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Combined Inhibition of CDK4/6 and PI3K/AKT/mTOR Pathways Induces a Synergistic Anti-Tumor Effect in Malignant Pleural Mesothelioma Cells[1]

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Abstract

Malignant pleural mesothelioma (MPM) is a progressive malignancy associated to the exposure of asbestos fibers. The most frequently inactivated tumor suppressor gene in MPM is *CDKN2A/ARF*, encoding for the cell cycle inhibitors $p16^{INK4a}$ and $p14^{ARF}$, deleted in about 70% of MPM cases. Considering the high frequency of alterations of this gene, we tested in MPM cells the efficacy of palbociclib (PD-0332991), a highly selective inhibitor of cyclin-dependent kinase (CDK) 4/6. The analyses were performed on a panel of MPM cell lines and on two primary culture cells from pleural effusion of patients with MPM. All the MPM cell lines, as well as the primary cultures, were sensitive to palbociclib with a significant blockade in G0/G1 phase of the cell cycle and with the acquisition of a senescent phenotype. Palbociclib reduced the phosphorylation levels of CDK6 and Rb, the expression of myc with a concomitant increased phosphorylation of AKT. Based on these results, we tested the efficacy of the combination of palbociclib with the PI3K inhibitors NVP-BEZ235 or NVP-BYL719. After palbociclib treatment, the sequential association with PI3K inhibitors synergistically hampered cell proliferation and strongly increased the percentage of senescent cells. In addition, AKT activation was repressed while p53 and p21 were up-regulated. Interestingly, two cycles of sequential drug administration produced irreversible growth arrest and senescent phenotype that were maintained even after drug withdrawal. These findings suggest that the sequential association of palbociclib with PI3K inhibitors may represent a valuable therapeutic option for the treatment of MPM.

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Abbreviations: MPM, Malignant Pleural Mesothelioma; BAP1, BRCA1 Associated Protein 1; CDK, Cyclin-dependent kinase; RB, Retinoblastoma Protein; SA-β-GAL, Senescence Associated β-Galactosidase.

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Introduction

Malignant pleural mesothelioma (MPM) is a progressive, poor prognosis malignancy of the pleura associated to the exposure to asbestos fibers. Due to the long latency period of this disease, the incidence of mesothelioma is expected to peak around the next 5–10 years [1]. Systemic chemotherapy remains one of the main treatments to prolong survival, nevertheless, the mean overall survival of MPM patients is limited to about 12 months and the median progression free survival is less than 6 months due to the intrinsic chemo-resistance of the disease. Based on the increasing incidence and the poor prognosis of MPM, novel therapeutic strategies are under investigation [2].

Several genetic alterations have been recently identified in MPM pathogenesis. However, MPM is being characterized mostly by the loss of tumor suppressor genes, rather than activation of oncogenes and an oncogenic driver is still lacking. The most frequently inactivated tumor suppressor genes in MPM are *CDKN2A/ARF*, deleted in about 70% of MPM cases, neurofibromin 2 (*NF2*), encoding for merlin and inactivated in 40–50% of MPM cases, and BRCA1 associated protein 1 (*BAP1*), gene associated to familial MPMs [3,4].

The *CDKN2A/ARF* tumor suppressor gene encodes for two cell cycle regulatory proteins: cyclin dependent kinase inhibitor 2A ($p16^{INK4a}$) and alternate reading frame ($p14^{ARF}$). *cdkn2a* and *arf* are regulated by separate promoters, differ in the first exon (exon 1alpha and 1beta respectively), share exons 2 and 3, and are translated from alternative reading frames [5]. $p16^{INK4a}$ inhibits the formation of the complex cyclin D1/CDK4/6 by binding to CDK4/6 and thereby maintaining retinoblastoma protein (Rb) in its hypophosphorylated active form, with consequent G1 cell cycle arrest. $p14^{ARF}$ binds to MDM2 and inhibits MDM2-induced degradation of p53, enhancing p53-dependent transactivation, cell cycle arrest and/or apoptosis. Deletion of the *CDKN2A/ARF* locus facilitates cell cycle progression, escape from apoptosis and immortalization.

