Original Article

Mitoxantrone in Combination with a DNA-PK Inhibitor: Possible Therapy of Promyelocytic Leukaemia Resistant Forms

(HL-60/MX2 / mitoxantrone / double-strand breaks / multiple drug resistance / DNA-PK / NU7026 inhibitor)

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Abstract. The aim of the study was to sensitize cells of human promyelocytic leukaemia HL-60/MX2 (resistant to mitoxantrone and further substances interacting with topoisomerase II) to the effect of mitoxantrone (MTX). We demonstrated that the main mechanism of the HL-60/MX2 cell atypical multiple drug resistance is not only their altered activity of topoisomerase II and reduced levels of topoisomerase II α and β proteins. The resistance of the HL-60/MX2 cells to MTX is associated with their increased ability to repair DNA double-strand breaks (DSBs) in these cells. The HL-60/MX2 cells, compared to HL-60 cells (which are sensitive to MTX effects), contain large amounts of DNA-PK, which is responsible for the main pathway of the DSB repair, non-homogenous end joining (NHEJ), and they also contain large amounts of further repair proteins Rad50 and Nbs1, which are important in both types of the repair processes (NHEJ as well as homologous re-combination). We demonstrated that specific DNA-PK inhibitor NU7026 reduced the amount of DNA-PK in HL60/MX2, thus preventing the DSB repair through the NHEJ pathway after the incubation with MTX and in this way essentially abolished the resistance of these cells to MTX.

Introduction

HL-60/MX2 is a mitoxantrone-resistant derivative of the human promyelocytic leukaemia cell line HL-60. HL-60/MX2 cells exert atypical multiple drug resistance (MDR) with the absence of P-glycoprotein over-expression, altered topoisomerase II catalytic activity, and reduced levels of topoisomerase II α and β proteins. HL-60/MX2 cells are cross-resistant to agents whose cytotoxicities result from interactions with the nuclear enzyme DNA topoisomerase II (topo II) – etoposide, teniposide, bisantrene, dactinomycin and anthracyclines (Harker et al., 1989). Mitoxantrone (MTX) is a commonly used anti-neoplastic agent for the treatment of acute myeloid leukaemia. Its interactions with topoisomerase II and DNA induce double-strand breaks (DSBs) and cause breakdown of the transcription and replication (Klener, 1996). The enzyme-mediated repair of the DSBs is another possible mechanism of the resistance to ionizing radiation and DSB-causing drugs. Two major pathways of the DNA double-strand break repair have been described as follows:

1/ Homologous recombination (HR), which is based on homologous sequences of the sister chromatid, homologous chromosome or ectopic homologous locations to align and ligate to correct DNA ends.

2/ Non-homogenous end joining (NHEJ), in which DNA ends are connected with little or no base-pairing at the junction (Dartsch and Gieseler, 2007).

Important components of these repair pathways are members of the phosphatidylinositol 3-kinase (PI 3-K)--related protein kinase family such as ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and

Rad3-related) kinases, and DNA-PK (DNA-dependent protein kinase) (Falck et al., 2005; Bekker-Jensen et al., 2006). The MRN complex (Nbs1, Mre 11, and Rad50) is essential to the DSB repair, telomere maintenance and cell-cycle checkpoint control. The MRN complex is a critical component in both (HR and NHEJ) repair pathways. An enhanced DNA repair response by the MNR complex is critical in driving chemo-resistance. The deficiency of ATM, MRE11, or Rad50 led to a 2–5-fold sensitization of leukaemia cells to gemcitabine, whereas the Nbs1 and H2AX deficiency did not cause this effect (Ewald et al., 2008). Abuzeid et al. (2009) suggested that the critical effects of Rad50 in the DNA repair and disruption of the Rad50 function sensitized squamous cell carcinoma to cisplatin chemotherapy.

