

## Research article

# Overcoming Palbociclib Resistance by Combined Treatment with PI3K/AKT/mTOR Inhibitors in Mesothelioma Cells

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## Abstract

Carcinogenesis of malignant pleural mesothelioma (MPM) is strictly associated with chronic exposure of mesothelial cells from the pleura to asbestos fibers and MPM incidence is expected to peak in the next years. Extensive genome analyses of patient-derived MPM tumors revealed that the most frequent mutational event involves the inactivation of the CDKN2A gene that encodes for the cell cycle inhibitors p16<sup>INK4a</sup> and p14<sup>ARF</sup> with consequent constitutive activation of CDK4/6 – cyclin D complexes. Therefore, inhibition of the latter complexes may represent a new option for the treatment of MPM patients. However, despite the efficacy of the specific CDK4/6 inhibitor palbociclib in blocking MPM cell proliferation, acquired resistance inevitably occurs. Herein, palbociclib-resistant clones isolated after stepwise exposure of MSTO-211H cells to gradually increasing drug concentrations, showed a reduction in Rb protein levels as well as in the cell cycle inhibitor p21<sup>waf1</sup>, accompanied by increased phosphorylation of AKT and p70S6K. Simultaneous treatment of resistant clones with both palbociclib and specific PI3K/AKT/mTOR inhibitors produced an additive effect in terms of reduction in cell growth, without any signs of senescence but with increased cell death. In MSTO-211H sensitive cells, this combination drug treatment significantly delayed the adaptation to palbociclib, suggesting that this treatment approach may prevent or at least retard the emergence of palbociclib resistance. Collectively, these results suggest that the combination of palbociclib with PI3K/AKT/mTOR inhibitors may overcome the acquisition of resistance to palbociclib treatment and could represent a potential therapeutic approach to treat cancers with acquired resistance to CDK4/6 inhibitors in the presence of activation of the AKT/mTOR signaling pathway.

## Keywords

Mesothelioma; Palbociclib; PI3K inhibitors; Senescence; CDK4/6; Drug resistance

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## 1. Introduction

Malignant pleural mesothelioma (MPM) is a malignancy originating from pleural mesothelial cells, strictly related to asbestos exposure. MPM is classified as rare tumor, however the incidence in Europe is expected to peak approximately after 2020, probably as a result of the long latency period between asbestos exposure and tumor development [1]. Chronic exposure to fibers of asbestos caused a progressive accumulation of deleterious genetic alterations in mesothelial cells including overexpression of VEGF and loss of function of some tumor suppressor genes, including BRCA-associated protein 1 (BAP-1), neurofibromatosis type 2 (NF-2), and CDKN2A [2, 3]. Whereas activation of oncogenes is still lacking along with the consequent absence of targeted strategies.

Currently, the main therapeutic options for patients with MPM are curative surgery or palliative chemotherapy based on doublet chemotherapy of cisplatin combined with pemetrexed [4, 5].

The median overall survival with platinum-based treatment does

not exceed 13–16 months, with the best outcome in patients with the epithelioid MPM subtype. Considering the absence of new therapeutic options, the knowledge of genetic alterations in MPM and the development of new therapeutic strategies are greatly needed.

The CDKN2A gene maps to chromosomal locus 9p21 and is the tumor suppressor gene most frequently inactivated in MPM, with 50–100% of incidence representing homozygous deletions [6]. CDKN2A encodes for p16<sup>INK4a</sup> and p14<sup>ARF</sup>, two key regulators of cell cycle progression: in particular, upon binding to CDK4/6 kinases, p16<sup>INK4a</sup> prevents the association with cyclin D, resulting in cell cycle arrest in the G<sub>1</sub> phase. CDK4/6 are necessary for the phosphorylation and inactivation of the retinoblastoma protein (Rb), which controls G<sub>1</sub>-S progression. In the presence of various stressors (e.g. oncogenic signaling and DNA damage), p16<sup>INK4a</sup> expression blocks inappropriate cellular division, and prolonged induction of p16<sup>INK4a</sup> leads to an irreversible cell cycle arrest with the induction of ‘cellular senescence’.