Palbociclib (PD-0332991) is an oral-available, highly selective inhibitor of CDK4/6 kinase activity that inhibits Rb phosphorylation and therefore prevents cellular DNA synthesis by inhibiting progression of the cell cycle from G1 to S phase. Currently, palbociclib is approved by the US FDA (Food and Drug Administration), for the treatment of estrogen positive metastatic breast cancer in association with letrozole. Palbociclib usually presents tolerable toxicity with mild neutropenia and thrombocytopenia as main adverse events. Considering the high frequency of deletion of *CDKN2A/ARF* in MPM, we investigated the effect of palbocilib on a panel of MPM cell lines and on cells obtained from pleural effusion of MPM patients.

One feature related to palbociclib treatment is the increased activation of the AKT/mTOR pathway, due to the increased phosphorylation of AKT, as recently reported by Zhang and coworkers [6] and confirmed in mesothelioma cells in our study. By inhibiting the TSC1–TSC2 complex, AKT activates the serine–threonine kinase mTOR, which exists in two distinct complexes, mTORC1 and mTORC2, upon binding with different regulatory proteins [7]. The PI3K/AKT/mTOR pathway plays a critical role in the control of cell growth, proliferation, metabolism, and migration, and is frequently deregulated in cancer cells, thus representing an attractive candidate for targeted cancer agents.

Thus, the present work was addressed to evaluate the antitumor potential of combining palbociclib with inhibitors of the PI3K/AKT/mTOR pathway in MPM cells. In particular, we tested the effect of the combination with NVP-BEZ235, a reversible competitive inhibitor of the ATP-binding site of both class I PI3K and mTOR [8], and NVP-BYL719, a specific inhibitor of the p110α subunit of class I PI3K [9].

Our findings demonstrated that, in comparison with individual treatments, the sequential association of palbociclib and PI3K/mTOR inhibitors enhanced the inhibition of cell proliferation (both in 2D and 3D cultures) and the induction of cell senescence; moreover, these effects were maintained after drug removal, suggesting a new therapeutic strategy to challenge the aggressive behavior of MPM.

Material and Methods

Cell Lines and Drugs

Human MPM cell lines MSTO-211H (biphasic histotype), H2452, H28 (both of epithelioid histotype), H2052 (sarcomatoid histotype) and MDA-MB-468 breast cancer cells were obtained from ATCC (Manassas, VA), cultured as recommended and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

ZS-LP e MG-LP primary cell lines were obtained from two patients (both male, 66 years for ZS-LP, 62 years for MG-LP) affected by mesothelioma biphasic histotype of stage T4 N0 for ZS-LP and T3 N0 for MG-LP, diagnosed at the Department of Pathology -University/Hospital of Parma. Patients were enrolled after informed consent to the employment of biologic samples for research purpose. The procedure was approved by the institutional review board for human studies (Ethical Committee) of the University-Hospital of Parma and in accord with principles listed in the Helsinki declaration. Pleural effusions were collected and transferred under sterile conditions. After centrifugation at 240 x g for 5 min at room temperature (RT), red blood cells were lysed and the pellet was suspended in fresh medium. ZS-LP e MG-LP cells were then cultured in RPMI supplemented with 2 mM glutamine, 10% FBS, non-essential amino acids (NEAA) and 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Daily microscopic observation of the cultures showed the growth of a population of adherent cells whose MPM phenotype was assessed by the immunocytochemical analysis of Calretinin, HBME-1 and panCytokeratin.

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

Western Blotting

Total cell lysates and Western blotting were performed as previously described [10].

Antibodies against p-Rb(Ser780), Rb, p-ERK1/2(Thr202/Tyr204), ERK1/2, p-AKT(Ser473), p-AKT(Thr308), p-AKT(Ser473), AKT, p-mTOR(Ser2448), mTOR, p-p70S6K(Thr389), p70S6K, $p21^{Waf1/Cip1}$, cyclin D1, CDK6, c-Myc, p-MDM2(Ser166) were from Cell Signaling Technology, Incorporated (Danvers, MA); anti-p53-(DO-1) and anti-p-CDK6(Tyr24) were from Santa Cruz