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ORIGINAL ARTICLE

LLL-3, a STAT3 inhibitor, represses BCR-ABL-positive cell proliferation, activates apoptosis and improves the effects of Imatinib mesylate

Andre Luiz Mencalha · B. Du Rocher · D. Salles · R. Binato · E. Abdelhay

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Abstract
Purpose The chimeric protein BCR-ABL, a constitutively active protein-tyrosine kinase, triggers downstream signalling proteins, such as STAT3, ultimately resulting in the survival of myeloid progenitors in BCR-ABL-positive leukemias. Here, we evaluated the effect of LLL-3, an inhibitor of STAT3 activity, on cell viability and its addictive effects with Imatinib mesylate (IM) treatment in BCR-ABL-positive cells.
Methods Viability of cell lines was determined using the WST-1 assay in response to drug treatment, either LLL-3 alone or in conjunction with IM. Annexin V-FITC/PI staining, sub-G1 DNA content and Caspase-3/7 activation assays were performed to evaluate apoptosis.
Results LLL-3 treatment decreased cell viability, triggered apoptosis and activated Caspases-3/7 in K562 cells. LLL-3 increases IM treatment to inhibited cell viability and activation of apoptosis in BCR-ABL-positive cell lines.
Conclusions LLL-3 reduced cell viability and induced apoptosis in K562 cells. Moreover, the observed addictive effects of co-treatment with IM and LLL-3 suggest this combination has therapeutic potential.

Keywords LLL-3 · STAT3 · BCR-ABL · Imatinib

A. L. Mencalha · B. Du Rocher · D. Salles
Instituto de Biofísica Carlos Chagas Filho,
CCS, UFRJ, Rio de Janeiro, RJ, Brazil

A. L. Mencalha (✉) · B. Du Rocher · D. Salles · R. Binato · E. Abdelhay
Laboratório de Células-tronco, Divisão de laboratórios do CEMO,
Instituto Nacional do Câncer (INCa), Praça da Cruz Vermelha,
23/6° Andar–ALA C, Rio de Janeiro, RJ 20230-130, Brazil
e-mail: mencalha@biof.ufrj.br; amencalha@inca.gov.br

Introduction

The chimeric BCR-ABL gene, generated by the genetic translocation known as the Philadelphia chromosome, encodes the BCR-ABL protein, which is characterised by a constitutively active ABL tyrosine kinase moiety [1]. BCR-ABL is present in 95% of patients with chronic myeloid leukemia (CML), in 20% of patients with acute lymphoblastic leukemia (ALL) and is rarely found in patients with acute myeloid leukemia (AML) [2]. BCR-ABL kinase activity promotes constitutive activation of downstream cellular signalling molecules, such as JAK/STAT [3], which results in uncontrolled cellular proliferation and inhibition of the apoptotic process [4].

The BCR-ABL protein represents a potential therapeutic target in CML and, to a lesser extend, other BCR-ABL-positive leukemias [5]. Imatinib mesylate (IM), an inhibitor of the tyrosine kinase activity of BCR-ABL, has been successfully used for treatment of CML patients in the chronic phase [6, 7]. However, Imatinib is less effective in advance phases of CML [8] and, a subset of patients has developed IM-resistance [9]. Thus, it is important to identify molecules downstream of BCR-ABL for the development of alternative treatment strategies.

Several approaches to reduce the IM-resistance mechanism and enhance the success of Imatinib therapy have been attempted [10]. Treatment with additional chemotherapeutics in combination with Imatinib mesylate has shown synergistic improvement of Imatinib effects and has also overridden Imatinib resistance [10, 11]. However, the specific molecular targets of the co-treatments are not well established.

Among the BCR-ABL target proteins, the signal transducer and activator of transcription 3 (STAT3) has been shown to be directly phosphorylated by BCR-ABL [12, 13].

Constitutive activation of STAT3 has been reported in a number of haematological neoplasias, such as leukemias and lymphomas [14–16], as well as in solid tumours of the breast, bladder, head, neck and prostate [17–20]. Therefore, the STAT3 protein has emerged as a promising molecular target for the treatment of various cancers [21].