It has been reported that restoration of p16<sup>INK4a</sup> expression in

mesothelioma cells with CDKN2A loss induced apoptosis [7, 8], pointing to the rationale of inhibiting the CDK4/6-Rb signaling in p16<sup>INK4a</sup> null mesothelioma cancer cells. Consistent with this hypothesis, pharmacological inhibition of CDK4/6 in cancer cells with inactivating CDKN2A mutation or loss of expression, suppressed cell growth in different tumor types [9–11] and currently some clinical trials are ongoing to evaluate the efficacy of CDK4/6 inhibition in different tumors [12].

As reported for other specific targeted agents, chronic pharmacological inhibition of CDK4/6 caused the acquisition of resistance. In particular, the emergence of Rb point mutations was reported under selective pressure of CDK4/6 inhibitors in breast cancer [13]; in a pre-clinical study, chronic exposure to palbociclib caused increased expression of Cyclin E in gastric cancer cells [14]; finally, Rb positive breast cancer cells developed resistance to palbociclib with up-regulation of MAPK signaling [15].

Recently, we reported that MPM and breast cancer cells negative for p16<sup>INK4a</sup>, were sensitive to palbociclib with G<sub>1</sub> arrest and the induction of a senescence phenotype [16] along with an impairment of glucose metabolism [17]; these effects were hampered by the addition of inhibitors of PI3K/AKT/mTOR axis, suggesting that coupling palbociclib with PI3K/AKT/mTOR inhibitors might be a valuable strategy for treatment of MPM and breast cancer.

In the present paper, we demonstrated that acquired resistance to palbociclib in MPM cells is a consequence of Rb loss associated with activation of AKT/mTOR pathway; dual inhibition of CDK4/6 and AKT/mTOR in resistant cells reduced cell growth without induction of senescence. Finally, to prevent the acquisition of resistance to palbociclib in p16<sup>INK4a</sup>-negative MPM cells, we demonstrated that the dual treatment with palbociclib and BEZ235, induced a more pronounced inhibition of cell growth compared to single drug treatment together with an increased rate of apoptotic cell death.

## 2. Material and Methods

### 2.1. Cell culture and drug treatment

The human MPM cell line MSTO-211H (biphasic histotype) was cultured as previously described [16]. Resistant cells were established from MSTO-211H by exposure to an initial dose of 1  $\mu$ M palbociclib and culturing surviving cells during three months with gradually increasing drug concentrations (from 1 to 10  $\mu$ M). Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### 2.2. Materials

Palbociclib (PD-0332991) was obtained from Selleckchem (Munich, Germany); NVP-BEZ235 and NVP-BYL719 (hereafter, referred to as BEZ235 and BYL719, respectively) were provided by Novartis Institutes for BioMedical Research (Basel, Switzerland). Drugs were prepared in water or DMSO; DMSO concentration never exceeded 0.1% (v/v); equal amounts of the solvent were added to control cells.

### 2.3. Western blotting

Western blot analysis was performed as previously described [18]. Antibodies against p-Rb(Ser780), Rb, p-AKT(Thr308), AKT, p-mTOR(Ser2448), mTOR, p-p70S6K(Thr389), p70S6K, p-ERK1/2(Thr202/Tyr204), ERK1/2, and p21<sup>Waf1</sup> were from Cell Signal-

ing Technology, Incorporated (Danvers, MA); anti- $\beta$ -actin (clone B11V08) was from BioVision (Milpitas, CA). HRP-conjugated secondary antibodies were from Pierce (Rockford, IL) and chemiluminescence system (Immobilion<sup>TM</sup> Western Chemiluminescent HRP Substrate) was from Millipore (Temecula, CA). The chemiluminescent signal was acquired by C-DiGit<sup>®</sup> Blot Scanner and the bands were quantified by Image Studio<sup>TM</sup> Software, LI-COR Biotechnology (Lincoln, NE).

### 2.4. Cell proliferation and cell death

Cell proliferation was evaluated as previously reported [17]. The nature of interaction between palbociclib and PI3K/mTOR inhibitors was calculated using the Bliss additivity model as previously described [19]. Cell death was analyzed by fluorescence microscopy after staining with Hoechst 3342 and Propidium Iodide (PI) [20].

### 2.5. $\beta$ -galactosidase staining

The evaluation of Senescence Associated  $\beta$ -Galactosidase (SA- $\beta$ -Gal) expression was performed as previously reported [16].

