Response to Rotenone Is Glucose-Sensitive in a Model of Human Acute Lymphoblastic Leukemia: Involvement of Oxidative Stress Mechanism, DJ-1, Parkin, and PINK-1 Proteins

Miguel Mendivil-Perez, Marlene Jimenez-Del-Rio, and Carlos Velez-Pardo

Neuroscience Research Group, Medical Research Institute, Faculty of Medicine, University of Antioquia (UdeA), Calle 70 No. 52-21 and Calle 62 No. 52-59, Building I, Room 412, Medellin, Colombia

Correspondence should be addressed to Carlos Velez-Pardo; carlos.velez@neurociencias.udea.edu.co

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1. Introduction

Acute lymphoblastic leukemia (ALL) is a type of hematologic disorder characterized by excess production of lymphoblast cells. ALL represents 12% of all leukemia cases, with a worldwide incidence projected to 1–4.75 per 100,000 people [1]. Despite significant improvements in the treatment of ALL, pediatric (~30%) and adult (50–70%) patients develop treatment-resistant disease. Furthermore, hyperglycemia during hyper-CVAD (fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone) chemotherapy is associated with poor outcomes of ALL in about 35% of patients [2]. However, whether the action of new therapeutic compounds is different in hyperglycemic conditions versus normoglycemia in ALL patients is still unknown. Because leukemia cells are metabolically flexible, this condition might potentiate ALL cells to develop treatment resistance. At present, evasion of apoptosis—a type of programmed cell death—is one of the eight essential alterations in cellular physiology leading to malignancy [3]. Apoptosis displays morphological and biochemical hallmarks [4, 5] involving chromatin condensation, nuclear fragmentation, rounding-up of the cell, reduction of cellular volume, and metabolic and energetic modifications of the mitochondria. Reactivation of apoptosis therefore appears as a prime strategy to eliminate cancer cells. Specifically, the identification of molecular pathways of cell death in ALL is an essential step towards therapeutic strategies. Before reaching this aim, the mechanism(s) must be unravelled.

Since mitochondria play essential roles in cellular metabolism, redox homeostasis, and the regulation of cell death, they represent an important target for anticancer therapy [6].
Interestingly, several natural compounds have been demonstrated to preferentially kill cancer cells with mitochondrial dysfunction [7]. Indeed, rotenone (ROT), a class 5 mitocan [8] that derives from the roots and backs of the Derris and Lonchorcarpus plant species, irreversibly binds to the Complex I (NADH: ubiquinone oxidoreductase) of the mitochondrial electron transport chain. This inhibition allows the reduction of molecular oxygen ($O_2$) to superoxide anion radical ($O_2^·$) by the iron-sulfur cluster N2, and subsequent generation of $H_2O_2$, most probably by the flavin mononucleotide (FMNH$^+$) of Complex I [9]. ROT induces apoptosis in several cancerous cells [10–13]. However, whether the activation of executor protease caspase-3 [13, 14] and the stress response c-Jun N-terminal protein kinase (JNK) pathway [12, 15, 16] occur in cancer cell lines exposed to ROT is not yet fully resolved. Moreover, ROT may or not be regulated by glucose [17] or may not [15] provoke cell death in pheochromocytoma PC12 cells. Therefore, the mechanism underlying ROT-induced apoptosis in cancer cells is not completely clear.

Recently, our group has provided evidence that oxidative stress (OS) generated by glucose-starvation (GS) induces apoptosis-inducing factor (AIF)- and caspase-3-dependent mitochondrial mechanisms of cell death in Jurkat cells (a model of human acute lymphoblastic leukemia) characterized by the activation of transcription factors such as nuclear factor-kappa B (NF-$κ$B), p53, c-Jun; activation of c-Jun N-terminal kinase, AIF translocation to nuclei, mitochondria depolarization, and nuclear fragmentation as evidence of caspase-3 activation [18]. These observations have led us to postulate minimal completeness of cell death signaling induced by OS as a mechanistic explanation of cancer cell demise [19]. However, whether ROT induces a similar cell death pathway in Jurkat cells and whether ROT action can be regulated by glucose is not yet clear.

