Together, these data indicate that the cytostatic effect of SHR6390 is mainly attributable to cell cycle arrest and cellular senescence through blockade of the CDK4/6-RB pathway.

3.3 | Pharmacokinetic/pharmacodynamic studies of SHR6390 in a COLO 205 tumor xenograft model

Given the prominent effects of SHR6390 in vitro, we next investigated its pharmacokinetic and pharmacodynamic profiles in a COLO 205 xenograft model. As shown in Figure 3A, after treatment with a single dose of 75 mg/kg, SHR6390 concentration reached a rapid peak in plasma (Tₘ₉₅ = .5 hours, Cₘ₉₅ = 2305 ng/mL), and exhibited a
FIGURE 2  SHR6390 inhibits the CDK4/6-RB pathway and induces G1-phase cell cycle arrest and cellular senescence in retinoblastoma (RB)-positive tumor cell lines. A, Cells were treated with 1000 nmol/L SHR6390 or palbociclib for 24 h. Total cell lysates were immunoblotted with indicated antibodies. B, Cells were treated with SHR6390 at the indicated concentrations for 24 h. Total cell lysates were analyzed using the indicated antibodies. C, Cells were treated with 1000 nmol/L SHR6390 for the specified times, and western blotting was performed using the indicated antibodies. D, Cells were treated with SHR6390 or palbociclib (pal) for 24 h, after which DNA content was assessed. E, Cells were treated with SHR6390 or palbociclib at 1000 nmol/L for 6 d, and the activity of SA-β-galactosidase was performed by SA-β-gal staining. Quantification by counting cells in 5 randomly chosen microscope fields (standard bar, 50 μm; error bars denote SD)
half-life \( t_{1/2} \) of approximately 3 hours. Drug concentration in tumor tissue reached a peak approximately 2 hours after administration; the resulting \( C_{\text{max}} \) was 11,067 ng/g, which is approximately 4.8-fold higher than the \( C_{\text{max}} \) in plasma, and the \( t_{1/2} \) was extended to 8.6 hours in tumor tissue. Even 34 hours after administration, the drug concentration in tumor tissue was still significantly higher than the corresponding IC\(_{50}\) value in vitro (150.2 nmol/L). In keeping with these data, p-Rb, the main target of SHR6390, was clearly suppressed in tumor tissue 2 hours after administration of SHR6390 (75 mg/kg), and this inhibitory effect lasted at least 34 hours (Figure 3B).

Consistent with these pharmacokinetic findings, once-daily oral administration of SHR6390 produced a dose-dependent inhibitory effect on COLO 205 xenografts, yielding tumor growth inhibition (TGI) values of 88%, 115% and 129% at doses of 37.5, 75 and 150 mg/kg, respectively. Moreover, SHR6390 at a dose of 75 mg/kg caused tumor regression in 4 of 6 tumor xenografts, and at a higher dose (150 mg/kg) caused tumor regression in all tumor xenografts. All of these treatments were well tolerated, and no additive body weight loss was observed in any group during the course of the experiment. (Figure 3C,D). By comparison, the same doses of palbociclib caused regression of 3 of 6 and 5 of 6 tumors, respectively (data not shown).

### 3.4 In vivo antitumor activity of SHR6390 alone in human xenograft models

Given the wide-spectrum cytotoxicity of SHR6390 in vitro, we further investigated the antitumor activity of SHR6390 against a panel of tumor xenografts. Once-daily oral administration of SHR6390 caused dose-dependent inhibition of tumor growth in all tested tumor models (Figure 4A-C). In the U-87 MG model, TGI values for SHR6390 were 53%, 86% and 133% at doses of 37.5, 75 and 150 mg/kg, respectively. Moreover, at the highest dose tested, SHR6390 caused regression of all tumor xenografts (Figure 4A). We further tested the effects of SHR6390 against a xenograft model prepared by subcutaneously inoculating Matrigel-embedded human estrogen-dependent breast cancer cells stably transfected with the aromatase gene (MCF7/ARO cells) into nude mice, an experimental model that represents postmenopausal breast cancer in many respects.\(^{24}\) SHR6390 at a dose of 75 mg/kg exerted moderate inhibitory effects on tumor growth in the MCF7/ARO model, exhibiting a TGI value of 39%. The corresponding TGI values for 150 mg/kg SHR6390 and palbociclib were 70% and 57%, respectively (Figure 4B). In the COLO 205 model, we further found that tumors did not rebound after termination of SHR6390 treatment; in the 75-mg/kg group, significant inhibition of tumor growth was
observed even 7 days after cessation of SHR6390. Notably, SHR6390 at 150 mg/kg caused tumor regression, an inhibitory effect that was retained for at least 12 days after termination of SHR6390 treatment (Figure 4C). All of these treatments were well tolerated, as evidenced by the absence of drug-related deaths and significant body weight loss in all groups during the course of the experiment (Figure 4A-C).