### 2.6. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6.00 software. Statistical significance of differences among data was estimated by two-tailed Student's t test or by ANOVA followed by Bonferroni's post-test and P values are indicated where appropriate.

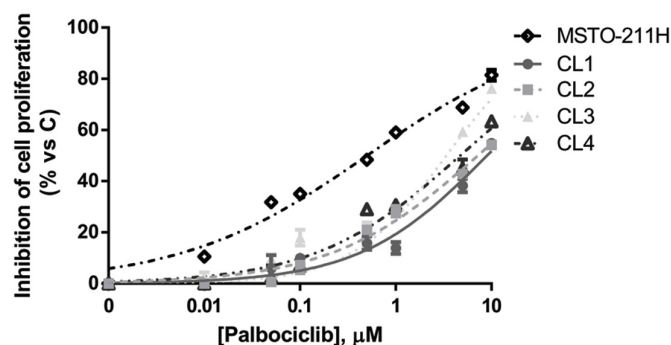
## 3. Results

### 3.1. Generation and characterization of palbociclib-resistant MPM cell clones

In order to generate palbociclib-resistant clones, human MPM MSTO-211H cells were cultured in gradually increasing drug concentrations from 1  $\mu$ M to 10  $\mu$ M. Four independent palbociclib-resistant clones (CL1-4) were isolated after three months of stepwise palbociclib selection (Fig. 1A); as shown in Fig. 1B, these clones displayed IC<sub>50</sub> values for palbociclib that were 6–18-fold higher than that of parental cells (3.3–9  $\mu$ M vs 0.5  $\mu$ M).

To investigate the molecular mechanism underlying the acquired resistance to palbociclib, the expression of several proteins involved in cell cycle regulation and signal transduction pathways were analyzed by Western blot analysis. As shown in Fig. 2, parental cells (MSTO-211H) were compared with the resistant clones exhibiting the highest IC<sub>50</sub> values for palbociclib (i.e. CL1 and CL2); these clones showed a significant reduction in Rb protein levels (0.4 and 0.2, respectively, relative to MSTO-211H cells) and phosphorylation associated with the reduction of P21<sup>Waf1</sup> levels (0.6 and 0.2, respectively). Moreover, CL1 and CL2 showed an up-regulation of the AKT/mTOR pathway, as indicated by the increased phosphorylation levels of AKT (3.2- and 2.7-fold, respectively) and p70S6K (2- and 2.5-fold, respectively), whereas total levels of both proteins did not change. In contrast, neither the phosphorylation nor the total expression of P44/42 proteins were changed in these clones as compared with the parental cells, suggesting that the acquisition of palbociclib-resistance was not associated with alterations in the MAPK signaling pathway.

A



B

CLONES	IC <sub>50</sub> , μM
MSTO-211H	0.5 ± 0.12
CL1	9 ± 1.09
CL2	7 ± 1.11
CL3	3.3 ± 1.23
CL4	5 ± 1.12

Fig. 1. Biological characteristics of palbociclib-resistant MSTO-211H clones. Parental MSTO-211H cells and their palbociclib-resistant clones CL1-4 were treated with increasing concentrations of palbociclib for 72 h. (A) Cell proliferation was assessed by the crystal violet assay and data are expressed as percentage of inhibition relative to control cells  $\pm$  SD. (B) EC<sub>50</sub> values reported in the summary table were calculated using GraphPad Prism 6.00 software. Data depicted are the mean values of three independent experiments.

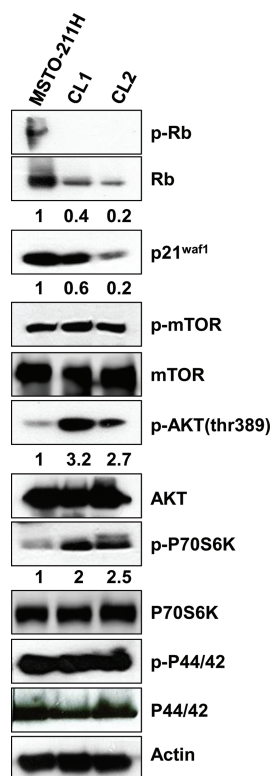


Fig. 2. Molecular characterization of palbociclib-resistant clones. Western blotting showing levels of indicated proteins in total cell lysates isolated from MSTO-211H, CL1 and CL2 cells. Data are representative of two independent experiments. The immunoreactive bands were quantified by densitometric analysis as ratio with  $\beta$ -actin and are expressed as fold decrease/increase vs MSTO-211H cells.