Interestingly, Zhang et al. [20] have shown that p53 increases the transcription of PARKIN gene both in vitro and in vivo under OS. In turn, Parkin contributes to the role of p53 in regulating glucose metabolism and antioxidant defense by activating NF-$κ$B [21, 22]. Surprisingly, PARKIN and other genes such as PINK-1 (Phosphatase and tensin homolog (PTEN)-induced novel kinase-1) and DJ-1 have been associated with leukemia [23, 24]. How these molecules interplay with p53, NF-$κ$B, and AIF in OS-induced apoptosis in Jurkat cells under different metabolic conditions (e.g., glucose) is not yet established.

On the assumption that a high-glucose milieu may interfere with the cell response to drugs, we investigated the ROT-induced toxicity in low (11mM, G11) and high (55mM, G55) glucose concentrations that mimic in vivo conditions of normoglycemia and hyperglycemia, respectively. To get insight, we sought (i) to investigate whether ROT induces apoptosis in Jurkat cell line; (ii) to determine whether ROT treatment induces OS through $O_2^·$/H$_2$O$_2$, caspase-3, AIF; and the activation of proapoptotic transcription factors NF-$κ$B, p53, and c-Jun. We also investigated (iii) whether ROT differentially alters DJ-1, Parkin, and PINK-1 protein expression in Jurkat cells in both glucose conditions. The present investigation suggests that combined therapy by using mitocan compounds and metformin might efficiently terminate leukemia cells via apoptosis in hyperglycemic ALL patients.

## 2. Materials and Methods

3,3’-dihexyloxacarbocyanine iodide (D$_2$OC$_6$(3), Catalog number D-273), ammonium pyrrolidinethiocarbamate (PTDC, Catalog number 548000), and 1,9-pyrazoloanthrone (SP600125, Catalog number 420119) were acquired from Calbiochem. Dichlorofluorescin diacetate (DCFH$_2$-DA) was obtained from Invitrogen. All other reagents were purchased from Sigma-Aldrich.

### 2.1. Jurkat T Leukemia Cell Culture

Jurkat clone E6-1 (ATCC Catalog number TIB-152) was cultured according to supplier’s indications.

### 2.2. Experiments with Jurkat Leukemia Cell Line

#### 2.2.1. Morphological Assessment of Cell Death by Fluorescent Microscopy

The cell suspension (1mL, final volume) was exposed to increasing ROT (0–100µM) concentrations freshly prepared in RPMI-1640 medium either supplemented with 11mM (hereafter 11G) or 55mM (hereafter 55G) glucose in the absence or presence of different products of interest for 1–24h at 37°C. Cells were incubated with 1µL acridine orange (AO, 0.1mg/mL)/ethidium bromide (EB, 0.1mg/mL)/Hoechst (H, 3µg/mL) staining. Quantiﬁcation of apoptotic morphology was performed by ﬂuorescent microscopy analysis. The apoptotic indexes were assessed 3 times in independent experiments blind to experimenter.

#### 2.2.2. Evaluation of Intracellular Reactive Oxygen Species (ROS)

Superoxide anion radicals were evaluated as described in Ref Quantiﬁcation of nitroblue tetrazolium positive cells (NBT$^+$) was performed blind to experimenter. The assessment was repeated 3 times in independent experiments. Hydrogen peroxide ($H_2O_2$) was determined with 2′,7′-dichlorofluorescin diacetate (5µM), using a ﬂow cytometer Beckman Coulter Epics XL and ﬂuorescent microscopy analysis, respectively. The assessment was repeated 3 times in independent experiments.

#### 2.2.3. Analysis of Mitochondrial Membrane Potential ($ΔΨ_m$) by Flow Cytometry and Fluorescent Microscopy

Jurkat cell line was treated as described above. Then, cells (1×10$^6$) were incubated with cationic lipophilic (10nM) and (1µM) DIOC$_6$ (3) final concentration for 20min at RT in the dark. Cells were then analyzed using ﬂow cytometer Beckman Coulter Epics XL and ﬂuorescent microscopy analysis, respectively. The assessment was repeated 3 times in independent experiments.

