The Protective Role of N,N’-Dimethylthiourea and Its Effect on Hydrogen Peroxide Level of Hela Kyoto Cells under Cisplatin Action

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The aim of the study is to sense the level of hydrogen peroxide (H2O2) in viable and apoptotic HeLa Kyoto cells under the action of cisplatin in presence of reactive oxygen species scavenger N,N’-dimethylthiourea (DMTU).

Materials and Methods. HeLa Kyoto cell line transfected with hydrogen peroxide sensor HyPer2 or pH sensor SypHer2 was incubated with cisplatin for 24 h with or without DMTU. The viability of the cells was determined by trypan blue staining technique and flow cytometry with apoptosis marker and vital dye. The response of the sensors was determined in viable cells and in cells in early apoptosis separately.

Results. A decrease in the percentage of viable cells upon exposure to cisplatin inhibited by the addition of DMTU to the incubation medium was shown. A significant increase in H2O2 level in HeLa Kyoto cells was observed upon drug exposure, while incubation with cisplatin and DMTU simultaneously resulted in a value close to the control one. The observed reaction was typical for both viable and apoptotic cells.

Conclusion. The protective role of DMTU and the involvement of H2O2 in tumor cells death under the cisplatin action was proved.

Key words: HeLa Kyoto; fluorescent sensor; HyPer2; SypHer2; cisplatin; hydrogen peroxide; N,N’-dimethylthiourea.

Introduction

Cisplatin is a platinum-containing chemotherapeutic drug successfully used in treatment of a wide range of tumors such as carcinomas, lymphomas, sarcomas, germinogenic tumors, etc. [1, 2].

Along with direct action on DNA, this agent causes the formation of reactive oxygen species (ROS), including hydrogen peroxide (H2O2) [3, 4]. Hydrogen peroxide is of particular interest among other ROS due to its participation in apoptosis of tumor cells. H2O2 mediates apoptosis by inhibiting the expression of Bcl-2 [5], as well as caspases activation and Bax expression [6–8]. In this connection, the study of hydrogen peroxide potential role in cisplatin-induced apoptosis of tumor cells is important for development of novel safe and efficient therapeutic strategies against oncological diseases.

The study of intracellular H2O2 level changes is associated with a number of difficulties in measurement procedure. Recently, specific fluorescent probes have been developed, sensitive to H2O2 due to the ability to fluoresce upon oxidation of H2O2. These probes have a number of limitations related to low selectivity or irreversibility of the reaction with H2O2 [9]. Genetically encoded sensor HyPer is free of these disadvantages. This sensor based on the peroxide sensitive domain Oxy-R and the yellow fluorescent protein has high selectivity and sensitivity, demonstrates direct expression in cells, and allows for easy targeting to various subcellular compartments [10]. Recently this sensor was applied to study hydrogen peroxide level in transfected tumor cells under the action of chemotherapy [11], photodynamic therapy [12], growth factors [13], and in macrophages during phagocytosis [14]. These studies employed fluorescence microscopy for assessment of the sensor reaction. Previously we proposed a novel approach based on flow cytometry to study the hydrogen peroxide level in cells expressing HyPer family sensor [15]. This approach allows estimating H2O2 level separately in viable cells and in cells in the stage of early apoptosis. The proposed approach was approbated in the study of cell death mechanisms of and sensor response under cisplatin and bleomycin action on tumor cells.
To confirm the connection of the sensor activation with cisplatin-induced cell death, it is necessary to induce the reduction of H$_2$O$_2$ content inside a cell, which should lead to an absence of sensor response and an increase in the percentage of viable cells upon addition of cisplatin. For this purpose, in this study, we employed ROS scavenger N,N'-dimethylthiourea (DMTU). DMTU is a highly efficient absorber of OH$^+$, $^{1}$O$_2$ [16, 17], and H$_2$O$_2$ [18], known to decrease the H$_2$O$_2$-induced damage of cultured endothelial cells [19], to reduce the cisplatin-induced tumor necrosis factor-alpha (TNF-$\alpha$) expression in macrophages in vitro, and to prevent the development of oxidative stress, activation of p53 and, as a consequence, apoptosis of renal and hepatic cells in vivo [20–23]. An important step is to carry out experiments on cytotoxicity of cisplatin under conditions of hydrogen peroxide removal from cells using DMTU. To confirm that DMTU, along with other ROS, reduces the hydrogen peroxide content under cisplatin action, in this study its level was controlled with the specific sensor HyPer2 in parallel with the cytotoxic tests. This approach allows the determination of the role of H$_2$O$_2$ in the process of cisplatin-induced cell death.

The aim of the study is to sense the level of hydrogen peroxide in viable and apoptotic HeLa Kyoto cells under the action of cisplatin in the presence of reactive oxygen species scavenger N,N'-dimethylthiourea.