### 3.2. Effects of the combination of palbociclib with PI3K inhibitors on cell proliferation in palbociclib-resistant clones

Considering the increased activation of the PI3K/AKT pathway in palbociclib-resistant clones, we evaluated whether palbociclib sensitivity could be restored by combining this drug with BEZ235, a dual PI3K and mTORC1-2 inhibitor, or with BYL719, a specific inhibitor of the  $\alpha$ -subunit of the PI3K enzyme. To assess the nature of the interactions between the drugs, we used the Bliss interaction model. As shown in Fig. 3A–B, the combination of palbociclib with BEZ235 or BYL719 in CL1 induced an additive effect, with a 40% growth inhibition after treatment with 1  $\mu$ M palbociclib, an anti-proliferative effect comparable to that produced by palbociclib alone in the parental cells (see Fig. 1A). As previously reported [16], treatment of MSTO-211H cells with palbociclib induced the appearance of approximately 30% senescent cells, that was increased to 80% after BEZ235 treatment [16]. Therefore, we sought to determine whether the combination of palbociclib with BEZ235 caused a similar effect in our resistant cells. As shown in Fig. 4, in the presence of 10  $\mu$ M palbociclib, CL1 cells displayed a significantly lower percentage of senescent cells in comparison with parental cells treated with palbociclib for 72 h. In addition, the combination of palbociclib with BEZ235 was not effective in promoting cellular senescence in CL1 cells, in contrast to the significant induction of senescence observed in parental cells. Interestingly, the combination of palbociclib with BEZ235 caused increased cell death when compared to the treatment with palbociclib alone.

### 3.3. Effects of dual treatment of palbociclib and BEZ235 in palbociclib-sensitive MSTO-211H cells

We finally evaluated the impact of a long-term exposure to palbociclib or to the combined treatment with BEZ235 in MSTO-211H parental cells. As shown in Fig. 5, this simultaneous treatment was significantly more effective than palbociclib alone, in inducing a prolonged cell growth arrest suggesting that this therapeutic approach may prevent or overcome the acquisition of resistance to palbociclib.

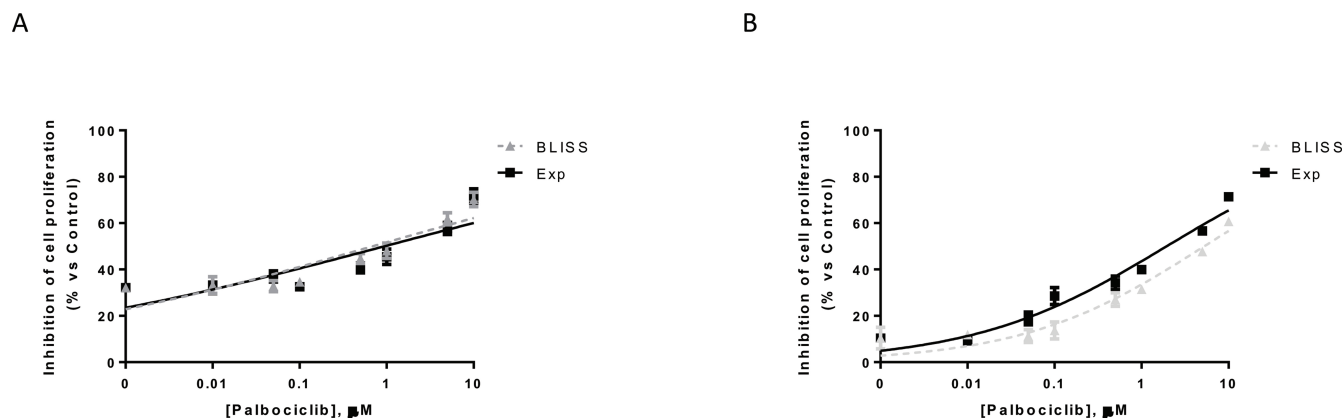


Fig. 3. Effect of simultaneous treatment with palbociclib and PI3K/mTOR inhibitors on cell proliferation. Growth inhibition curves of the effects of the combined treatment with both palbociclib along with 20 nM BEZ235 (A) or 2  $\mu$ M BYL719 (B) vs theoretical Bliss additivity curve for CL1 cells. Cells were treated with the drugs for 3 days and then, cell growth was assessed using the crystal violet assay as described in Materials and Methods. Data are expressed as percent inhibition of cell proliferation vs control cells.