### 2.3. Immunocytochemistry Detection of Transcription Factor NF-$κ$B, p53, c-Jun, Caspase-3, Apoptosis-Inducing Factor (AIF), DJ-1, Parkin, and PINK-1

The Santa Cruz Biotechnology (SCB) supplier protocol goat ABC staining System was followed.
(Catalog number sc-2023) was followed for the immunocytochemistry using primary goat polyclonal antibodies NF-κB p65 (C-20)-G (Catalog number sc-372-G), p53 (FL-393) (Catalog number sc-6243-G), p-(Ser73)-c-Jun (Catalog number sc-7981), caspase-3 (Catalog number sc-22171) and AIF (Catalog number sc-9417) and SCB protocol goat ABC staining System (Catalog number SC-2018) was followed for the immunoocytochemistry using primary antibodies DJ-1 (FL189) (Catalog number sc-32874), Parkin (H300) (Catalog number sc-30130) and PINK-1 (H300) (Catalog number sc-33796). The cells were immune-stained and diaminobenzidine positive (DAB⁺) cells were quantified blind to experimenter.

2.4. Detection of DJ-1, Parkin, and PINK-1 by Flow Cytometry. After each treatment with or without ROT in GI1 or G55, cells (1 × 10⁶) were fixed in 80% ethanol and stored at −20°C overnight. Then after that, cells were washed with PBS and permeabilized with 0.2% triton X-100 plus 1.5% bovine serum albumin (BSA) in phosphate buffer solution (PBS) for 30 min. Cells were washed and incubated with 20 μg/mL (diluted in PBS containing 0.1% BSA) primary antibody DJ-1 (FL189) (Catalog number sc-32874), Parkin (H300) (Catalog number sc-30130), and PINK-1 (H300) (Catalog number sc-33796) for 2 h at 37°C under agitation. Subsequently, the cells were washed and incubated with FITC-conjugated rabbit-antigoat-IgG antibody (Catalog number sc-2018) was followed for the immunocytochemistry using primary antibodies DJ-1, Parkin, and PINK-1 by Flow Cytometry. 2.5. Evaluation of Signal Inhibitors on Cells Exposed to Rotenone. The cell suspension (1 mL, final volume) was treated with (50 μM) ROT in glucose (11G) and inhibitor reagents at the concentration listed in Table 1 at 37°C for 24 h. After this time, cells were evaluated for apoptotic features (nuclei morphology and ΔΨₘ) by fluorescence microscopy or flow cytometry, as described in previous sections. The assessment was repeated 3 times in independent experiments.

2.6. Statistical Analysis. Data are means ± S.D. of three independent experiments. One-way ANOVA analyses with Bonferroni or Games-Howell post hoc comparison were calculated using SPSS 18 software. A P value of * P < 0.05 and ** P < 0.001 was considered significant.

2.7. Photomicrography. The light microscopy or fluorescent photomicrographs were taken using a Zeiss (Axiostart 50) microscope equipped with a Canon PowerShot G5 digital camera.

Table 1: Oxidative stress and signalling molecules (NF-κB, c-Jun, p53, and caspase-3) are involved in rotenone (ROT)-induced apoptosis in Jurkat T cells.

| Treatment/assay | AO/EB/H (%) | PI/ΔDOC₃(3) (%) |
|-----------------|-------------|-----------------|
| Untreated       | 1 ± 0*      | 98 ± 1**        |
| ROT (50 μM)     | 50 ± 3**    | 51 ± 2**        |
| PDTCT (10 nM)   | 1 ± 0**     | 97 ± 1**        |
| PDTCT (10 nM) + ROT (50 μM) | 9 ± 3** | 89 ± 3** |
| PFT (50 nM)     | 1 ± 0**     | 98 ± 1**        |
| PFT (50 nM) + ROT (50 μM) | 11 ± 2**  | 87 ± 3**       |
| SP600125 (1 μM) | 2 ± 1**     | 97 ± 1**        |
| SP600125 (1 μM) + ROT (50 μM) | 6 ± 2**  | 93 ± 3**       |
| NSCI (10 μM)    | 1 ± 0**     | 98 ± 1**        |
| NSCI (10 μM) + ROT (50 μM) | 5 ± 1**  | 94 ± 2**       |
| NAC (1 mM)      | 1 ± 0**     | 98 ± 1**        |
| NAC (1 mM) + ROT (50 μM) | 10 ± 3**  | 89 ± 1**       |

Jurkat cells (1 × 10⁶ cells/mL) were exposed to ROT (50 μM) in absence or presence of PDTCT (10 nM), pifithrin-α (PFT, 50 nM), SP600125 (1 μM) and NSCI (50 μM) at 37°C 24 h. Cells were then evaluated for apoptotic nuclei morphology and mitochondrial membrane potential, as described in Material and Methods. The percentage of positive AO/EB/Hoechst staining and PI/ΔDOC₃(3) of Jurkat cells treated with or without ROT is expressed as a mean percentage ± S.D. from three independent experiments. * P < 0.001.

