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Lithium, a classic drug in psychiatry, improves nilotinib-mediated antileukemic effects

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ABSTRACT

Although Tyrosine kinase inhibitors (TKIs) that target Bcr-Abl play a key role in Chronic Myeloid Leukemia (CML) therapy, they do not eradicate CML-initiating cells, which lead to the emergence of drug resistance. Here we used the lithium, a GSK-3 inhibitor, to attempt to potentiate the effects of nilotinib against leukemia cells. For this purpose, a K562 leukemia cell line and bone marrow cells from untreated Chronic Myeloid Leukemia (CML) patients, prior to any exposure to TKIs, were used as a model. Our results demonstrated that the combination of lithium + nilotinib (L + N) induced K562-cell death and cleaved caspase-3 when compared to lithium or nilotinib alone, accompanied by GSK-3β phosphorylation and Bcr-Abl oncoprotein levels reduction. Interestingly, these events were related to autophagy induction, expressed by increased LC3II protein levels in the group treated with L + N. Furthermore, the clonogenic capacity of progenitor cells from CML patients was drastically reduced by L + N, as well as lithium and nilotinib when used separately. The number of cell aggregates (clusters), were increased by all treatments (L + N, lithium, and nilotinib). This pioneering research has demonstrated that lithium might be of therapeutic value when targeting Bcr-Abl cells with nilotinib because it triggers cell death in addition to exerting classical antiproliferative effects, opening new perspectives for novel target and therapeutic approaches to eradicate CML.

1. Introduction

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder and the first human cancer associated with consistent chromosomal abnormality, the Philadelphia chromosome (Ph), which results from the reciprocal translocation of chromosomes 9 and 22 [1,2]. This translocation fuses the *c-Abl* gene (human homolog of the Abelson murine leukemia virus) with the *Bcr* gene (breakpoint cluster region), forming the *Bcr-Abl* [3,4] complex that encodes a tyrosine kinase oncoprotein with an important role in the CML pathogenesis [5].

The cytoplasmic localization of Bcr-Abl allows the activation of several signaling pathways in leukemia cells [6], such as Ras [7], nuclear factor kB (NF-kB) [8], STAT-5 [9] and Phosphatidylinositol 3-kinase/Protein kinase B (PI3-k/Akt) [10]. The activation of Akt is responsible for regulating the activity of several targets, such as Bad, IkB kinase [11], mammalian target of rapamycin(mTOR) [12], mouse double minute 2 homolog (MDM2) [13], and Forkhead box (FOXO) [14]. One of the mechanisms by which Akt promotes cell survival occurs is due to the inactivation of the machinery components of apoptosis, such as the inhibition of Bad [15], which blocks caspase-dependent cell death [16,17]. Thus, resistance to apoptosis is one of the striking characteristics of Bcr-Abl cells, and this oncoprotein is considered one potent anti-apoptotic molecule [5].

Although tyrosine kinase inhibitors (TKIs) have strong antiproliferative effects, they fail to induce cell death in leukemic stem cells. This therapeutic failure could explain, at least in part, why leukemic Bcr-Abl stem cells are present in the bone marrow of patients with a complete cytogenetic response to imatinib mesylate treatment for more than five years [18,19]. Therefore, the high resistance to TKIs stimulates studies involving combined therapy with other agents [20] in an effort to sensitize Ph^+ stem/progenitor cells.

Recently, glycogen synthase kinase-3 (GSK-3) has been implicated

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in leukemia pathophysiology, suggesting a potential target for the leukemic cells eradication. Notably, GSK-3 interacts with multiple signaling pathways important for cell survival, proliferation, and death [21–23]. Dolnikov et al. [22] have demonstrated that the administration of a GSK-3 inhibitor to mice transplanted with hematopoietic stem cells can augment hematopoietic repopulation. Reddiconto et al. [24] reported that targeting GSK-3 promotes imatinib-mediated death by apoptosis in $CD34^+$ leukemia progenitor cells while preserving normal stem cells.

Lithium Carbonate (lithium), a monovalent cation used for bipolar disorders for more than 60 years is a potent GSK-3β and α isoforms inhibitor [25]. Many therapeutic uses have been proposed for this cation in hematology since the 1980s, such as idiopathic, infectious, and iatrogenic neutropenia [26]. In this study, our goal was to improve the nilotinib effects against CML by lithium, as studies have encouraged the use of nilotinib as the first-line in CML chronic phase. Nilotinib is more potent than imatinib mesylate, resulting in rapid cytogenetic and molecular response and less resistance rate when compared to imatinib [27–29]. However, nilotinib side effects might limit its therapeutic uses, denoting the importance of studies involving its combination with other drugs aiming to improve its efficacy with fewer doses.

Our data demonstrated that lithium in combination with nilotinib (L + N) induces death in leukemia cells. Reduced Bcr-Abl oncoprotein, autophagy induction, GSK-3β phosphorylation, and CML bone marrow progenitor cells clonogenicity impairment were verified in groups treated with L + N supporting our suggestion that targeting GSK-3 might be an important strategy to improve nilotinib effects.

2. Material and methods

2.1. Drugs

Lithium carbonate (Li_2CO_3), Z-VAD-FMK, Necrostatin-1 (Nec-1), Ferrostatin-1 (Fer-1), Bafilomycin (BAF A1), and Chloroquine (CQ) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nilotinib was gently supplied by Novartis and stored at 10 mM in DMSO at −20 °C. Lithium was diluted in distilled water and stored at room temperature protected from light.