The NHEJ pathway requires the activity of DNA-PK, a serine/threonine kinase consisting of two components, a 460 kDa polypeptide catalytic subunit (DNA-PKcs) and autoimmune antigen Ku, which is composed of polypeptides of about 70 (Ku70) and 80 kDa (Ku80). Numerous studies demonstrated that cells lacking DNA-PK are hypersensitive to ionizing radiation and cross-linking agents and tend to cause defective DSB repair (Shen et al., 1998; Chin et al., 2005; Kim et al., 2009). This fact supports the concept that a specific DNA-PK inhibitor could prove very useful in combinations with topo II poisons in the leukaemia therapy. NU7026 (2--(morpholin-4-yl)-benzo[h]chomen-4-one), a specific inhibitor of DNA-PK, was evaluated here. NU7026 is a competitive and highly selective inhibitor of DNA-PK and it is inactive towards ATM and ATR (Veuger et al., 2003). In the work presented here, we describe differences between HL-60 and HL-60/MX2 cell lines and the effects of MTX in combination with NU7026 on the cell growth and cell-cycle phase distribution. The levels of selected DNA damage repair proteins were also studied.

Material and Methods

Cell culture

HL-60 and HL-60/MX2 (American Type Culture Collection) were incubated as suspension cultures in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% foetal bovine serum, 0.05% L-glutamine, 150 UI/ml penicillin, 50 µg/ml streptomycin (all from Sigma-Aldrich) in a humidified incubator at 37 °C under controlled 5% CO_2 atmosphere. The cultures were divided every other day by dilution to a concentration of 2×10^5 cells/ml. Cells were counted with a haemocytometer and the cell membrane integrity was determined by using the trypan blue exclusion technique. Cells in a maximum range of 20 passages were used for this study.

Chemicals

Mitoxantrone dihydrochloride and NU7026 were purchased from Sigma-Aldrich. NU7026 was dissolved in dimethylsulphoxide (DMSO) and diluted with PBS before use. The final concentration of DMSO in the culture was lower than 0.2 %.

Viability of cells

Cell viability was assessed with trypan blue. To establish the concentrations applicable to further studies, we incubated both lines with 5 µM, 10 µM and 20 µM NU7026 and assessed the amounts of cells in groups every 24, 72 and 144 h. To investigate the effect of MTX and NU7026 on the cell growth we used the same technique and incubated a control group, a group with 1 nM MTX, a group with 1 nM MTX and 5 µM NU7026 and a group with 1 nM MTX and 10 µM NU7026.

Electrophoresis and Western blot analysis

Twenty-four hours after the incubation of HL-60 and HL-60-/MX2 cells with MTX and NU7026 or with both of them, the cells were washed with PBS and lysed. Whole-cell extracts were prepared by lysis in 500 µl of lysis buffer (137 mM NaCl; 10% glycerol; 1% n-octyl--β-glucopyranoside; 50 mM NaF; 20 mM Tris, pH = 8; 1 mM Na_3VO_4; 1 tablet of protease inhibitors Complete™ Mini, Roche, Grenzach-Wyhlen, Germany). The lysates containing equal amounts of protein (30 µg) were loaded onto a 10% SDS polyacrylamide gel (8% gel for DNA-PK). After the electrophoresis, proteins were transferred to a PVDF membrane and hybridized with an appropriate antibody: anti-Nibrin/Nbs1 clone EE15 from Upstate (Millipore, Billerica, MA); and anti-DNA-dependent protein kinase (catalytic subunit, clone 42 – psc), anti-RAD50, anti-β-actin from Sigma-Aldrich. After washing, the blots were incubated with secondary peroxidase-conjugated antibody (Dako, High Wycombe, UK) and the signal was developed with a chemoluminescence detection kit (Boehringer, Mannheim, Germany) and exposure of film (Foma, Hradec Králové, Czech Republic).

Cell cycle analysis

Twenty-four hours after the incubation, the cells were washed with cold PBS, fixed with 70% ethanol and stained with propidium iodide (PI) in Vindelov's solution for 30 min at 37 °C. Fluorescence (DNA content) was measured with a Coulter Electronic apparatus (Beckman Coulter, Inc., Nottingham, MD). At least 10,000 cells analysed in each sample served to determine the percentage of cells in each phase of the cell cycle, by using the Multicycle AV software.

Statistical analysis

The results were statistically evaluated with one-way ANOVA followed by multiple comparisons with the Scheffé's test. The statistical significance of differences was considered at a significance level of 0.05. The values represent mean ± SD (standard deviation of the mean) of three independent experiments.