3. Results

3.1. Rotenone (ROT) Induces Nuclei Morphology Distinctive of Apoptosis in Jurkat T Cells Associated with Superoxide Anion Radical (O₂⁻)/Hydrogen Peroxide (H₂O₂) Generation and Impairment of Mitochondrial Membrane Potential (ΔΨₘ). We initially evaluated the effect of ROT in Jurkat cells under standard culture conditions (glucose medium containing 11 mM glucose, GI1). As shown in Figure 1, ROT induced generation of O₂⁻ (Figure 1(a)) in a concentration-dependent fashion up to 50 μM (from 25% to 58%), declining to ~20% FORMz at 100 μM (Figure 1(g)). On the contrary, ROT generated H₂O₂ (Figure 1(b)) almost constantly (48–55% DCF⁺) up to 50 μM, but its generation was reduced by ~30% at 100 μM (Figures 1(e), 1(g)). Concurrently, ROT provoked loss of ΔΨₘ (Figures 1(c) and 1(f)) and nuclei condensation/fragmentation (Figure 1(d)) in Jurkat cells in a concentration fashion (Figures 1(f) and 1(g)), as assessed by fluorescence microscopy (Figures 1(c) and 1(d)) and flow cytometry (Figures 1(f), 1(g), and 6(a)), respectively. Because 50 μM ROT provoked ~50% nuclei fragmentation/ΔΨₘ depolarization and it is an end-point for O₂⁻/H₂O₂ generation, this concentration was selected for further experiments. Of note, the antioxidant N-acetyl-cysteine (NAC, 1 mM) significantly reduced the proapoptotic effect of ROT in Jurkat cells (Table 1).

3.2. ROT Induces Apoptosis in Jurkat T Cells Associated with NF-κB, c-Jun, p53 and c-Jun Transcription Factors, Caspase-3 Activation, and Apoptosis-Inducing Factor (AIF). To evaluate the participation of signaling molecules in ROT-induced apoptosis, Jurkat cells were either preincubated one hour...
Figure 1: Continued.
with PDTC (10 nM, an inhibitor of NF-κB), PFT (50 nM, a specific inhibitor of p53), SP600125 (1 μM, a specific inhibitor of c-Jun N-terminal kinase), or NSCI (10 μM, a specific inhibitor of caspase-3) prior to incubation with ROT (50 μM) in G11 for 24 h. All the specific pharmacological inhibitors moderately reduced the ROT-induced apoptotic effect and the ΔΨm compared to control (Table 1). The participation of the transcription factors, caspase-3, and AIF was also evaluated by immunohistochemistry. As shown in Figure 2, ROT clearly induced DAB⁺ nuclei staining of the active form of NF-κB (Figure 2(b)), p53 (Figure 2(d)), c-Jun (Figure 2(f)), CASP-3 (Figure 2(h)), and AIF (Figure 2(j)) compared to untreated cells (Figures 2(a), 2(c), 2(e), 2(g), and 2(i)) with a different percentage of detection (Figure 2, insets). Noticeably, the morphology of the stained DAB⁺ cells corresponded exactly to the nuclei fragmentation observed under fluorescence microscopy (see Figures 1(d) and 2(b), 2(d), 2(f), 2(h), and 2(j)).

### 3.3. ROT Induces DJ-1 Cytoplasmic Accumulation and Parkin Overexpression but Has No Influence on PINK-1 Protein

Next, we examined whether DJ-1, Parkin, and PINK-1 were involved in ROT-induced cell death. As shown in Figure 3, the expression or the presence of Parkin monomers rather than aggregates, compared to untreated cells (Figure 4(c)). PINK-1 protein levels were unaffected in cells under ROT exposure (Figure 4(e)).
Rotenone induces activation of the transcription factors, apoptosis-inducing factor, and caspase-3 in Jurkat T cells. Leukemia cells were left untreated (a), (c), (e), (g), and (i) or exposed to (50 μM) ROT (b), (d), (f), (h), and (j) in G11 medium for 24 h. Cells were stained with anti-NF-κB-p65 (a) and (b)), anti-p53 (c) and (d)), anti-c-Jun ((e) and (f)), anti-caspase-3 (g) and (h)), and anti-AIF ((i) and (j)), antibodies according to procedure described in Materials and Methods. Notice that positive nuclei (dark brown color) reflect their nuclear translocation/activation and appear to correlate with the apoptotic nuclear morphology. Magnification 1000x ((a)–(j)). Insets: percentage of positive DAB staining.