Natural derivative products, peptidomimetics, platinum compounds and small molecules have been used to inhibit STAT3 activity [22]. Treatment with cathecois, quinolinones, anthracenes and anthraquinone derivatives have been used extensively to inhibit STAT3 activity in cancers [23, 24]. Although STAT3 inhibitors have shown some promise for some cancers, they have not been well evaluated in the context of CML.

Here, we tested the effects of an anthracene derived compound, herein referred to as LLL-3, in BCR-ABL and STAT3 expressing cell lines. LLL-3 preferentially inhibited the proliferation of the STAT3-expressing cell line DU145, but did not affect the proliferation of MCF-7, a cell line with low expression of STAT3 [25]. The viability of the K562 cell line treated with LLL-3 was reduced in a time- and dose-dependent manner. In combination with Imatinib, LLL-3 has shown improved inhibition of cell growth in BCR-ABL-positive cell lines. Moreover, LLL-3 enhanced Imatinib-induced apoptosis of K562 cells. Our results suggest that LLL-3 may have therapeutic potential in treating STAT3-positive cancers, and as an addition to Imatinib therapy in the treatment of diseases that involve the BCR-ABL oncoprotein.

Materials and methods

Cell lines

All cell lines were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (Hyclone), 100 U/ml penicillin (Invitrogen), 100 mg/mL streptomycin (Invitrogen) at 37°C in 5% CO2. K562, established from a CML patient in blast crisis, was used as a BCR-ABL-positive cell line. MBA, megakaryocytic cell line, is stably transfected with BCR-ABL [26]. Mo7e, megakaryocytic cell line, was used as negative BCR-ABL cell line [26]. DU145, a prostate cancer cell line, was used as a STAT3-overexpressing cell line [25]. MCF-7, established from a breast cancer tumour, was used as a low STAT3-expressing cell line [25].

Drug treatment

Cell lines were exposed to different doses of LLL-3 and IM dissolved in Dimethyl sulphoxide (DMSO; Sigma–Aldrich). The number of viable cells was determined at 24 and 48 h post-treatment. DMSO-treated cells were used as vehicle controls. LLL-3 was kindly provided by Dr. Pui-Kai Li from The Ohio State University (Columbus, OH, USA). The concentration of drug necessary to achieve 50% cellular growth inhibition was denoted as the IC_{50}.

Viability determination

The viability of cells was measured using a 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) assay (Roche). Approximately 2×10^5cells/mL K562 and MBA cells were treated with 40 μM of LLL-3. Adherent cells lines, such as DU145 and MCF-7, were seeded at 5×10^3 cells per 100 μL/well. Cells were plated into 96-well microplates (Corning) for 24 h, and treated with LLL-3. After 24 h, 10 μL WST-1 was added to each well, and plates were incubated at 37°C for an additional 2 h. Plates were read on a microplate reader (Bio-Rad, model 550) at 450 nm with a reference wavelength at 630 nm. Data analyses and graphing were performed in GraphPad Prism version 4 (GraphPad Software, Inc.).

Sub-G1 DNA content and annexin V staining

Sub-G1 DNA distribution was analysed using propidium iodide (PI; Sigma–Aldrich) as described by Nicoletti et al. [27]. The cells in culture were subjected to different treatments and were washed in phosphate-saline buffer, reconstituted in 300 μL of hypotonic buffer (0.1% Sodium citrate, 0.1% Triton-X, 100 μg/ml RNase, 50 μg/mL PI) and incubated for 30 min at 4°C. Apoptosis was evaluated via analysis of Annexin V-FITC staining (Becton, Dickinson and Company). Briefly, K562 cells treated with LLL-3 and IM drugs were harvested in 500 μL of Binding buffer (10 mM Hepes [pH 7.4], 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2), stained with 1 μL of Fluorescein-labelled Annexin V-FITC (5 μl), followed by incubation for 20 min in the dark. Propidium iodide (1.5 μg/mL) was added to the incubated tubes prior to FACS analysis. Ten thousand events were collected from each sample in a FACSCalibur Flow Cytometer (Becton, Dickinson and Company). Annexin V-FITC (+), PI (-) cells were considered apoptotic events. The data were analysed using Cell Quest software (Becton, Dickinson and Company).