Materials and Methods

Cell lines. The study was performed with HeLa Kyoto, the human cervical carcinoma cell line transfected with cytoplasmic sensor HyPer2 (a HyPer modification with an extended dynamic range). This sensor has a high sensitivity to changes in the hydrogen peroxide level in cells [13], however, like most fluorescent proteins, is pH-sensitive, which necessitates the intracellular pH control. In this connection, the HeLa Kyoto cell line transfected with hydrogen peroxide insensitive HyPer2 analogue SypHer2 [24], was used. Cell lines were obtained from the M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences.

The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (PanEco, Russia), supplemented with 2 mM glutamine and 10% fetal bovine serum (HyClone, USA) in a CO$_2$ incubator at 37°C in a humidified atmosphere with 5% CO$_2$.

Staining with trypan blue. Cells were seeded into 12-well plates at 1·10$^5$ cells per well a day before the experiment. On the next day, they were treated with 16.6 μM cisplatin, the concentration corresponding to 2-fold IC$_{50}$ (IC$_{50}$ causes inhibition of 50% of cells) [25] in the presence or absence of 15 mM DMTU (Sigma-Aldrich, USA). Cells without addition of cisplatin to incubation solution served as a control group. After 24 h of incubation with the drug, the medium with the cells was taken into centrifuge tubes, the cells were washed off from the well with cold PBS solution twice. Annexin binding buffer in the amount of 1.5·10$^6$ cells/ml was added to the cells precipitated by centrifugation for 5 min with Refrigerated Centrifuge 5810 (Eppendorf, Germany) at 900 rpm. 1.5·10$^5$ cells were transferred to tubes for flow cytometry.

Flow cytometry with apoptosis markers. Cells were plated on 12-well plates at 1·10$^5$ cells per well a day before the experiment. On the next day, they were exposed to 16.6 μM cisplatin in the presence or absence of 15 mM DMTU. Cells without addition of cisplatin to incubation solution served as control cells. After 24 h of incubation with the drug, the medium with the cells was taken into centrifuge tubes, the cells were washed off from the well with cold PBS solution twice. Annexin binding buffer in the amount of 1.5·10$^6$ cells/ml was added to the cells precipitated by centrifugation for 5 min with Refrigerated Centrifuge 5810 (Eppendorf, Germany) at 900 rpm. 1.5·10$^5$ cells were transferred to tubes for flow cytometry.

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To analyze the fluorescence of sensors in viable cells and cells in the early apoptosis the approach [15] was used. This approach allows for simultaneous assessment of cell death pathways and hydrogen peroxide content in each cell using the marker for Annexin V labeled with phycocerythrin (PE), the vital dye 7-amino-actinomycin D (7-AAD) and the sensor for H$_2$O$_2$. To analyze the pathways of cell death PE Annexin V Apoposis Detection Kit I (BD Biosciences, USA) was used. PE Annexin V and 7-AAD in the amount of 5 μl each were added to each probe, each probe shaken for 1–2 s with minicentrifuge/ vortex Microspin FV-2400 (BioSan, Latvia) and incubated in the dark at 25°C for 15 min.

Probes were transferred to the cold, 400 μl of Annexin binding buffer was added to the cells prior to fluorescence detection. The change in fluorescence intensities of dyes and the response of genetically encoded sensors were recorded with a flow cytometer FACSCalibur (Becton Dickinson, USA) employing laser excitation at the wavelength of 488 nm and three detection channels with filters 530/30 nm (fluorescence of HyPer2 and SypHer2, FL1 channel), 585/42 nm (fluorescence of PE Annexin V, FL2 channel), 670 LP (fluorescence 7-AAD, FL3 channel). In each probe 1.5·10$^4$ cells were analyzed.

Data processing. For assessing cell viability by trypan blue staining 5 experiments were carried out on the HeLa Kyoto–HyPer2 cells and 3 experiments on the HeLa Kyoto–SypHer2 cells. Data was processed using Excel 2016 software (Microsoft, USA) and presented as mean values and standard deviations. To determine the statistical significance of differences between groups a one-factor analysis of variance with the post-hoc Bonferroni test for multiple comparisons in Statistica 6.0 (StatSoft, USA) was used. To calculate the percentage of viable, early apoptotic and dead cells, as well as median fluorescence intensities of the sensors obtained.
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by flow cytometry, BD CellQuest™ Pro Software (Becton Dickinson, USA) was used.

Results

The results of staining of HeLa Kyoto–HyPer2 cells with trypan blue (Figure 1 (a)) demonstrated a statistically significant decrease in the percentage of viable cells after cisplatin treatment from 74.2±9.2 to 29.1±15.6% (p<0.0001) as compared to control (cisplatin untreated cells). However, this effect was not observed when DMTU was added to the incubation medium simultaneously with cisplatin, which resulted in a statistically significant (p<0.001) increase in the number of living cells up to 71.5±13.2% as compared to cells treated with cisplatin only. It should be noted that the addition of DMTU to untreated cisplatin cells did not adversely affect the viability of this cell line amounting to 77.7±8.1%.