ROT (50% apoptotic nuclei: 10% stage I, 40% stage II nuclei morphology), as evaluated by triple AO/EB and Hoechst staining techniques (Figure 1(d)). These observations were confirmed by DNA fragmentation (Figure 5(b)) and ΔΨₘ depolarization (Figure 5(c)), as evaluated by flow cytometer. Of note, the ΔΨₘ was significantly preserved in cells exposed to ROT in G55 compared with cells exposed to ROT in G11. We then established generation of ROS, activation of signaling (NF-κB, p53), executor (caspase-3, AIF), and mitochondrial maintenance (DJ-1, Parkin, and PINK-1) molecules under G55 condition. As noted in Figure 5(d), cells exposed to ROT generated high levels of H₂O₂, shown as MFI, irrespective of glucose medium concentration. Likewise, cells under G55 + ROT showed a low percentage of NF-κB (Figure 6(b)), p53 (Figure 6(d)), c-Jun (Figure 6(f)), caspase-3 (Figure 6(h)) activation, and AIF (Figure 6(j)) compared with cells under G55 alone (Figures 6(a), 6(c), 6(e), 6(g), and 6(i)). Furthermore, cells exposed to G55 plus ROT also displayed low percentage of DJ-1 (Figures 4(b) and 7(b)) and Parkin (Figures 4(d) and 7(d)), evaluated as DAB⁺ cells and...
MFI units, respectively. PINK-1 showed similar percentages of DAB+ cells and MFI units in cells under G55 and ROT (Figures 4(f) and 7(f)).

3.5. Metformin Sensitizes Jurkat Cells against ROT-Induced Toxicity. Since metformin, a recognized glucose-lowering drug, has been shown to strongly interfere with survival and proliferation on Jurkat cells [25], we wanted to know whether metformin affected the toxicity of ROT in Jurkat cells under G55. As shown in Figure 8(e), metformin increases ~3-fold ROT-induced apoptosis in Jurkat cells in G55, as assessed by AO/EB/Hoechst staining (Figures 8(a)–8(d)), DNA fragmentation (Figure 8(f)), and ΔΨm depolarization assay (Figure 8(g)).