Caspase-3/7 activation

To verify activation of Caspases-3/7, we used the Caspase-3/7-Glo Assay (Promega) to evaluate the effects of LLL-3 on K562 cells. The assay was performed according the manufacturer's recommendations. Caspase activation was also assessed via STAT3 inhibition using a phospho-peptide (pTyr-Pep) [28]. Caspase-3/7 activity was measured in the Veritas Luminometer (Promega).

Statistical analysis

Data are expressed as means ± standard deviation (SD). The significance of differences between control and treated groups was evaluated using one-way analysis of variance (ANOVA). Experimental tests were performed at least three times. Differences were considered to be significant when $P < 0.05$.

Results

LLL-3 inhibits proliferation of BCR-ABL positive cells and improves Imatinib effects to decrease viability of BCR-ABL expressing cell lines

To test the efficacy of the LLL-3 molecule in inhibiting BCR-ABL cell line growth and its additive effect with IM, we compared cellular viability between treated and untreated cells and co-treated K562 cells. The proliferation of the K562 cell line was inhibited following 40 μM of LLL-3 in a dose- and time-dependent manner at 24 h (IC_{50} 37 μM) and 48 h (IC_{50} 6.3 μM) (Fig. 1a, b). We also tested the potential of LLL-3 in the survival of cells with high levels of STAT3 activation. LLL-3 dramatically inhibited the survival of DU145 cells (IC_{50} 11.3 μM), which also exhibit high STAT3 expression compared to MCF-7 (IC_{50} 150 μM) cells that present low levels of STAT3 expression (Fig. 1c, d). The observed growth inhibition of a STAT3-dependent cell line by LLL-3 was in agreement with previous results by Bhasin [25]. When LLL-3 demonstrated a capacity to inhibit K562 cell growth, we tested whether LLL-3 could enhance IM efficiency to decrease cellular proliferation. Co-treatment of 40 μM LLL-3 and 1 μM IM increased the percentage of nonviable K562 cells by 21% at 24 h and 22% at 48 h compared to IM treatment alone (Fig. 2a, b). Additionally, we tested LLL-3 co-treatment with IM in BCR-ABL-positive and negative cells, MBA