3.5 Combined SHR6390 and endocrine therapy exerts synergistic antiproliferative effects on estrogen receptor-positive breast cancer cell lines

The combination of endocrine therapy and CDK4/6-selective inhibitors has proven to be an effective approach for improving antitumor activity against ER-positive breast cancers.25 Here, we found that SHR6390 exerted synergistic effects on ER-positive MCF7, T-47D and aromatase overexpressing MCF7/ARO breast cancer cell lines when combined with tamoxifen, a selective ER modulator, exhibiting CI values of .75, .53 and .64, respectively (Figure 5A). A subsequent investigation of the combined effects of SHR6390 and fulvestrant, a selective ER degrader, in breast cancer cell lines revealed that this combination resulted in synergistic inhibition of MCF7 cell proliferation, with a CI value of .42. Consistent with results obtained using parental cells, combined treatment with SHR6390 and fulvestrant also exerted synergistic cytotoxicity against tamoxifen-resistant MCF7/TR cells, with a CI value of .66 (Figure 5B). Taken together, these results suggest that SHR6390 combined with antiestrogen therapy exerts synergistic effects against ER-positive breast cancer cells, including those resistant to tamoxifen.

3.6 Combined SHR6390 and endocrine therapy exerts synergistic antitumor efficacy in estrogen receptor-positive breast tumor xenografts

Given the synergistic cytotoxicity of SHR6390 and endocrine therapy in vitro, we next investigated the efficacy of this combined treatment regimen in ER-positive MCF7 xenografts. As shown in Figure 6A, regression of tumor xenografts was observed in all co-treatment groups. Moreover, SHR6390 combined with fulvestrant produced an antitumor efficacy that was significantly superior to that of the corresponding single agents (P < .01). In MCF7/ARO xenografts, representing postmenopausal breast cancer, treatment with 20 mg/kg letrozole resulted in moderate inhibition of tumor growth, with a TGI value of 20.7%, whereas the addition of 100 mg/kg SHR6390 significantly enhanced the inhibitory potency compared with each single agent, as evidenced by the TGI value of 45.5% (P < .05; Figure 6B). The combination of SHR6390 with endocrine
therapy was generally well-tolerated, and no additive body weight loss was observed in any group during the course of the experiment.

4 | DISCUSSION

Dysregulated cell cycle machinery has been identified as an essential driver of malignant behavior. The cyclin-CDK-RB pathway is an important regulator of the cell cycle; consequently, targeting this pathway is a promising strategy for treating cancers. First-generation CDK inhibitors were nonselective, and exhibited limited antitumor activity and excessive toxicity. The next generation of inhibitors was developed to selectively target the ATP-binding site of the CDK4-cyclin D complex, and also target CDK6 (a closely related but likely redundant protein) but none of the other CDK. Although efficacious in the clinic, current CDK4/6 inhibitors...
inhibitor treatment regimens require a dosing holiday owing to severe neutropenia as well as tumor recurrence and emergence of drug resistance.\textsuperscript{15,28} Therefore, there has been a continuing desire to develop novel CDK4/6 inhibitors with improved pharmacological characteristics or potential for inclusion in rational combination regimens.

CDK4/6 inhibitors primarily exert their efficacy by repressing RB phosphorylation, a potential factor for predicting responsiveness to CDK4/6 inhibitors.\textsuperscript{29,30} Here, we assessed the antitumor activity of SHR6390, a novel CDK4/6 inhibitor, in various tumor models of RB-positive cell lines derived from different tissues of origin. Consistent with its role as a selective CDK4/6 inhibitor, SHR6390 dramatically reduced the expression of RB and p-RB, effects that were associated with a block in the progression of the cell cycle from G\textsubscript{1}-phase to S-phase and subsequent cellular senescence and inhibition of cell proliferation. In contrast, SHR6390 had no effect on cell cycle progression or proliferation in RB-deficient tumor cells. Consistent with the observation that not all tumor cell lines that retain RB are sensitive to CDK4/6 inhibitors\textsuperscript{29} and a previous report that RB-positive Calu-3 cell line was insensitive to CDK inhibitors,\textsuperscript{31} we found that SHR6390 exerted little cytotoxicity against Calu-3 cell line (IC\textsubscript{50} > 5000 nmol/L). P16 mutation without co-occurring D-cyclin activating features (DCAF) mutations in Calu-3 cells, may correlate with relative insensitivity to SHR6390, as reported by Gong et al\textsuperscript{32} Consistent with a previous report that sensitivity of tumor cells to CDK4/6 inhibitor was inversely correlated with p16 expression,\textsuperscript{29} much lower levels of p16 in the sensitive group were generally expressed (Figure S1). Collectively, this suggested that RB expression should be a prerequisite for sensitivity to CDK4/6 inhibition. However, RB expression alone was not sufficient to confer a high level of sensitivity to CDK4/6 inhibitor. P16 expression level in RB-positive cells could be used as a molecular marker for predicting response to CDK4/6 inhibitor. Some other potential predictors of CDK4/6 inhibitor efficacy have been reported, including hormone receptor status,\textsuperscript{33} CDK4/6 levels\textsuperscript{34,35} and the cyclin E-CDK2 axis.\textsuperscript{30,36} However, to date, no single biomarker, except ER\textsuperscript{R}/HER2\textsuperscript{R} status, has been identified for use in clinical trials. More in-depth and comprehensive research along these lines and further clinical validation of candidate biomarkers will be required.