4. Discussion

Several data suggest that ROT induces death through enhancing O2−/H2O2 production by mitochondrial complex I [9] in cancer cells [13, 26]; however, the mechanism by which ROT provokes cell death remains controversial (i.e., OS versus the impairment of ΔΨm and decline of energy production). Here, we provide in vitro evidence supporting a role for OS in ROT-induced apoptosis in Jurkat cells under 2 different glucose (G) milieus: 11 mM (G11) and 55 mM (G55) glucose, as a model of normoglycemia and hyperglycemia in ALL, respectively. Mechanistically, ROT-induced apoptosis complies with the model of minimal completeness of cell death signaling [19]. Effectively, we confirm that ROT (1–100 μM) produces O2−/H2O2 in Jurkat cells according to the reduction of NBT to formazan and DFCH2 oxidation to fluorescent DCF assay, respectively. Furthermore, ROT generated O2− in a concentration-dependent fashion up to 50 μM with a marked decrease at 100 μM; however, H2O2 was constantly produced up to 50 μM with a significant reduction at 100 μM. Why did O2− and H2O2 levels decrease at 100 μM ROT concentration compared with lower ROT concentrations? A possible explanation is that 100 μM ROT is high enough concentration to generate high amounts of O2−, which can dismutate enzymatically or nonenzymatically into H2O2 at early ROT exposure with no apparent effect on nuclei morphology and ΔΨm. Once produced, H2O2 triggers nuclei and ΔΨm damage. By 24 h, O2−/H2O2 have been exhausted, destroyed, or decomposed concomitantly with maximal percentage of cell nuclei condensation/fragmentation and loss of ΔΨm. Because 50 μM ROT provoked ~50% nuclei fragmentation/ΔΨm depolarization, and it was an end-point for O2−/H2O2 generation, this concentration was selected for testing. Interestingly, 50 μM ROT generates high amounts of ROS in G11 and G55. This observation implies that ROT produces O2− and H2O2 independently of glucose concentration. However, the specific behavior of effector (NF-κB, p53, c-Jun), executor (caspase-3, AIF), antioxidant (DJ-1), and mitochondrial related (Parkin, PINK-1) molecules changed according to milieu condition. We found that ROT induces chromatin condensation (stage I) and nuclei fragmentation (stage II) concomitantly with loss of ΔΨm, nuclear localization...
Figure 4: Glucose modulates the response of DJ-1, Parkin, and PINK-1 in Jurkat cells exposed to ROT. Leukemia cells were left untreated or exposed to (50 μM) ROT in G11 or G55 medium for 24 h. Cells were stained with anti-DJ-1 ((a) and (b)), anti-Parkin ((c) and (d)), and anti-PINK-1 ((e) and (f)) antibodies, as described in Materials and Methods. Results are expressed as mean fluorescence intensity (MFI) of Jurkat cells and were compared between treatments from three independent experiments. * P < 0.05 versus respective control determined by the Student t-test.
Figure 5: Continued.
of AIF, NF-κB, p53, c-Jun, and caspase-3 markers and apoptotic nuclear morphology in G11. Furthermore, while ROT induced cytoplasmic DJ-1 protein accumulation and the upregulation of Parkin, PLINK-1 protein kinase was unaffected. In contrast, when glucose was present in excess (G55), cell death and/or dysfunctional markers were dramatically (>50%) reduced. Taken together, our data suggest that ROT induces apoptosis in Jurkat cells more efficiently in low (G11) than in high (G55) glucose environment by OS molecular mechanism. Several data support this conclusion. First, ROT-induced apoptosis in Jurkat cells was significantly reduced by the antioxidant NAC [26]. Second, H$_2$O$_2$ was constantly produced by ROT exposure (up to 50 μM for 24 h). Currently, H$_2$O$_2$ is considered an intracellular second messenger in several signalling pathways by activating kinases. Interestingly, some reports have demonstrated that H$_2$O$_2$ induces NF-κB activation in Jurkat cells either through the spleen tyrosine kinase (Syk) [27] or the IκBα kinase complex (IKK) [28]. Noticeably, Jurkat cells treated with ROT induced p65-DAB$^+$ nuclei, as an indicator of p65 activation and translocation to the nuclei. Moreover, pharmacological inhibition of NF-κB with PDTC significantly inhibited the apoptotic morphology under ROT exposure. These data suggest that ROT induces activation and translocation of the NF-κB (p65) probably via the aforementioned H$_2$O$_2$-induced mechanisms, thus implicating the activation of the transcription factor NF-κB in ROT-induced cell demise. In accordance with other scientific reports (e.g., [29]), our data suggest that NF-κB functions as a sensor of OS linked to cell death signaling. Third, it has been shown that NF-κB is able to upregulate p53 expression in cells exposed to H$_2$O$_2$ [30]. Accordingly, it is found that ROT induces p53 DAB$^+$ cells with evident morphology of apoptotic nuclei. This observation implies p53 as an important molecule in ROT-induced apoptosis. This conclusion is further supported by the fact that PFT, a specific inhibitor of p53, was able to significantly reduce ROT-induced apoptotic morphology and ΔΨ$_m$ depolarization. Our observations suggest an association between NF-κB and p53 in Jurkat cells under OS. Finally, inhibition of JNK, reduced activation of c-Jun, and low percentage of cell death in presence of ROT indicates that c-Jun activation is also required for ROT-induced cell death [31]. Collectively, these data suggest that NF-κB, p53, JNK, and c-Jun are critical proapoptotic factors in ROT-induced apoptosis in Jurkat cells.