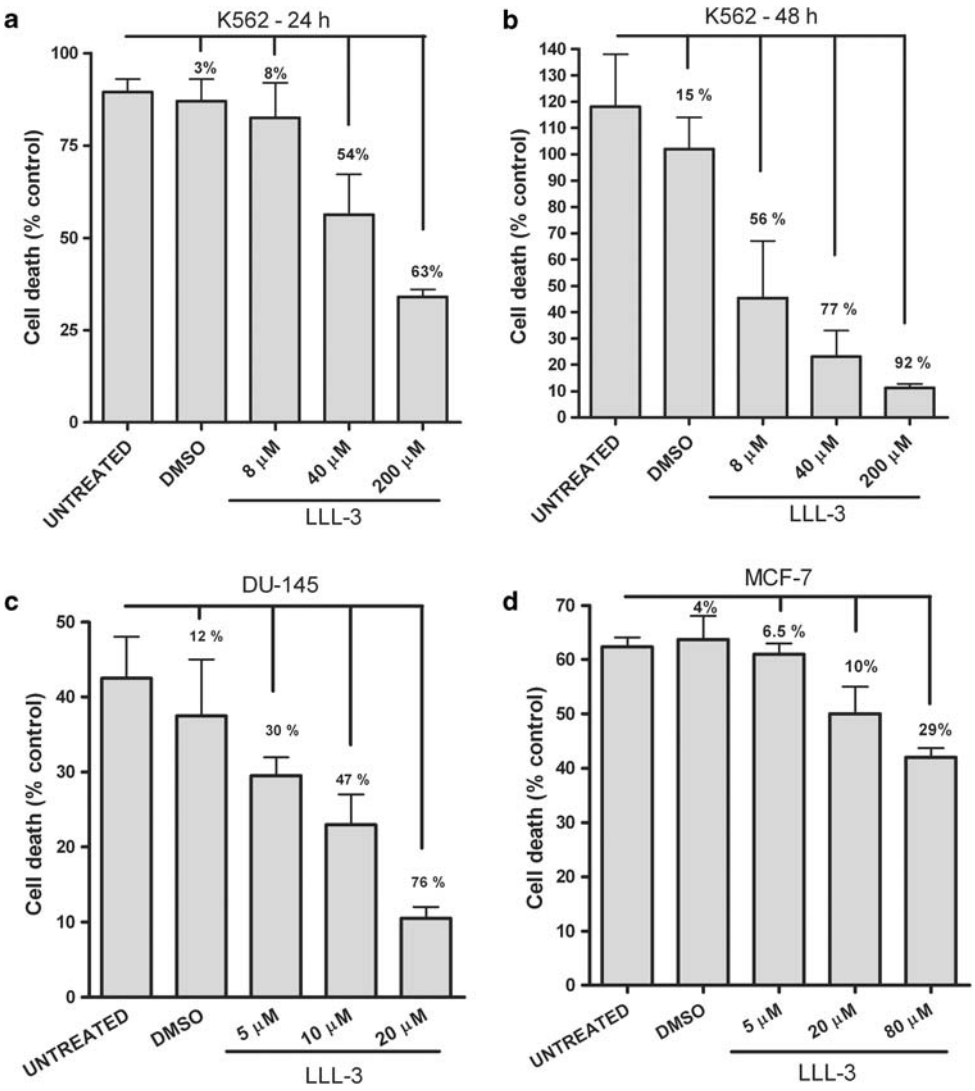

Fig. 1 LLL-3 inhibits K562 viability. The K562 cell line was treated with a range of doses of LLL-3 (200, 40 and 8 μM) (**a**). Viability effects were assessed at 24 and 48 h after drug addition (**a**, **b**). Comparative viability effects of LLL-3 between DU145, a STAT3 positive cell, and MCF-7, a lower or negative STAT3 cell, after 24 h of treatment (**c**, **d**). The data represents the means of independent experiments ± standard deviation. The difference between the treated and untreated cells in (**a**) is significant ($P < 0.05$) with 40 μM of LLL-3, in (**c**) is significant ($P < 0.05$) with 10 μM of LLL-3 and, in (**d**) is significant ($P < 0.05$) with 80 μM of LLL-3

