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Dual inhibition of PI3K/mTOR signaling in chemoresistant AML primary cells

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ABSTRACT

A main cause of treatment failure for AML patients is resistance to chemotherapy. Survival of AML cells may depend on mechanisms that elude conventional drugs action and/or on the presence of leukemia initiating cells at diagnosis, and their persistence after therapy. MDR1 gene is an ATP-dependent drug efflux pump known to be a risk factor for the emergence of resistance, when combined to unstable cytogenetic profile of AML patients.

In the present study, we analyzed the sensitivity to conventional chemotherapeutic drugs of 26 samples of primary blasts collected from AML patients at diagnosis. Detection of cell viability and apoptosis allowed to identify two group of samples, one resistant and one sensitive to *in vitro* treatment. The cells were then analyzed for the presence and the activity of P-glycoprotein. A comparative analysis showed that resistant samples exhibited a high level of MDR1 mRNA as well as of P-glycoprotein content and activity. Moreover, they also displayed high PI3K signaling. Therefore, we checked whether the association with signaling inhibitors might resensitize resistant samples to chemo-drugs. The combination showed a very potent cytotoxic effect, possibly through down modulation of MDR1, which was maintained also when primary blasts were co-cultured with human stromal cells. Remarkably, dual PI3K/mTOR inactivation was cytotoxic also to leukemia initiating cells. All together, our findings indicate that signaling activation profiling associated to gene expression can be very useful to stratify patients and improve therapy.

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1. Introduction

Acute myeloid leukemia (AML) is a progressive myeloid neoplasm associated with maturation arrest and accumulation of myeloblasts in peripheral blood and bone marrow (Döhner et al., 2015; Mohammadiasl et al., 2016). Although approximately 35% of AML patients are currently treated, disease relapse and drug resistance are still big challenges (Saultz and Garzon, 2016), so that 10–40% of patients show primary resistance or early relapse, and it is commonly reckoned that chemotherapy alone will not ameliorate the outcome in the future. Primary therapy resistance implies failure to achieve complete remission (Thol et al., 2015). Efflux of drug through the ATP-binding cassette transporters (ABC) such as the P-glycoprotein (P-gp) encoded by the multidrug resistance gene1 (MDR1) is one of the most important mechanisms of resistance in AML (Broxterman et al., 2000). MDR expression increases with age, being P-gp expressed in 47% of elderly AML cases and in 34% of younger patients. Although its expression as a prognostic value for survival is still a matter of debate, it is well-known that high P-gp expression at diagnosis, combined with unstable cytogenetic profiles or epigenetic mutations in hematopoietic progenitors, correlates with poor prognosis due to resistance (van der Heuvel-Eibrink et al., 2001). However, hitherto several efforts to block P-gp/ABC transporter by the use of inhibitors failed due to dose-limiting toxicities and off-target effects (Mahadevan and List, 2004).