Apoptosis is a morphological phenomenon as an outcome of the biochemical process taking place at the mitochondria [5]. To avoid potential confusion about the mode of cell death in Jurkat cells with other techniques as reported by Mattes [32], we used one of the most reliable methods to directly visualize apoptotic morphology—the acridine orange/ethidium bromide assay in combination with Hoechst staining. ROT-induced morphological apoptotic features (i.e., stage I and II nuclei fragmentation) in Jurkat cells. These observations suggest that ROT induces apoptosis via 2 pathways which run in parallel: AIF-dependent and caspase-3-dependent mechanisms associated with mitochondrial depolarization. This assumption is supported by a significant reduction of apoptosis in Jurkat cells treated with specific caspase-3 inhibitor NSCI, and caspase-3/AIF immunohistochemical positive nuclei (DAB$^+$) staining. These results comply with the notion that caspase-3 is required for DNA fragmentation and some of the typical morphological changes of cells undergoing apoptosis such as shrinkage, blebbing,
and the appearance of nuclei fragmentation in packed round masses, while AIF is required for chromatin fragmentation (for a review, see [33]). Remarkably, we observed neither cytoplasmic vacuolization, nor swelling of the cell, nor mild clumping of nuclear chromatin on cells treated with ROT. Our findings strongly suggest that Jurkat cells die by apoptosis rather than by other modes of cell death. In conclusion, ROT-induced apoptosis proceeds in a domino-like mechanism triggered by $O_2^{\cdot-}/H_2O_2$, which in turn activates (≫) kinases $> NFKB, JNK (>c-Jun) > p53 > \Delta\Psi_m >$ caspase-3, AIF $> chromatin condensation (stage I)$, and nuclei fragmentation (stage II).

ROT induces massive cytoplasmic aggregation of DJ-1 in Jurkat cells cultured in G11 compared to untreated cells in either G11 or G55 milieu. These findings are in agreement with the notion that $H_2O_2$ may be able to modify the structural...
disposition of DJ-1 [34]. Indeed, it has been reported that 
$\text{H}_2\text{O}_2$ is able to oxidizing Cysteine$_{106}$ residue into cysteine 
sulfonate (Cys$_{106}$-SO$_3^-$), thereby provoking DJ-1 aggregates 
[35]. However, whether this oxidative modification has a 
functional role in DJ-1 is unknown. Despite this drawback, 
several data suggest that Cys$_{106}$ is required for DJ-1 to 
confer cellular protection against OS [36]. Current evidence 
suggest that under basal conditions, DJ-1 (intact Cys$_{106}$) 
might function as an antioxidant protein by either scavenging 
relatively low $\text{H}_2\text{O}_2$ concentrations or performing other 
prosurvival ancillary functions (e.g., as chaperone). Under 
moderate $\text{H}_2\text{O}_2$ exposure, oxidized DJ-1 (cysteine-sulfinate 
Cys$_{106}$-SO$_2^-$) protein might function as an inhibitor of 
either proapoptotic proteins (e.g., apoptosis signal regulating 
kinase-1, ASK-1; p53) by preventing buildup of $\text{H}_2\text{O}_2$ in mito-
chondria [37] or of the upregulation of $\gamma$-glutamylcysteine 
ligase—the rate-limiting enzyme in the glutathione (GSH) 
biosynthetic pathway. This last assumption is compatible with 
our observation that NAC, an antioxidant and precursor 
of GSH, protects Jurkat cells against ROT. However, under 
extreme or constant $\text{H}_2\text{O}_2$ generation, such as by ROT, 
other oxidation of DJ-1 (cysteine-sulfonate Cys$_{106}$-SO$_3^-$) 
elicits loss of structure and aggregation [34]. Because DJ-1 is 
a sensitive protein towards oxidation by $\text{H}_2\text{O}_2$, this protein 
may constitute a marker of cellular stress.

Accumulating evidence suggests that Parkin and PINK-1 
may play a role in maintaining mitochondrial function and 
in preventing OS [38]. We found that ROT/$\text{H}_2\text{O}_2$ upreg-
ulates Parkin expression [39], most probably through p53 
transcriptional regulation [20]. Our results indicate that ROT 
induces the differential expression of Parkin and PINK-1 
proteins. What role does Parkin play in ROT-induced 
apoptosis? Because phosphorylation of Parkin by PINK-1 
activates Parkin E3 ligase function, which in turn activates 
NF-κB signaling through the IκB kinase/NF-κB pathway 
[21, 22], we speculate that the PINK-1/Parkin interaction 
might amplify the NF-κB/p53 death signal axis. Therefore, 
ROT induces a vicious cycle wherein $\text{H}_2\text{O}_2$ indirectly triggers 
PINK-1, which in turn activates (>) Parkin > NF-κB > p53 
> Parkin. Our findings suggest that PINK-1/Parkin might be 
important target proteins to accelerate cell death in Jurkat 
cells when treated with pro-oxidant drugs.

