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Ribociclib, a Cdk4/Cdk6 kinase inhibitor, enhances glucocorticoid sensitivity in B-acute lymphoblastic leukemia (B-All)

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ABSTRACT

Dysregulation of the cyclin D1-CDK4/CDK6 complex is frequently observed in almost all human cancer and contributes to aberrant cell proliferation and consequent tumorigenesis. Although many reports described the importance of CDK4/CDK6 in different set of human tumors, only few studies have been performed on leukemia.

By gene expression analysis performed in a cohort of childhood patients affected by B-acute lymphoblastic leukemia (B-ALL) we found that both CDK4 and CDK6 are highly expressed. Moreover, reverse phase protein array (RPPA) analysis showed that cyclin D1 levels are higher in patients undergoing relapse. Starting from these considerations, we evaluated the effect of dual inhibition of CDK4/CDK6 in B-ALL and if this inhibition could enhance cytotoxic killing of leukemia cells after combination treatment with dexamethasone. We treated B-ALL cell lines with ribociclib, a highly specific CDK4/6 inhibitor. As expected, treatment with ribociclib induced growth inhibition of B-ALL cell lines, accompanied by strong cell cycle arrest in G1 phase, along with a dose-dependent decrease in phosphorylated retinoblastoma protein. Ribociclib exposure strongly synergizes with dexamethasone in SEM and RCH-ACV, two dexamethasone-resistant cell lines, along with a strong decrease in proliferation and a significant increase in apoptotic cell death. These results were also confirmed on primary cultures derived from bone marrow of pediatric patients affected by B-ALL. Immunoblot analysis showed a significant increase in glucocorticoid receptor (GR) along with some of its target genes, after combined treatment with ribociclib and dexamethasone. Altogether our findings support the concept that pharmacologic inhibition of CDK4/CDK6 may represent a useful therapeutic strategy to control cell proliferation in B-ALL and provide new insight in understanding potential mechanism of glucocorticoid resistance.

1. Introduction

Unrestrained cell proliferation and cell cycle deregulation are common features in almost all human cancer. Cyclin Dependent Kinases (CDKs) are the major regulator of cell cycle transition and increased CDKs activation and signaling have been described in hyperproliferating malignant cells [1,2]. Among the CDKs that tightly control cell cycle progression, cyclin D-dependent kinases CDK4 and CDK6 are considered the most important oncogenic drivers [3]. CDK4 and CDK6 have high homology, present the same biochemical properties and play redundant roles in cell cycle regulation, but are expressed in different tissues and their alteration are associated with different types of human tumors. In particular CDK6 is mainly expressed in hematopoietic cells and has been described as a driver in MLL-rearranged and FLT-IDT myeloid leukemia and infant B-ALL with MLL rearrangement [4].

During G1-S transition, the cyclin D1-CDK4/CDK6 complex phosphorylates retinoblastoma protein (pRB) that becomes inactivated and consequently induces the release of the transcription factor E2F1 which drives the cells through the cell cycle. Thus, persistent CDK4/6 activity gives the cells the capability to enter the cell cycle continuously [2,3]. In addition to retinoblastoma proteins other substrates such as SMAD2, SMAD3, FOXM1 and MEP50 have been described to be direct phosphorylation targets of cyclin D1-CDK4/CDK6 complex. In particular, phosphorylation of SMAD2/3 by CDK4/CDK6 induces the inhibition of SMAD signaling which slows down cell proliferation by inducing the expression of p15 and p21 and represses the transcription of MYC [5,6]. While, as concern FOXM1, phosphorylation by cyclin D-dependent kinase induces a stabilization of FOXM1 [7] transcription factor

Abbreviations: B-ALL, B-acute lymphoblastic leukemia; Dex, dexamethasone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ribo, ribociclib; RPPA, reverse phase protein array; PI, propidium iodide

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counteracting the repression of cell cycle genes by its competitor FOXO3a, and pushing its transcriptional program to promote G1-S, G2-M transitions [8].

Many alterations of the Cyclin D1-CDK4/6 axes regulation have been described in different types of tumors. In particular amplification or overexpression of cyclin D1, amplification of CDK4, activating mutation of CDK4 and loss of CDK4/CDK6 inhibitors p16INK4a and have been described in many cancer types and deletion and methylation of their genes, CDKN2a and CDKN2b respectively, are frequently observed in B-ALL [2,9]. Moreover CDK6 is a direct target of MLL-AF9 driven AML [4,10] and of infant MLL-rearranged B-cell acute lymphoblastic leukemia [4,11].

B-ALL represents one of the most frequently diagnosed cancer in children and despite remarkable progress in chemotherapeutic protocols that has been made for the treatment of these malignancies, therapy is not yet effective in all cases [12].

Standard treatment for newly diagnosed childhood B-ALL patients includes chemotherapy treatment and although glucocorticoids (GC) are the most effective and commonly used drug in the treatment of acute lymphoblastic leukemia, their effect is often hindered by the development of resistance. Responsiveness to GC represents a prognostic marker in B-ALL and since poor responders have the worst outcome compared to good responders they are classified as high-risk patients [13].