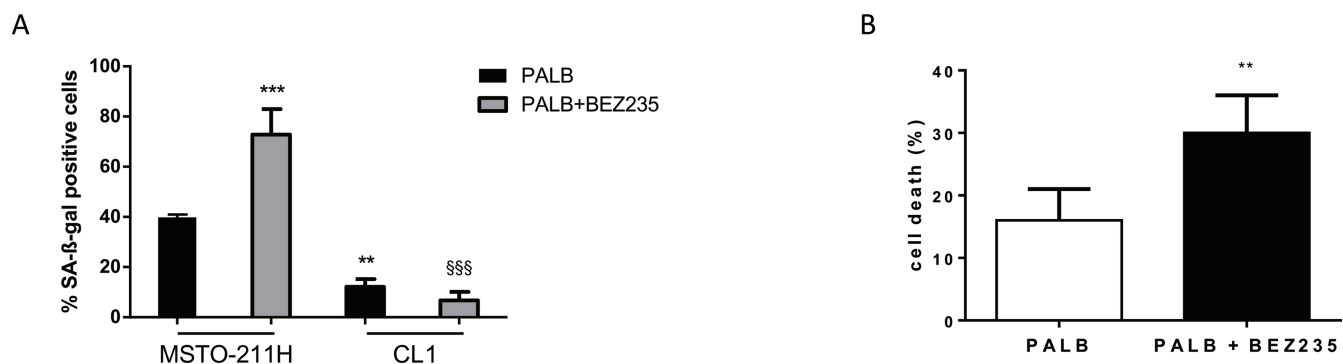


Fig. 4. Effect of simultaneous treatment of palbociclib and BEZ235 on cellular senescence. MSTO-211H cells were treated with the combination of 0.5  $\mu$ M palbociclib and 50 nM BEZ235 and CL1 cells with the combination of 10  $\mu$ M palbociclib and 50 nM BEZ235 for 3 days. After these drug treatments, senescent (A) or dead (B) cells were determined after staining as described in Materials and Methods. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 vs PALB MSTO-211H treated cells; §§§ $p$  < 0.001 vs PALB-BEZ235 MSTO-211H treated cells, Student's  $t$  test or one-way Anova analysis of variance followed by Bonferroni's post-test.

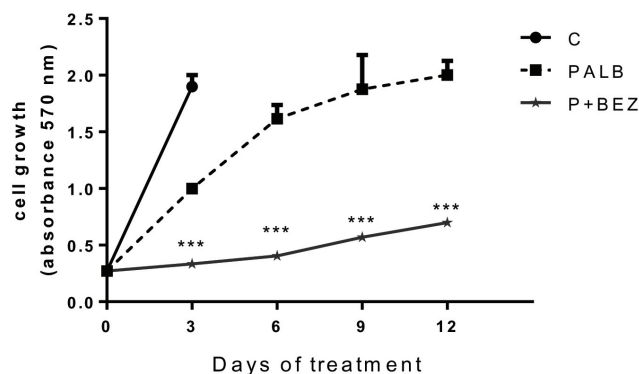


Fig. 5. Evaluation of prolonged treatment of palbociclib, BEZ235 or their combination in MSTO-211H cells. Parental MSTO-211H cells were treated with 1  $\mu$ M palbociclib or palbociclib combined with 50 nM BEZ235. Drugs were refreshed in the growth medium every 3 days. Cell viability was monitored by crystal violet assay. The data reported are representative of two independent experiments. \*\*\* $p$  < 0.001 vs PALB treated cells, two-way Anova analysis of variance followed by Bonferroni's post-test.