Results

Results based on the Western blot method presented in Fig. 1 show the levels of repair protein DNA PK and its subunits Ku 80, Rad50 and Nbsl in both types of the cells and the effects of cell incubation with MTX (10–500 nM) on the amounts of these proteins. The cells were incubated for 1 h with MTX and thereafter, MTX was removed by rinsing and the cells were subjected to further incubation for one hour and lysis. We demonstrated that cells resistant to MTX (HL-60MX2) contain larger amounts of DNA-PK, Rad50 and Nbs1 compared to the HL-60 cells. Large amounts of these proteins were apparent in the HL-60MX2 cells 2 h after the beginning of the incubation with all the MTX concentrations compared to the HL-60 cells, where only DNA-PK increased after the lowest dose used of 10 nM. The increase in Rad50 and Nbs1 was obvious after a dose of 100 and 500 nM MTX. The amounts of Ku80 exerted changes neither in controls nor after the exposure to MTX (Fig. 1).

In the next experiment, we tested MTX effects in doses of 1 to 20 nM. The cells were incubated with MTX for the entire duration of the experiment (i.e. for 144 h). Figure 2 shows MTX effects on the growth curve of the HL-60 cells and of their mitoxantrone-resistant form HL60MX2. In the HL-60 cells we observed cytotoxic effects of the dose of 20 nM and cytostatic effects of low MTX doses (1 and 2 nM). We demonstrated a statistically significant decrease ($P < 0.05$) in the number of live cells 72 and 144 h after the beginning of the incubation with 1 and 2 nM MTX, respectively, compared to controls. However, the number of the cells was statistically significantly higher (after the incubation with 1 and 2 nM MTX) compared to the incubation with 20 nM MTX. In the HL60/MX2 cells, there was an essentially less considerable effect of MTX. The dose of 20 nM exerted cytostatic effects only. We demonstrated a statistically significant decrease in the number of living cells after a dose of 20 nM compared to controls and group after incubation with 1 nM MTX (72 h and 144 h).

Given the DNA-PK concentration, the specific DNA-PK inhibitor, NU7026, was employed to suppress the repair capacity of the cells. Preliminary experiments indicated that the 144-h incubation of the two types of
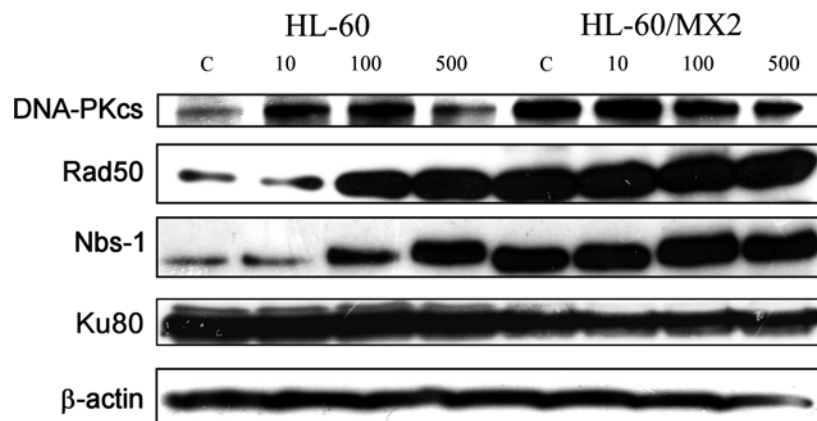