Specific inhibition of cyclin D/CDK4/CDK6 axis could be an attractive strategy to improve the effect of common chemotherapy on B-ALL patients. Although many reports described the usefulness of CDK4/CDK6 inhibition against a broad range of carcinomas, few studies have been performed on leukemia. Many CDK4/CDK6 specific inhibitors are currently tested in clinical trials for several tumors as single agents or in combination with other chemotherapeutics. Among that, ribociclib is an orally bioavailable small molecule that binds and inhibits with high selectivity the ATP cleft of CDK4 and CDK6 [14]. Results from both preclinical and clinical studies showed that not only ribociclib was effective alone and in combination with other drugs but important results have been obtained in delay the development of therapy resistance [14,15].

In this paper, we found that cyclin D1, CDK4 and CDK6 are generally highly expressed in pediatric B-ALL and thus we sought to evaluate the dual inhibition of CDK4/CDK6 in B-ALL and the combination with dexamethasone. We confirmed the sensitivity of B-ALL cell lines to the specific CDK4/6 inhibitor ribociclib and we found synergistic activity with glucocorticoids. The synergistic effect of ribociclib-dexamethasone combination was also confirmed on primary cultures derived from pediatric patients affected by B-ALL.

Our findings support the concept that pharmacologic inhibition of CDK4/CDK6 may represent a useful therapeutic strategy to control cell proliferation in B-ALL and provide new insights in understanding potential mechanism of glucocorticoid resistance.

2. Materials and methods

2.1. Cell culture

The human leukemia cell lines RS 4;11 (ACC-604), NALM6 (ACC-128), SEM (ACC-546), RCH-ACV (ACC-548) were purchased from DSMZ. Cells were cultured in RPMI 1640 (Life Technologies, Italy) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM; Life Technologies, Italy), penicillin (100 U/ml; Life Technologies, Italy) and streptomycin (100 µg/ml; Life Technologies, Italy), and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Primary leukemia cell cultures

B-ALL patient samples were obtained after informed consent following the tenets of the Declaration of Helsinki. The study was approved by the ethical committee board of the University of Padova, the Padova Academic Hospital and the Italian Association of Pediatric Onco-Hematology (AIEOP). Diagnosis was made according to standard cytomorphology, cytochemistry and immunophenotypic criteria (30). B-ALL primary cell lines were obtained after hemolysis of red blood cells, from bone marrow samples collected at the time of diagnosis.

2.3. Gene expression analysis

For gene expression analysis, samples (GEO accession n. **GSE13204** [16], **GSE71935** [17]) were normalized by RMA [18] using R Bioconductor (www.rproject.org); known batch effect due to different synthesis protocols was removed using the sva package in R [19], genes were collapsed for max probe value. CDK4 and CDK6 expression for all samples (N = 299) was shown as boxplots.

2.4. Reverse phase protein arrays (RPPA)

Cyclin D1 expression was investigated in 111 pediatric patients affected by B-ALL by reverse phase protein arrays (RPPA) analysis as previously described [20]. Briefly, cells were lysed in an appropriate lysis buffer with proteases and phosphatases inhibitors, serially diluted into four-points dilution curves and printed on nitrocellulose-coated glass slides with the 2470 Aushon Arrayer (Aushon Biosystems). Slides were stained with Cyclin D1 (BD Transduction Laboratories) primary antibody using the CSA kit (Dako Cytomation) and signal was revealed using DAB. Stained slides were analyzed using MicroVigene software (VigeneTech Inc, Boston, MA). Cyclin D1 expression was compared between 84 not relapsed and 27 relapsed patients by unpaired *t* test with Welch's correction using Prism (GraphPad Software 7.0.3, Inc., La Jolla, CA, USA).

2.5. Cell growth inhibition assay

Cells were treated in 96-well plated with ribociclib (Selleck, Munich, Germany) for 72 h. Different concentrations of drug were added to each well in triplicate. Cell viability was assayed by MTT test as previously described [21]. The GI_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

2.6. Combined drug treatment

Cells were treated with scalar dilutions of dexamethasone (Sigma-Aldrich, Milano, Italy) in the presence or absence of ribociclib, at fixed concentration ratio. After 72 h cell viability was measured by MTT and drug combination effect was evaluated using CompuSyn software (ComboSyn Inc, Paramus, NJ; www.combosyn.com) based on the method of the combination index (CI) described by Chou [22]. Synergy, additivity and antagonism were defined by Combination Index of (CI) < 1, CI = 1, or CI > 1, respectively.

2.7. Measurement of cell proliferation

Cell proliferation was determined by trypan blue exclusion assay plating SEM and RCH-ACV ($2.5 * 10^5$ cells/well) in 6 wells plate a day before treatment with ribociclib 5 µM and 10 µM. The following day, the starting cell number was calculated (T = 0 h) and until the third day, every 24 h cell number was calculated in continuous presence of the drug.

2.8. Annexin-V/PI assay

Surface exposure of phosphatidylserine on apoptotic cells was measured by flow cytometry with a Coulter Cytomics FC500 (Beckman Coulter, CA, USA) by adding Annexin-V conjugated to fluorescein isothiocyanate (FITC) to cells according to the manufacturer's instructions