## 4. Discussion

The most relevant genetic alterations reported in MPM patients do not involve classical druggable oncogenic drivers, and chemotherapy remains the main therapeutic option for these patients [21]. Therefore, identification of novel targets and the development of alternative pharmacological therapies are urgently needed [22]. As previously reported [2], the genetic alteration most frequently detected in MPM patients is the genomic deletion of the CDKN2A/ARF gene encoding for the cell cycle suppressors p16<sup>INK4a</sup> and p14<sup>ARF</sup>, with consequent activation of CDK4/6 complexes and enhanced cell cycle progression. *In vivo* inactivation of CDKN2A [23–25] with consequent loss of both p16<sup>INK4a</sup> and p14<sup>ARF</sup> leads to the escape from cellular senescence, a physiological process that confers protection against tumor development by inducing growth arrest of cells with acquired, potentially deleterious, genetic alterations. Furthermore, genetic or epigenetic loss of CDKN2A is one of the most frequent events in neoplastic lesions [26], suggesting that loss of senescence is a prerequisite for cancer development.

It has been previously shown that pharmacological inhibition of CDK4/6 caused induction of cellular quiescence or senescence, depending on the tumor cell type [10, 27, 28]. As reported in our previous study, treatment of MPM cells expressing an active Rb



pathway with the specific CDK4/6 inhibitor palbociclib significantly reduced cell growth and promoted senescence [16].

Despite the clinical response evidence, acquired palbociclib resistance is common, and some of the mechanisms that lead to the inactivity of palbociclib have been described in breast cancer and hepatocellular carcinoma cells involving loss of Rb protein (as a consequence of genetic alterations or epigenetic mechanisms), amplification of Cyclin D1 and CDK6, and constitutive activation of cyclin E-CDK2 complex [29, 30].

Recently, activation of the MAPK signaling axis in preclinical models of breast cancer has been reported as a mechanism of palbociclib resistance and the addition of a specific inhibitor of the MAPK cascade restored drug sensitivity [15]. Several drug combination strategies have been proposed for palbociclib, in order to enhance the efficacy of the drug or overcome drug resistance [31]; for example, CDK4/6 inhibition coupled to other targeted agents can alter cellular metabolism, depleting anti-oxidants, increasing reactive oxygen species and finally inducing apoptosis [31].

In our present study, we isolated palbociclib-resistant clones from human MPM cells after stepwise selection to gradually increasing concentrations of the drug. In these clones, we observed a reduction in Rb phosphorylation/expression, which presumably accounts for the acquisition of drug resistance. In addition to down-regulation of Rb, palbociclib-resistant MSTO-211H-derived clones showed decreased levels of p21<sup>waf1</sup> protein and an increased activation of the AKT/mTOR signaling pathway in comparison with parental cells.

Activation of the PI3K/AKT/mTOR signaling axis has been reported as a mechanism of acquired resistance to palbociclib in breast cancer models [32, 33], although it occurred independently by Rb loss. In our experimental system, acquisition of resistance in MPM cells is presumably a consequence of both losses of Rb function and AKT/mTOR activation, confirming the co-existence of multiple mechanisms of palbociclib resistance, which may depend on the tumor type.

We demonstrated that the addition of specific agents targeting the PI3K/AKT/mTOR signaling can overcome the acquisition of palbociclib resistance, with reduction of cell growth and induction of cell death. In our previous paper on MPM cells we demonstrated that palbociclib was able to induce senescence and that addition of a PI3K/AKT/mTOR inhibitor significantly increased the percentage of senescent cells, with values close to 80-90% [16]. In contrast, in palbociclib resistant clones we failed to observe any sign of senescence either in the presence of palbociclib or after the addition of the PI3K/AKT inhibitor; the lack of senescence might be a consequence of loss of Rb and p21<sup>waf1</sup> expression. A recent study has shown that induction of senescence after CDK4/6 inhibition is Rb-dependent in melanoma cells [34], pointing to the critical role of the Rb pathway as a key regulator of senescence induction in cancer cells.

Collectively, the current findings suggest that the combination of CDK4/6 inhibitors and PI3K/AKT/mTOR signaling pathway inhibitors may have different effects in MPM cells depending on the activation status of the Rb pathway; in addition, our study suggests that this drug combination might represent a potential therapeutic approach to treat cancers with acquired resistance to CDK4/6 inhibitors in the presence of activation of the AKT/mTOR signaling pathway.

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## Conflict of Interest

The authors declare no conflict of interest.

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