Fig. 1. Changes in selected repair proteins in HL-60 and HL-60/MX2 cells after 1-h incubation with MTX (10, 100 and 500 nM). Representative Western blots are shown. β-Actin was used as loading control.
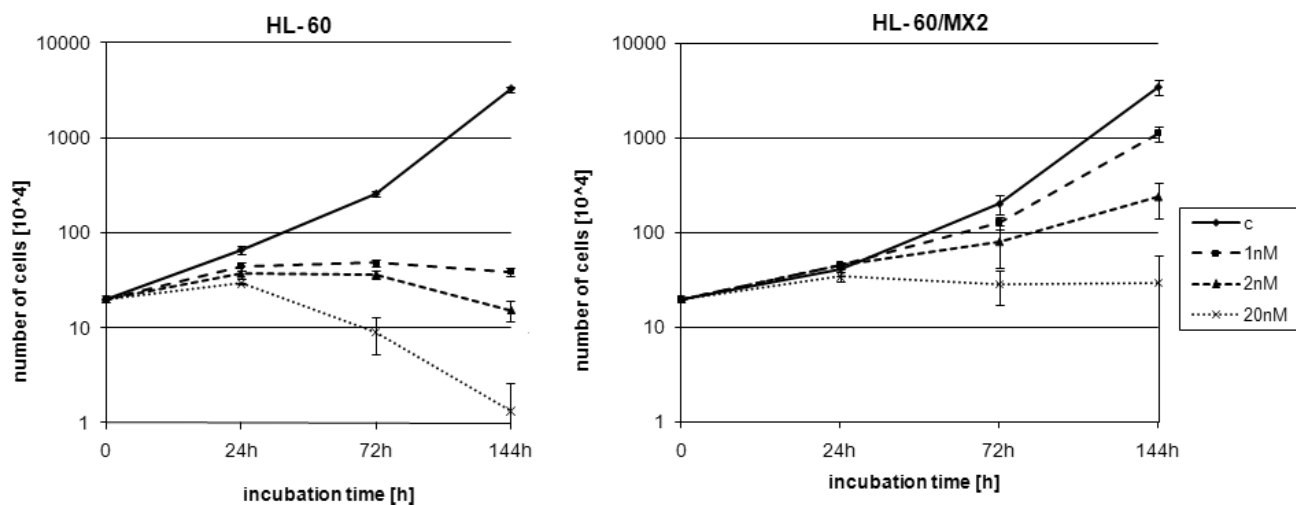

Fig.2. Changes in proliferation of HL-60 and HL-60/MX2 after 144-h incubation with MTX (1, 2 and 20 nM). Mean values from three independent experiments ± SD are given.

cells with 20 μM NU7026 exerted cytostatic effects and the cell growth was essentially discontinued, whereas after the incubation at lower doses (5 and 10 μM), there were only minimum effects on the cell growth. Thus, in further experiments, we used the concentration of 10 μM NU7026. We studied the effects of 1 nM MTX and 10 μM NU7026 and those of the combinations of these two substances. The cells were incubated with these noxious agents for the entire duration of the experiment, i.e. for 144 h. MTX at 1 nM discontinued growth of the HL-60 cells, but only limited that of HL-60/MX2. NU7026 at 10 μM potentiated the MTX effect in HL-60 cells, and in HL-60/MX2 cells it essentially eliminated the resistance to MTX (Fig. 3). A statistically significant decrease in the number of HL-60/MX2 cells after incubation with the combination compared to 1 nM MTX (72 and 144 h) was observed.

To establish the amount of DNA-PK and its involvement by incubation with MTX and NU7026, we employed the same arrangement of the experiment as shown in Fig. 3 and incubated the cells with MTX and NU7026 for 24 h. In the HL-60/MX2 cells incubated with NU7026, there was a decrease in the DNA-PK amounts (which was increased by intact HL-60/MX2 controls compared to HL-60 controls). The incubation of HL-60/MX2 cells with MTX did not affect the DNA-PK level compared to controls. The combination of MTX and NU7026 caused a considerable decrease in DNA-PK in the resistant HL-60/MX2 cells compared to controls as well as to the group affected with MTX (Fig. 4).

Discussion

Understanding the mechanisms through which the cells become resistant to anti-cancer drugs is of principal importance for the choice of a suitable therapeutic strategy. The resistance of HL-60-MX2 cells to mitoxantrone is usually considered in association with a
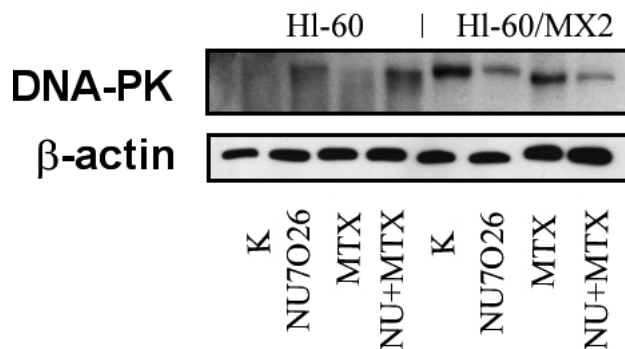