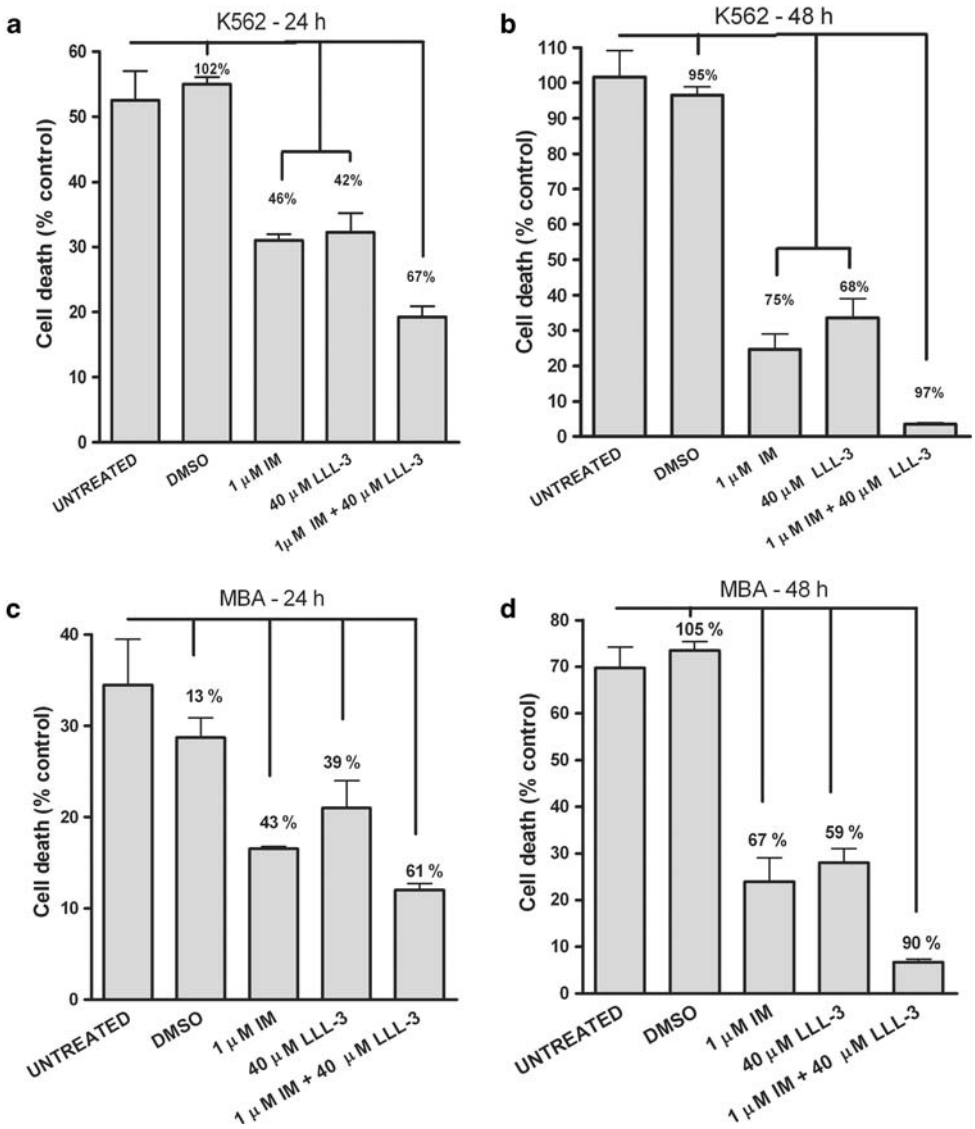

Fig. 2 LLL-3 improves IM effects in BCR-ABL positive cell lines. K562 (**a**, **b**) and MBA (**c**, **d**) cells were used as BCR-ABL-positive cell lines. Cells were treated with 40 μM of LLL-3, 1 μM of IM or both drugs, and synergism was assessed after 24 h (**a**, **c**) and 48 h (**b**, **d**). Percentages at the top of the *bars* show the amount of cell death compared to untreated cells. The data represent the means of three independent experiments ± standard deviation. The difference between single-treated and LLL-3/IM treated in (**a**, **b**, **c**, **d**) is significant ($P < 0.05$)

and Mo7e, respectively. For this, the MBA cell line was treated with 40 μM LLL-3 jointly with 1 μM IM, which resulted in decrease in the percentage of proliferation by 18% at 24 h and 23% at 48 h compared to IM treatment alone (Fig. 2c, d). In the BCR-ABL-negative cell line, Mo7e, both LLL-3 neither IM had no effect in the cell viability (data not shown). These preliminary results suggest that LLL-3 is a potent proliferation inhibitor of cells with high expression of STAT3 such as DU145 cell line. Moreover, LLL-3 inhibited the growth of K562 cells, suggesting that co-treatment with LLL-3 and IM may further inhibit proliferation of BCR-ABL-positive cells.

LLL-3 acts additionally with IM to promote apoptosis in the K562 cell line

To investigate whether LLL-3 treatment decreased K562 viability by initiating apoptosis, we assessed markers of apoptosis through Annexin V-FITC cell staining and sub-G1 DNA content analysis. We also tested if LLL-3 acted additionally with IM in promoting apoptosis. A total of 28.5% (±2.3) of K562 cells incubated with 40 μM of LLL-3 for 24 h showed positive staining for Annexin V-FITC (Fig. 3b). In addition, K562 cells treated with 1 μM of IM for 24 h resulted in 18.2% (±4.7) Annexin V-FITC-positive cells (Fig. 3c). Interestingly, K562 co-treated with 40 μM LLL-3 and 1 μM IM for 24 h resulted in 47.5% (±3.6) Annexin V-FITC positive cells (Fig. 3d). The content of sub-G1 DNA, a determinate of cell-cycle progression, is another indicator of apoptotic events [29]. LLL-3 or IM treatment increased the amount of sub-G1 DNA by 26% (±3.7) and 16% (±5.1), respectively, over a period of 24 h compared with untreated K562 cells (Fig. 4a). LLL-3 administered together with IM increased the sub-G1 DNA content by 25% (41% ± 6.3 compared to the IM treatment alone (Fig. 4a). These observations suggest that treatment