Endocrine therapy has considerably improved survival in breast cancer patients over the past several decades. However, resistance to these therapies often develops and is a leading cause of treatment failure.\textsuperscript{37} Several molecular mechanisms have been proposed to account for endocrine resistance, most of which center on the ER or estrogen signaling networks.\textsuperscript{38-40} Despite the fact that mechanisms of various endocrine therapies are complicated and incompletely elucidated, there is a widely held consensus that CDK4/6 cell cycle pathways offer significant opportunities for therapeutic intervention in endocrine-resistant breast tumors.\textsuperscript{41,42} In agreement with this, we found that SHR6390 alone significantly inhibited RB phosphorylation and cell proliferation in the tamoxifen-resistant MCF7/TR cell line, where it exhibited the same potency as that against the parent cell line. A previous study suggested that CDK4/6 inhibitors may be effective against HER2-positive breast cancer.\textsuperscript{43} More recently, an elegant study using a transgenic mouse model demonstrated that activation of cyclin D/CDK4 mediates resistance to HER2.\textsuperscript{44} In the current study, we found that SHR6390 exhibited even greater potency against the trastuzumab-resistant BT-474/T cell line than against parental BT-474 cells. Our previous study showed that enhanced expression of epidermal growth factor receptor (EGFR), as well as elevated levels of p-AKT and p-ERK may be responsible for the resistance to trastuzumab in BT-474/T cells.\textsuperscript{18} These results are compatible with those of a previous study suggesting that CDK4/6 blockade reduced TSC2 phosphorylation, leading to attenuation of mTORC1 activity, an effect that was shown to relieve feedback inhibition of EGFR family kinases and thus sensitized the respective cells to EGFR/HER2 blockade. These observations indicate that SHR6390 is a good alternative option for breast cancer, including those forms that are resistant to endocrine and anti-HER2 therapies.

Our preclinical results suggest that SHR6390 exerts its antitumor activity mainly through specific effects on RB and, thus, may ultimately produce a wider spectrum of activity. Compared with palbociclib, SHR6390 generally exhibited the same or slightly better antitumor efficacy in tumor xenograft models. SHR6390 also induced tumor regression in some xenograft models, including COLO 205, U-87 MG and MCF7, in which no cell death was observed in vitro. This finding may indicate that the cell growth/death balance is altered in vivo and that tumor regression may reveal an endogenous apoptosis rate. Similarly, palbociclib treatment has been reported to exert tumor regression effects in some tumor xenograft models that are distinct from the cell cycle inhibitory effects observed in vitro.\textsuperscript{45} Importantly, we found that tumors did not rebound after treatment with 150 mg/kg SHR6390, measured 12 days after termination of SHR6390 treatment, suggesting sustained target inhibition. Consistent with this, p-RB, the main target of SHR6390, was clearly suppressed in tumor tissue 2 hours after administration of SHR6390 (75 mg/kg) and total inhibition was maintained for at least 34 hours, whereas p-RB levels partly recovered to normal after treatment with palbociclib on this schedule (data not shown). These results suggest that an intermittent schedule, which may decrease the toxicity and risk of inducing drug resistance, would be an alternative regimen for SHR6390 in the clinic. Dosing schedules, with correlative pharmacodynamics that avoid potential feedback mechanisms, warrant further investigation.

Selective CDK4/6 inhibitors together with other targeted agents or chemotherapy are also being investigated in the setting of advanced cancers.\textsuperscript{13,14} Although combination therapy has yielded better results in clinical studies performed to date, identifying the most promising and best-tolerated CDK4/6 inhibitor combination therapies is a significant challenge.\textsuperscript{46,47} In this context, the combination of SHR6390 with endocrine therapy, including tamoxifen and fulvestrant, exerted synergistic antitumor activity in ER-positive breast cancer compared with each monotherapy. SHR6390 combined with fulvestrant also exhibited synergistic effects against
tamoxifen-resistant MCF7/TR cells. Similarly, whereas SHR6390 alone exerted moderate antitumor activity in MCF7/ARO xenografts, which simulate postmenopausal breast cancer in several respects, including the absence of ovarian function and the lack of feedback regulation of estrogen production by gonadotropins, the antitumor activity of SHR6390 was significantly enhanced when combined with the aromatase inhibitor letrozole. Taken together, these results indicate that SHR6390 alone or combined with endocrine therapy may be a promising strategy for both premenopausal and postmenopausal breast cancer.

Collectively, our results demonstrate that SHR6390, a novel orally available CDK4/6 inhibitor, has wide-spectrum, potent antitumor activity against RB-positive cancers both in vitro and in vivo, with evidence of sustained target inhibition. SHR6390 can overcome acquired drug resistance to trastuzumab or tamoxifen, and when combined with endocrine therapy, exerts synergistic effects against breast cancer. In conclusion, the current study should benefit further clinical evaluations and, together with ongoing phase II clinical trials, suggests expanded clinical use of SHR6390 as part of personalized treatment strategies.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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