Fig. 4. Changes in DNA-PK in HL-60 and HL-60/MX2 cells after 24-h incubation with MTX (1 nM), NU7026 (10 μM) and with their combination. Representative Western blots are shown. β-Actin was used as loading control.

change in the topoisomerase II catalytic activity and reduction in the topoisomerase II α and β amounts. Harker et al. (1995) demonstrated that in the drug-resistant cell line HL-60/MX2, the topo II catalytic activity was reduced by a factor of 2. The use of a reverse transcription-PCR assay demonstrated the presence of very low levels of topo II β mRNA in HL-60/MX2 cells, representing less than 1 % of that found in the HL-60 cells. It is hard to explain the 35-fold differences in mitoxantrone sensitivity (Harker et al., 1989) of the HL-60 and HL-60/MX2 cell lines based on a 2-fold difference in the cellular topo II catalytic activity. It is thus obvious that other mechanisms are also responsible for the resistance of these cells to mitoxantrone.

DNA topoisomerases I/II kill tumour cells through origination of irreparable DNA double-strand breaks. The DSB repair is triggered through the activation of protein kinases ATM, ATR and DNA-PK (Bekker-Jensen et al., 2006). Changes in repair processes can
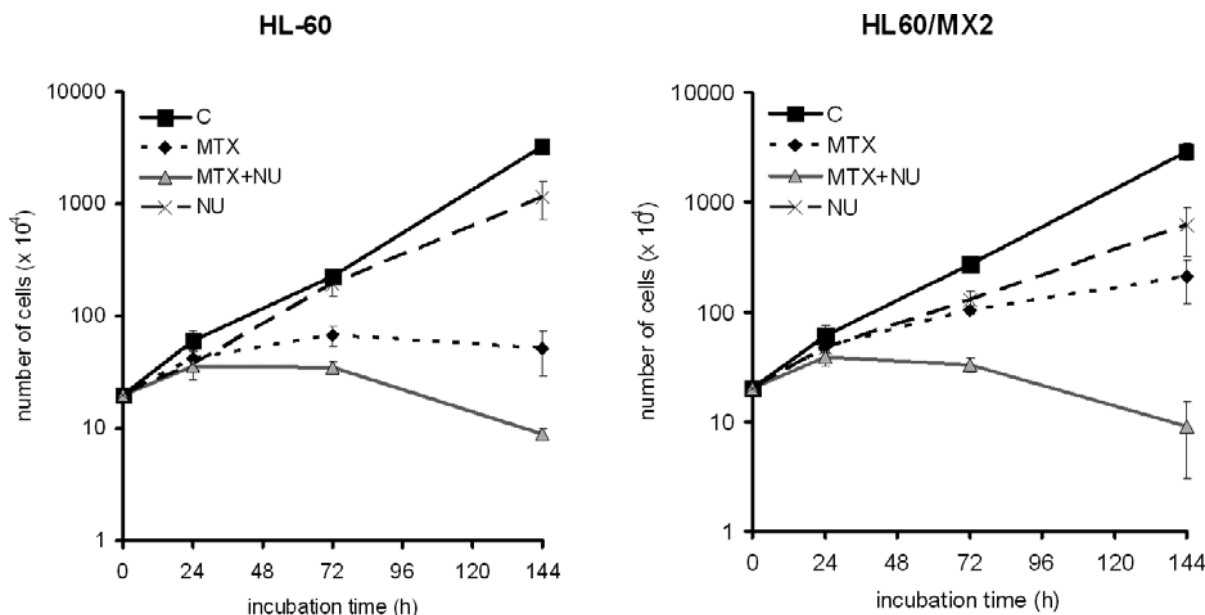

Fig. 3. Changes in proliferation of HL-60 and HL-60/MX2 after 144-h incubation with MTX (1 nM), NU7026 (10 μM) and with their combination. Mean values from three independent experiments ± SD are given.