Recently, it has been shown that glucose starvation (GS) 
induces OS and apoptosis (∼85%) through AIF- and caspase-
3 dependent mechanisms [18]. Here, we found that excess 
glucose (G55) significantly reduces ROT-induced apoptosis 
(∼20%) in Jurkat cells. Accordingly, the signaling death 
markers (NF-κB, p53), executor (caspase-3, AIF), antioxidant 
(DJ-1), and mitochondria maintenance (Parkin, PINK-1) 
proteins diminished (>50%) compared to cells treated with 
ROT in G11. Our results suggest that high glucose promotes 
stress resistance against ROT/$\text{H}_2\text{O}_2$-induced apoptosis in 
Jurkat cells. How do cells manage to become resistant to 
ROT-induced apoptosis in G55? When cells are exposed to

**Figure 7:** High glucose reduces DJ-1 aggregation in Jurkat T cells exposed to ROT. Leukemia cells were left untreated ((a), (c), and (e)) or exposed to 50 μM ROT ((b), (d), and (f)) in G55 medium for 24 h. Cells were stained with anti-DJ-1 ((a) and (b)), anti-Parkin ((c) and (d)), and anti-PINK-1 ((e) and (f)) antibodies, as described in *Materials and Methods*. Notice that positive aggregates (dark brown color) reflect their cytoplasmic localization. Magnification 800x ((a)–(f)). Insets: percentage of positive DAB staining.
high OS stimuli, glucose can be routed through the pentose phosphate pathway (PPP) to generate the reducing agent NADPH, which preserves the intracellular levels of GSH, one of the most abundant intracellular antioxidant (for a review, see reference [40]). For this reason, cells with an active PPP are protected against OS and ROS-generating xenobiotics such as ROT. Therefore, alteration in energy metabolism by availability of glucose strictly correlated with degree of resistance to class 5 mitocan. However, whether high glucose affects the response of ALL cells to other mitocans (e.g., class 1–4, 6–8) [8] is still unknown. Further investigation is warranted to solve this issue. Interestingly, we found that the glucose-lowering drug metformin sensitizes Jurkat cells to ROT in G55 milieu. Grimaldi et al. [41] have found that metformin alone induced apoptosis and autophagy in ALL cells including Jurkat cells. Others have reported that metformin exerts its antidiabetic effects through inhibition of complex I of the mitochondrial respiratory chain [42].
However, we found that cells exposed to metformin alone were morphologically and metabolically normal, according to AO/EB/Hoechst staining, DNA fragmentation, and ΔΨm assay in both glucose conditions. Our data indicate therefore that the toxic effect of metformin may not involve inhibition of Complex I in Jurkat cells, at least under the present experimental conditions. Noticeably, metformin in presence of ROT in G55 dramatically induces apoptosis and
nonapoptosis cell death. These data suggest that metformin in combination with Complex-I inhibitors might be a useful therapeutic strategy in hyperglycemic ALL patients [43].

In summary, we provide mechanistic evidence explaining the toxic effect of ROT in Jurkat cells under 2 different glucose conditions (Figure 9). ROT induces O$_2^•^-$/H$_2$O$_2$ generation, mitochondrial depolarization, NF-κB, p53, and c-Jun transcription factor activation, AIF nuclear translocation, caspase-3 activation, chromatin condensation (stage I) and nuclei fragmentation (stage II), DJ-1 cytoplasmic aggregates, and upregulation of Parkin, typical characteristics of apoptosis in Jurkat cells cultured in G11 milieu. However, those apoptosis markers were significantly reduced in cells cultured in G55 under similar OS stimuli. Hyperglycemia in patients with leukemia is associated with increased hospital mortality. Here, we provide insight into the signaling and metabolic alterations related with drug response to metabolic milieu analogous to diabetic and non diabetic situation in patients with ALL. Taken together, our data suggest that combined therapy by using mitochondrial targeted damaging compounds and regulation of glucose (e.g., by metformin) might efficiently terminate leukemia cells via apoptosis in hyperglycemic conditions.

Conflict of Interests
The authors declare that they have no conflict of interests concerning this paper.

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