2.2. K562 cell line and treatments

The human leukemia cell line K562 was obtained from the cell bank of Rio de Janeiro Federal University, Rio de Janeiro, RJ, Brazil. The cells were cultured at 37 °C in 5% CO_2 and maintained in RPMI-1640 culture medium, from Sigma-Aldrich (St. Louis, MO, USA), supplemented with 10% FBS, 1% penicillin/streptomycin (1000 IU), and 0.1% fungizone (Life Technologies). Cells were treated with 20 mM lithium, 5 μM nilotinib, or combination of the both (L + N) for 24 h. The concentration of lithium (20mM) is a commonly used for *in vitro* studies [25,26].

2.3. Cytotoxicity and cell death assays

To assess K562 cell viability and death measurements, 10^5 cells were treated with lithium, nilotinib, and combination of L + N as indicated above. For determination of Sub-G1 fraction by flow cytometry, after treatment, K562 cells were fixed in ice-cold 70% ethanol in phosphate-buffered saline (PBS) and then centrifuged, pelleted, and resuspended in PBS containing 0.25 μg/mL of RNase A and 25 μg/mL of propidium iodide (PI). To evaluate specify modalities of cell death we used the inhibitors of caspase, z-VAD-FMK (20 μM), inhibitor of necroptosis, Nec-1 (20μM), and inhibitor of ferroptosis Fer-1 (6 μM), respectively. These inhibitors were used at concentrations that did not induce cell death. Overall, 10.000 events were acquired in FL2 channel using a FACS Calibur cytometer (Becton-Dickinson, Mountain View, CA, USA) analyzed using FlowJo software.

For apoptosis studies, K562 cell line was treated with the appropriate compounds as indicated above and then, stained with fluorescein isothiocyanate (FITC) conjugated to AnnexinV and PI according to the manufacturer's instructions (AnnexinV/FITC Apoptosis Detection Kit, BD Pharmigen, San Diego, CA, USA). The population of $AnnexinV^-PI^-$, $AnnexinV^+PI^-$, $AnnexinV^+PI^+$, and Annexin V^-/PI^+ cells was evaluated by flow cytometry. Data were collected in a FACS Calibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA). In total, 10.000 events were acquired in FL1/FL2 channels using a FACS Calibur cytometer (Becton-Dickinson, Mountain View, CA, USA) analyzed using FlowJo software.

To confirm apoptosis inducted by the combination of L + N, cleaved caspase-3 was analyzed following treatment of K562 cells for 24 h. Treated cells were washed with PBS and fixed in 2% paraformaldehyde in PBS (v/v) for 30 min. The cells were then permeabilized with PBS containing 0.01% saponin and 1% BSA. Cells were then incubated with cleaved caspase-3 (Asp175) and Alexa Fluor 488-conjugated antibody in the dark at room temperature for 1 h. Cells were harvested, resuspended in PBS and analyzed.10.000 events were acquired in FL1 channel using a FACS Calibur cytometer (Becton-Dickinson, Mountain View, CA, USA) analyzed using FlowJo software.

2.4. GSK-3β, Bcr-Abl, and LC3II protein levels by western blotting assay

After treatment, K562 cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM Na_3VO_4) with a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA) for 30 min on ice. After centrifugation (13,200 rpm for 5 min at 4 °C), the protein supernatants were collected, quantified by the Bradford method (Bio-Rad, Richmond, CA), and total protein content (60–80 μg) was separated by SDS-PAGE and transferred onto 0.45 μm PVDF membranes. Membranes were blocked with 5% non-fat dry milk for 1 h and incubated overnight with primary antibodies: total and phosphor anti-GSK-3β, and anti-LC3II (1:1000, from Cell Signalling Technologies, Beverly, MA, USA); total anti-Bcr-Abl (1:1000, Santa Cruz Biotechnology); anti-β-actin or anti-α-tubulin antibodies (1:10000), (Sigma-Aldrich, Saint Louis, MO, USA). The next day membranes were washed with Tris-Buffered Saline Tween (TBS-T) and incubated with horseradish peroxidase-linked with specific secondary antibodies (1:1000, Santa Cruz Biotechnology), visualized with ECL (GE Healthcare) and images were recorded by using a Chemidoc apparatus Uvitec, (Cleaver Scientific, UK).

2.5. Clonogenic assay from bone marrow cells of leukemia patients post diagnosis, prior to any exposure to TKIs

The progenitor cells from bone marrow of five untreated LMC patients of the UNICAMP Clinical Hospital/Hemocentro, prior to any exposure to TKIs were separated by 30 min centrifugation at 400g in Ficoll-Hypaque (1.077g/ml). The cells from the interface were washed three times with RPMI-1640 (Sigma. St Louis. MO. USA) and counted for colony formation in presence of lithium (20 mM), nilotinib (5 μM) or combined treatment (L + N) in semisolid methyl cellulose medium (10^3cell/mL; MethoCult™ H4435, Stem Cell Technologies Inc.). All patients provided their informed written consent, and all experiments were performed in accordance with the ethical and care guidelines and were approved by the local ethics committee of the University of Campinas (UNICAMP), and were adherent to the Declaration of Helsinki. After 11 days of culture, the colony forming unit to granulocytes (CFU-G), macrophages (CFU-M), granulocytes-macrophages (CFU-GM), burst forming unit-erythroid (BFU-E), and clusters were counted using an inverted microscope at 40x magnifications. Colonies were considered when more than 50 cells were observed [30].