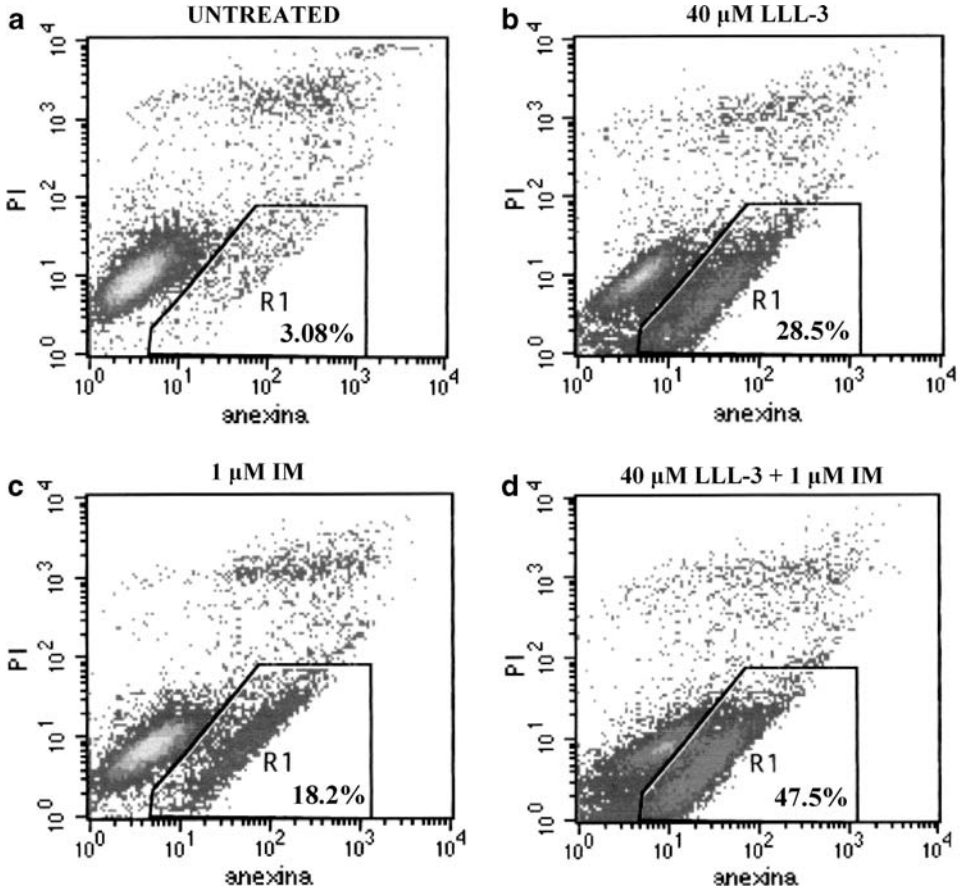

Fig. 3 Percentages of Annexin V-FITC staining positive cells after LLL-3 and IM treatment. The K562 cell line left untreated (**a**). K562 cells treated for 24 h with 40 μM of LLL-3 (**b**) ($P < 0.01$) or 1 μM of IM (**c**) ($P < 0.01$). K562 cells co-treated with 40 μM of LLL-3 and 1 μM of IM (**d**) for 24 h ($P < 0.05$)

