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Disulfiram/copper causes ROS levels alteration, cell cycle inhibition, and apoptosis in acute myeloid leukaemia cell lines with modulation in the expression of related genes

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ABSTRACT

The majority of acute myeloid leukaemia (AML) patients will die from their disease or therapy-related complications. There is an inevitable need to improve the survival of AML patients. Previous studies show that disulfiram (DSF), an anti-alcoholism drug with a low toxicity profile, demonstrates anticancer behaviors. Here, we evaluated the cytotoxicity and mechanistic action of DSF on the AML cell lines KG-1, NB4, and U937. The microculture tetrazolium test revealed that DSF alone or in combination with copper (Cu) is highly toxic to the AML cells at concentrations lower than those achievable in the clinical setting, with Cu increasing the DSF-induced inhibition of metabolic activity. Flow cytometric analysis and QRT-PCR indicated that in the two cell lines, NB4 and U-937, DSF/Cu increased reactive oxygen species (ROS) levels in association with the induction of superoxide dismutase 2 (SOD2) expression and suppression of catalase (CAT). In the KG-1 cell line, DSF/Cu reduced the ROS levels in agreement with the induction of CAT expression. The cell cycle and apoptosis assessment by flow cytometry demonstrated that DSF/Cu induced G0/G1 cell cycle arrest and apoptosis. These were associated with the increased expression of FOXO tumor suppressors, decreased expression of the MYC oncogene and the modulation of their known target genes related to the cell cycle and apoptosis. Therefore, DSF/Cu caused the disturbance of the ROS balance, cell cycle arrest and apoptosis in AML cells in coordination with the modulation in expression of their related genes. These results propose the possible use of DSF in AML therapies.

1. Introduction

Acute myeloid leukaemia (AML) is the most common myeloid leukaemia with a peak incidence in the 7th decade of life. Despite all the exciting advances in the realizing of AML molecular pathogenesis, these evolutions have, so far, not been translated into clinical practice and the prospects for AML patients have remained dismal [1]. Thus far, AML patients are still treated with the cytotoxic chemotherapy including, anthracycline and cytosine arabinoside. The majority of adult AML patients will perish from their disease or therapy related complications. In addition, in spite of successes in treatment of acute promyelocytc leukemia (APL), a particular case of AML with sensitivity to the all - trans retinoic acid (ATRA) and Arsenic trioxide (ATO), relapsed and refractory disease due to resistance still takes place among APL patients [1,2]. The development of new agents to bring to the market is an overlong and costly procedure. Moreover, any newly recognized purpose for an existing drug would facilitate the rapid assessment of phase II clinical trials because of formerly known pharmacokinetics and safety profiles established for these drugs in humans [3].

Disulfiram (DSF), tetraethylthiuram disulfide, is a FDA-approved anti-alcoholism drug for over 60 years with a very good safety record at FDA recommended doses [4]. It has demonstrated anticancer behavior both in vitro and in vivo [4]. In our previous in vivo investigations, the intravenous injection of DSF in PLGA-PEG nanoparticles has shown a significant decrease in the growth rate of breast cancer tumors [5,6]. DSF cytotoxicity appears to be exerted by two main ways, copper (Cu) dependent and nondependent [7]. DSF shows potentiation towards a number of chemotherapeutic agents and radiation, as well as protection of the normal cells of the kidney, gut and bone marrow from cytotoxic drugs while increasing their therapeutic index [7–11]. DSF/Cu could

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induce apoptosis by the alteration of the ROS levels and the inhibition of both aldehyde dehydrogenase and NFκB activity [10,12]. Disulfiram inhibits the activity of P-glycoprotein (P-gp) which mediates drug sensitivity [13]. Nevertheless, the detailed anticancer mechanisms of DSF are still not fully elucidated.

The elevation of the ROS levels and alterations in the expression of the genes that govern cell division and cell death contribute to the development of cancer [14–16]. Increased generation of ROS in cancer cells has a crucial role in unimpeded cell cycle progression and cell proliferation, and interruption of apoptosis signalling and cell survival [14]. Cell-cycle proteins are closely engaged in the checkpoint control mechanisms and instructions of apoptosis [17]. The genes involved in the ROS balance, cell cycle regulation and apoptosis exert a main influence on the reaction of neoplastic cells to different cytotoxic agents [14,15,18]. Considering the undeniable need to investigate more efficient and less toxic therapies for AML cases [1], the effects and mechanism of action of DSF were studied on three AML cell lines KG-1 (myeloblastic phenotype) [19], NB4 (promyelocytic phenotype) [20], and U-937 (myelomonocytic phenotype) [21]; in respect to the metabolic activity, ROS levels, cell cycle progression, apoptosis and expression of the related genes.

2. Materials and methods

2.1. Cell culture and treatment

Three AML cell lines, KG-1, NB4, and U-937, were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Biosera, UK) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Stock solutions of DSF (Merck, Germany) were freshly prepared prior to the use at a concentration of 100 mM by dissolving the compound in high quality anhydrous dimethylsulfoxide (DMSO) (Merck). A stock solution of copper (II) chloride ($CuCl_2$) (Merck) at a concentration of 100 mM was prepared by dissolving the compound in H_2O.