cause resistance to cytostatic substances or ionizing radiation. Shen et al. (1998) compared the levels of DNA-PK in the HL-60 cells with that in the line HL-60/ADR, which was resistant to adriamycin. They demonstrated that in the resistant line, the amount of mRNA DNA-PK is increased by a factor of 20 to 25 and the enzymatic activity is increased by a factor of 3. The antisense RNA transfection reduced the protein expression of DNA-PK to 50 % in HL-60/ADR and partially reversed the drug resistance. In our work, we studied the differences found between DNA-PK amounts in the HL-60 line and mitoxantrone-resistant HL-60/MX2 line. We demonstrated that the HL-60/MX2 line resistant to MTX had increased amounts of DNA-PK compared to the HL-60 cells. Ader et al. (2002) also found twice as high DNA-PK activity in radioresistant cell lines compared to lines sensitive to ionizing radiation. We also supported the association between the resistance of HL-60/MX2 cells to mitoxantrone and DNA-PK based on an increase in DNA-PK after the incubation of the HL-60/MX2 cells with MTX. This increase did not occur with the HL-60 cells. The DNA-PK inhibitor NU7026 alone reduced the DNA-PK amount in the HL-60/MX2 cells and DNA-PK essentially disappeared after 24-h incubation of the HL-60/MX2 cells with the combination of MTX and NU7026. Bentle et al. (2007) demonstrated that NHEJ is the key factor in the cell survival after the exposure to a new chemotherapeutic agent, β-lapachone. Glioblastoma cells of the line MO59K contained DNA-PK, whereas in MO59J cells DNA-PK was missing. The DNA-PK inhibitor NU7026 sensitized etoposide and lapachone effects in the cell line containing DNA-PK only, i.e. in the MO59K cells (Bentle et al., 2007; Hisatomi et al., 2011). Adult leukaemia-lymphoma T cells are leukaemic cells exerting high DNA-PKcs expression, the disease being aggressive and non-curable by standard therapeutic approaches. A new substance, NK314, was described, which exerts a double capability of inhibiting topoisomerase II α and DNA-PK, thus being a potential drug against this type of leukaemia (Hisatomi et al., 2011).

We demonstrated that the HL-60 as well as HL-60/MX2 cells are accumulated in G2 phase after their incubation with 1 nM MTX. There was no difference in the amount of cells in G2 phase after the incubation with MTX between these cell lines. The amount of DNA-PK does not affect this process. DNA-PK consists of three key components, KU70/80 and DNA-PKcs. In contrast to DNA-PK and its increase in the resistant cell line, we demonstrated no differences between the amounts of the Ku80 component in the two cell lines, as well as no effect of exposure to MTX on Ku80. Ader et al. (2002) also demonstrated that whereas DNA-PK was enhanced in the two radioresistant lines, the Ku expression remained unaltered. Muñoz et al. (2001) demonstrated the Ku antigen to be responsible for the release of cells from the cell-cycle G2 phase. Ku-deficient cells are furthermore arrested in the G2 phase after being affected by DNA topoisomerase II inhibitors.

DNA-PK is responsible for the DSB repair through NHEJ and occurs during the entire cell cycle; the MRN complex (MRE11/Rad50/Nbs1) participates in the DSB detection and in both NHEJ and HR repair pathways. Our results indicate that elevated amounts of repair proteins Rad50 and Nbs1 can also participate in the resistance of the HL-60/MX2 cells. The levels of these repair proteins in the HL-60 cells increased after the incubation with high MTX doses only, whereas HL-60/MTX cells exerted high amounts of Rad50 and Nbs1 proteins and were not affected by the incubation with MTX. Ewald et al. (2008) demonstrated that ATM, Mre11 or Rad50-deficient cells exerted sensitivity to gemcitabine higher by factors of 2 to 5, whereas the Nbs1 amounts did not affect the sensitivity to the cytostatic agent.

We can conclude that in the resistance of leukaemic cells to inhibitors of DNA topoisomerase II, not only the inhibiting activity against topoisomerase II α but also the amount of DNA-PK and other repair enzymes (such as Rad50), which is elevated in resistant cell lines, are of importance.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the contents and writing of the paper.

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