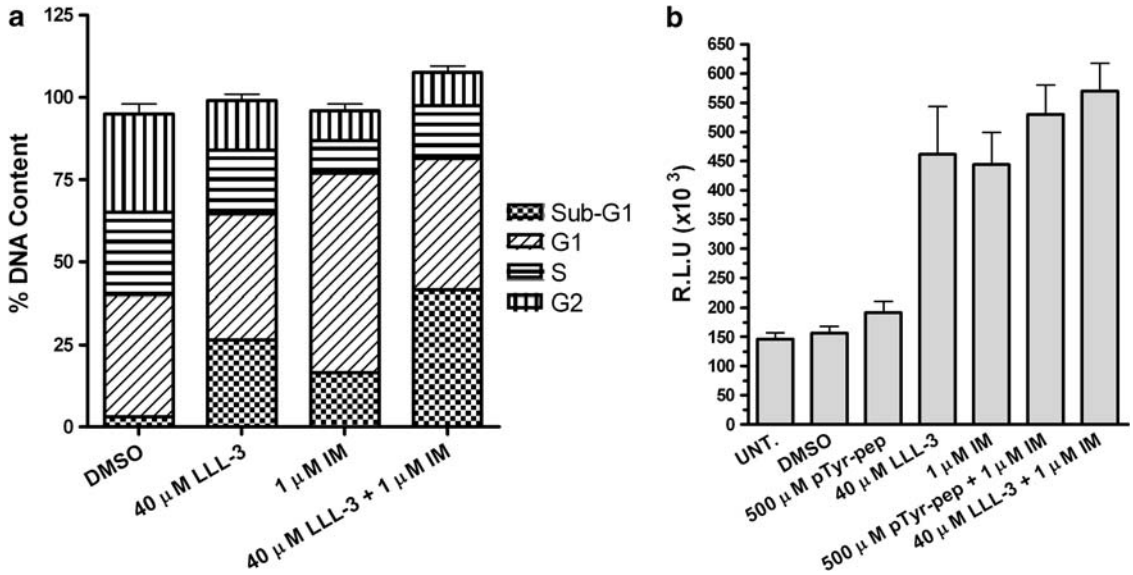

Fig. 4 LLL-3 increases sub-G1 DNA content and actives Caspase-3/7 in K562 cells. Sub-G1 DNA was assessed by flow cytometry analysis to estimate apoptotic events. The graph represents the mean percentage of cells in the indicated phases of the cell cycle (**a**), in three independents experiments. Caspase-3/7 activation was determined in response to 40 μM of LLL-3 ($P < 0.05$), 1 μM of IM ($P < 0.05$), blockade of the STAT3 peptide or in combination of this drugs (**b**). Responses are noted in Relative Luminescent Units (RLU)

with LLL-3, alone or in combination with IM, initiates apoptosis.

LLL-3 and STAT3 blocking peptide activates apoptosis and initiates Caspase-3/7 protein in K562 cell line

In an effort to determine if LLL-3 promotes Caspase-3/7 activation, we evaluated Caspase-3/7 activity using a luminescence assay. LLL-3 or IM treatment significantly increased the luminescent signal, which indirectly indicates Caspase-3/7 activation. We also blocked STAT3 activity with a tyrosine phosphoryledpeptide (pTyr-pep) and measured Caspase-3/7 activation by luminescent assays. Blocking STAT3 with 500 μM of pTyr-peptide slightly showed to activate Caspase-3/7 (Fig. 4b). These assays suggest that LLL-3 may activate apoptosis via Caspase-3/7 substrate cleavage, and STAT3 inhibition also activates Caspases-3/7 in the K562 cell line but in not the same magnitude as LLL-3 (Fig. 4b). Additionally, combination of 40 μM of LLL-3 or 500 μM of pTyr-pep with 1 μM of IM increased Caspase-3/7 compared to single IM treatment (Fig. 4b).

Discussion

Although Imatinib has been extensively used for CML treatment, several studies have attempted to increase the effectiveness of Imatinib therapy by combining it with other drugs in an effort to prevent activation of cellular mechanisms of drug resistance [30]. Conventional chemotherapeutics such as daunorubicin, cytosine arabinoside and interferon-alpha have been shown to act synergistically when co-treating with Imatinib in BCR-ABL expressing cells compared to Imatinib therapy alone [10]. Additionally, BCR-ABL downstream-activated signalling pathways have been tested as targets for drug inhibition in combination with Imatinib [10]. Specifically, JAK2, PI3K and Ras inhibitors have enhanced the antiproliferative effects of Imatinib against BCR-ABL positive cell lines and induced apoptosis even in IM-resistant cell lines [10].

LLL-3 is a small molecule that is based on anthracene-backbone derivatives, and is structurally similar to a commercially available compound, STA-21 [25]. STA-21 has been successfully used to inhibit the growth of STAT3 expressing cell lines, such as MDA-MB-468, MDA-MB-435s, DU145 and PC3, by docking into the STAT3 SH2 domain [31]. LLL-3 is synthetically easier to generate than STA-21, and retains the functional STA-21 characteristics such as docking in the STAT3 SH2 domain and has the capacity to inhibit the growth of the aforementioned cell lines at similar doses of STA-21 [25].