2.2. Microculture tetrazolium test (MTT)

The suppressive consequence of DSF, $CuCl_2$, and their combination on metabolic activity were assessed using the MTT (Sigma, USA). Concisely, 1.5×10^4 cells were placed into each well of 96-well plates and incubated with different concentration of DSF and/or 1 μM $CuCl_2$ for 24 and 48 h. $CuCl_2$ concentration used in this study is physiological [10]. Subsequently, 10 μl of MTT stock solution (5 mg/ml medium) was combined to each well, and the cells were incubated at 37 °C for 4 h. DMSO was used to solubilize Formazan crystals. An ELISA reader at a wavelength of 570 nm took the measurement of absorbance. The untreated cells were determined as the control group. Three replicates per treatment were evaluated and each experiment was repeated three times.

2.3. Intracellular ROS detection

2′,7′-Dichlorodihydro-fluorescein diacetate (DCFH-DA) (Sigma) was used for the detection of the intracellular ROS. Shortly before performing the test, DCFH-DA was dissolved in DMSO to make a stock solution. After a 4 h incubation with 1 μM of $CuCl_2$ or with 0.2 μM of DSF plus 1 μM of $CuCl_2$, the cells were removed from the growth media via centrifugation and exposed to a pre-warmed PBS containing DCFH-DA with a final concentration of 10 μM for 1 h at 37 °C. Next, the cells were washed two times with PBS before being resuspended in the PBS. Finally, fluorescence intensities of the samples were detected using the flow cytometer in the FITC channel. The data was analyzed with FlowJo software (FlowJo, USA).

2.4. Cell cycle analysis

The cell cycle was ascertained after a 24 h incubation of KG1, NB4, and U937 cells with 1 μM of $CuCl_2$ alone or the designated concentrations of DSF plus 1 μM of $CuCl_2$. Subsequently, 1×10^6 cells from the control (untreated) and treated cells were harvested, washed twice with cold phosphate-buffered saline (PBS), fixed in 70% ethanol (Merck) and then stored at −20 °C for 6 h. Next, fixed cells were incubated with propidium iodide (PI) (20 μg/mL) (Sigma), 0.1% Triton X-100 (Merck) and RNase A (100 μg/mL) (Sigma) for DNA staining and RNA degradation. After a 30 min incubation, the DNA content of the samples were evaluated by the flow cytometer (Partec PAS-III, Germany), and the data analysis was performed using FloMax software (Partec, Germany).

2.5. Apoptosis assessment

The KG1, NB4, and U937 cells were incubated with 1 μM of $CuCl_2$ or the designated concentrations of DSF plus 1 μM of $CuCl_2$ for 24 h. A negative control was prepared by the incubation of the cells in the absence of DSF and $CuCl_2$. Then, 1×10^6 cells were washed with PBS and the cell pellets were resuspended in the labeling solution of the Annexin-V-Flous/PI staining kit (Roche, Germany). After a 15 min incubation, the samples were analyzed on the flow cytometer using a 488 nm excitation and a 515 nm bandpass filter for fluorescein detection and a 617 nm filter for PI detection. Cells marked only with annexin-v-fluorescein were sorted as cells undergoing early apoptosis and the annexin-v-fluorescein and PI double– marked cells were sorted as cells in late apoptosis. In conjunction with this, the morphological features shown by laser light scattering were used as auxiliary parameters [22].

2.6. Gene expression analysis by quantitative reverse transcription PCR (QRT-PCR)

The total RNA from the cell lines were isolated after a 24 h drug treatment using the TRI reagent (Sigma) according to manufacturer's recommendation. The quantity of the RNA samples were measured spectrophotometrically by Nanodrop ND-1000 (Nanodrop Technologies, USA). For the synthesis of the complementary DNA (cDNA), 1 μg of total RNA was reverse transcribed using the Prime Script RT reagent kit (Takara Bio, USA). QRT-PCRs were carried out on the LightCycler® 96 System (Roche Diagnostics, Germany) using RealQ Plus Master Mix Green (Ampliqon, Denmark). 10 μl of the Master Mix, 2 μl of cDNA samples, 0.5 μl of forward and reverse primers (5 pmol), and 7 μl of nuclease-free water (Qiagen, Germany) were mixed to conduct PCR in a 20 μl of reaction volume. PCRs were performed as the following steps: first, 15 min at 95 °C as an initial activation and then 40 cycles including a denaturation step for 15 s at 95 °C and a combined annealing/extension step for 1 min at 60 °C. The sequences of primers used are listed in Table 2. The target gene expression levels were normalized to hypoxanthine phosphoribosyl transferase1 (HPRT1) levels in the same reaction, and the fold change in the relative expression of each target mRNA was calculated on the basis of the comparative Ct ($2^{-\Delta\Delta CT}$) method.

2.7. Statistical analysis

The results are the means ± standard deviation (SD) of three independent experiments. The significance of the differences between the experimental variables were calculated using the Student *t*-test (Microsoft Excel, Microsoft Corp.), and a P value of less than 0.05 was considered statistically significant.