Mutated progenitor cells, the so-called leukemic initiating cells (LICs), which constitute a subpopulation able to live in very harsh conditions such as hypoxic environment (Leibovitch and Topisirovic, 2018) in a quiescence phase similar to hematopoietic stem cells (HSCs) (Ratti et al., 2018), are another source of resistance to chemotherapy and disease relapse (Vergez et al., 2011). This subpopulation is more therapy-resistant than other cell populations (Terwijn et al., 2014) and several studies showed that in particular the $CD34+/CD38^-$ compartment is very important in clinical setting, because of mutations described with low frequency at diagnosis, which are found highly represented at relapse (Griessinger et al., 2016). These mutations are possibly connected with the aberrant activation of the PI3K/AKT/mTOR pathway that has been identified as a feature of LICs in acute leukemia (Kharas et al., 2010; Follo et al., 2013; Martelli et al., 2014; McCubrey and Cocco, 2014). PI3K/AKT/mTOR signaling controls proliferation, differentiation and survival of hematopoietic cells (Martelli et al., 2010; Ricciardi et al., 2017; Hermida et al., 2017). Under normal conditions, PI3K activation is triggered through extracellular binding of ligands, which in turn induce the activation of corresponding receptor tyrosine kinases (Ruzzene et al., 2017; Payrastre and Cocco, 2015; Yang et al., 2013). PI3K activation leads to the conversion of phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP3), which in turn recruits AKT and phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane resulting in AKT phosphorylation byPDK1 at Thr308. For full activation, AKT is also phosphorylated by mTORC2 at Ser473 (Ruzzene et al., 2017; Toker and Marmiroli, 2014). PI3K signaling is negatively modulated by dephosphorylation of PIP3 by the lipid phosphatases PTEN and SHP1/2 (Ciuffreda et al., 2014). Because several key elements of this pathway have been found mutated in cancer, much emphasis has been placed on developing drugs which target PI3K signaling (Fransecky et al., 2015; Fragoso and Barata, 2017; Ruvolo, 2017; Park et al., 2010), leading to a number of molecules being trialled in several malignancies, including AML. While it is well established that over 60% of AML patients show increased activity of PI3K/AKT signaling (Park et al., 2010; Bertacchini et al., 2014, 2015), its relationship with resistance to therapy is not completely settled (Abrams et al., 2017; Marmiroli et al., 2015). Previous studies by us and others showed that aberrant signaling by the abovementioned pathway modulates expression of the MDR1 gene in acute leukemia (Tazzari et al., 2007; Seo et al., 2010). We report here that in primary cells collected at diagnosis from AML patients, characterized by detectable MDR1 expression, combined treatment with signaling inhibitors and conventional chemotherapy results in a strong cytotoxic effect, able to kill also leukemia initiating cells. These results once again point out the importance of extensive patient profiling for signaling activation, MDR1 expression and LICs to identify specific subgroups of patients and select the best therapeutic protocols.

2. Materials and methods

Cell culture-DMSO-stored primary blast cells samples, previously collected from patients with newly diagnosed AML, were obtained from bone marrow or peripheral blood (Bertacchini et al., 2014). Mononuclear cells were isolated using the Ficoll–Hypaque system. Only samples with Trypan blue-positive cells < 20% and blast count > 70% were used in this study. The human leukemic cell lines HL60 and CEM, and the human stromal cell line HS-5 were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA), the cell sublines HL60-R and CEM-R were a generous gift of Dr. Cianfriglia and were obtained as described. Both primary AML cells and leukemia cell lines were cultured in RPMI 1640 with 10% FBS while HS-5 cell line was cultured in DMEM with 10% FBS, at 37 °C in humidified atmosphere with 5% CO_2. Cells were treated with Etoposide 10 mM, Cytarabine 1 mM, PF-04691502 500 nM and NVP-BEZ235 as indicated in the figures. Viability of AML cells treated with inhibitors was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay as previously described (Mediani et al., 2015).

Protein extraction, immunoblotting and RPPA-Cells were extracted by addition of Lysis buffer (20 mM Tris-Cl, pH 7.0, 1% NP-40, 150 mM NaCl, 10% glycerol, 10 mM EDTA, 20 mM NaF, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, and freshly added Sigma-Aldrich Protease Inhibitor Cocktail) at 4 °C for 10 min as described previously (Maraldi et al., 1993). Lysates were cleared by centrifugation and used for immunoprecipitation experiments, as described (Lucarelli et al., 2002). In brief, total lysates were boiled in SDS sample buffer, resolved by SDS-PAGE, then immunoblotted on Immobilon-P membranes (Millipore, MA), probed by Western blotting with the indicated antibodies, and detected by the Supersignal substrate chemiluminescence detection kit (Euroclone, Milan, IT) on a Kodak Image Station 440CF with the Kodak 1D Image software.

For RPPA analysis, array assembly, printing, staining and analysis were performed as reported previously (Maraldi et al., 2011; Bertacchini et al., 2014; Serafin et al., 2017). Briefly, cells were lysed on ice for 20 min in TPER Reagent (Pierce, Rockford, IL),