Anthracene derivatives have shown anti-tumour activity clinically [32]. However, their clinical use is frequently limited by the emergence of drug resistance during therapy [32]. For example, HL-37, an anthracene derivative, exhibited potent anti-cancer activity in both K562 and multidrug-resistant K562 cell lines. HL-37 induces K562 cell apoptosis through Caspase activation [32].

In this context, our work explored the potential of LLL-3 as a therapeutic molecule in BCR-ABL cell lines, alone or concomitantly with Imatinib treatment. LLL-3 has been shown to inhibit the proliferation of K562 cells in a dose- and time-dependently manner. Additionally, simultaneous LLL-3 and IM treatment decreases the growth of BCR-ABL expressing cell lines, such as K562 and MBA, compared to LLL-3 or IM treatment alone. We also compared the potential of LLL-3 to decrease both STAT3-positive and STAT3-negative cell proliferation. As demonstrated by Bhasin and co-workers, our results showed that the survival of DU145, a STAT3-activated cell line, was dramatically reduced after treatment with low doses of LLL-3, and the MCF-7 cell line, a lesser STAT3-expressing cell line, exhibited inhibition of proliferation with high doses of LLL-3 [25]. This suggests that LLL-3 could be used for growth inhibition in cells that exhibit high levels of activated STAT3 and also for BCR-ABL-positive cell lines.

Recent studies have focused on understanding and deciphering mechanisms of Imatinib resistance in an effort to override the relapse of Imatinib treatment through combination therapies [33]. Some studies have linked STAT3 activation with chemoresistance and radioresistance [34]. Constitutive phosphorylation of STAT3 has recently been linked to K562 resistance to Imatinib, in bone marrow conditioned medium [35]. Moreover, some data suggest that STAT3 activation can compensate for BCR-ABL signals and thus represents a potential BCR-ABL-independent mechanism of Imatinib resistance [35]. Hence, inhibitors of STAT3 have potential in the treatment of cancer and prevention of resistance in malignancies.

STAT3 activation also occurs transiently in normal cells, although its constitutive activation has been reported in several cancers [14–21]. In this context, therapeutic approaches that target STAT3 might kill cancer cells with a minimal effect on normal cells. In this study, we also tested a peptide that specifically inhibits STAT3 [28]. The K562 cell line incubated with 500 μM of STAT3-blocking peptide (pTyr-pep) shows low apoptosis in a Caspase-3/7-dependent manner. However, LLL-3 triggers Caspase-3/7 activation in elevated levels. Thus, LLL-3 may be useful to suppress cancer cell viability by activating the apoptotic cascade.

This data suggest that a small molecule, LLL-3, was more effectiveness in Caspase-3/7 activation than pTyr-pep. The efficiency of the LLL-3 in actives Caspase-3/7 might be related with the higher cell permeability and affinity of the LLL-3 to docking in SH2-STAT3 domain than peptide.

Moreover, LLL-3 could be docking at similar SH2 domain of other STAT members activated by BCR-ABL signalling.

Several studies have shown that STAT5 is also target of BCR-ABL signalling [36]. STAT5, as well STAT3, have been described as key regulators of proliferation and apoptosis in myriad of malignancies, including CML [12–19, 36]. The LLL-3 molecule designers, suggested that LLL-3 dock with STAT3-SH2 domain at Arg-609 and Ile-634 amino acids [25]. However, they did not evaluate if LLL-3 dock at STAT5-SH2 domain. In the STAT5 protein, the Ile-634 residue was not conserved in the same position as found in STAT3 protein. Hence, more detailed study evaluated the potential of LLL-3 dock and inhibit other molecular targets.

Turning off BCR-ABL activation using IM leads to an increase of apoptosis in the K562 cell line. Additionally, the combination of LLL-3 with IM significantly increased K562 apoptotic percentage. Hence, we hypothesise that LLL-3 could improve the eradication of BCR-ABL cells during IM therapy, thus preventing drug resistance by increasing the apoptotic ratio.

Taken together, these preliminary analyses suggest that LLL-3 may improve treatment of CML and STAT3 constitutive activated cancers, although a more detailed study is needed. Drug cocktails that target distinct signalling pathways known to be activated by BCR-ABL would increase the effectiveness of Imatinib therapy at later phases of CML and may also prevent the development of Imatinib resistance.

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