Staining of HeLa Kyoto–SypHer2 cells with trypan blue (Figure 1 (b)) showed similar results: addition of cisplatin leads to a statistically significant decrease in the percentage of viable cells from 82.5±7.3 to 26.5±17.6% (p<0.01) as compared to cisplatin untreated cells. When DMTU was added to the incubation medium simultaneously with cisplatin, a statistically significant increase in the percentage of viable cells was observed up to 68.7±13.7% (p<0.05) as compared with cisplatin-only treated cells, which indicates the elimination of the cisplatin effect by DMTU.

Further flow cytometry with the staining of cells with an apoptosis marker and a vital dye enabled the identification of three cell populations: viable cells that are negative for 7-AAD and PE Annexin V, cells in the early apoptosis that are positive for PE Annexin V and negative for 7-AAD, and cells in late apoptosis or necrosis (dead cells) that are positive for both dyes, as well as the ones that are 7-AAD positive cells and negative for PE Annexin V (Figures 2, 3).

Flow cytometry showed that the addition of cisplatin to HeLa Kyoto–HyPer2 cells line leads to a significant increase in the number of dead cells, from 17.7 to 73.6% (Figure 2 (a), (b)), without increasing the number of cells passing the stage of early apoptosis: 10.8% in the control and 9.9% in cells treated with cisplatin only. The number of viable cells after addition of cisplatin decreases for more than 50%: from 71.5 to 16.6%. It was shown that the simultaneous incubation of cells with cisplatin and DMTU leads to an increase in the percentage of viable cells from 16.6 to 76.8% (Figure 2 (b), (d)), while the percentage of cells in early apoptosis changes insignificantly and is equal to that in the control. Treatment with DMTU of the cells that were not exposed to cisplatin did not adversely affect the viability of this cell line.

Staining of the control cell line HeLa Kyoto–SypHer2 with 7-AAD and PE Annexin V demonstrated similar results (see Figure 3): treatment of cells with DMTU and cisplatin caused an increase in the percentage of viable cells from 11.9 to 61.8% and decrease in the percentage of cells passing the early apoptosis from 24.4 to 8.1%. Treatment with DMTU of the cells that were not exposed to cisplatin also did not affect the viability of the cell line.

Employment of the apoptosis marker and the vital dye allowed to construct fluorescence histograms of HyPer2 sensors (left column) and SypHer2 (right column) for the whole cell population (Figure 4 (a), (b)), and separately for living cells (Figure 4 (c), (d)) and for cells in stage of early apoptosis (Figure 4 (e), (f)).

The histogram of HyPer2 fluorescence (see Figure 4 (a)) plotted for all cells shows an increase in the fluorescence intensity of cisplatin-treated cells relative to control cells, which decreases with the addition of DMTU. Similar histograms plotted for the

Figure 1. The percentage of viable cells after exposure with 16.6 μM cisplatin and/or 15 mM DMTU for 24 h (results of staining with trypan blue): (a) HeLa Kyoto–HyPer2; * p<0.001, ** p<0.0001; (b) HeLa Kyoto–SypHer2; * p<0.05, ** p<0.01
cell line containing SypHer2 (see Figure 4 (b)) shows a decrease in fluorescence intensity of cisplatin-treated cells as compared to untreated control cells; upon DMTU addition the fluorescence intensity returns to the values of the control cells. A part of the cell population characterized by low fluorescence intensity of the sensors (up to ∼100 a.u., see Figure 4 (a), (b)), that was strongly stained with 7-AAD, was identified as dead cells and was excluded from the analysis (data not shown). In the histograms constructed for living cells and cells in the early apoptosis, a region for estimating the median fluorescence intensity of the sensors (M1) was selected. This allowed us to estimate the difference in the fluorescence of different cell probes.

The addition of DMTU resulted in a slight decrease in the median fluorescence intensity of the sensor from 221 to 215 a.u. in viable HeLa Kyoto-HyPer2 cells, while in viable cells containing SypHer2 it caused a slight increase in the median fluorescence intensity of the sensor from 389 to 414 a.u. (Figure 4 (c), (d)). The addition of cisplatin to cells containing HyPer2 resulted in a twofold increase in the median fluorescence intensity up to 461 a.u., while in the cell line containing SypHer2 the incubation with cisplatin caused a decrease in the median fluorescence intensity of the

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**Figure 2. Distribution of HeLa Kyoto–HyPer2 cells over fluorescence intensity of 7-AAD and PE Annexin V:**
(a) without addition of cisplatin and DMTU; (b) after exposure to 16.6 μM cisplatin; (c) after exposure to 15 mM DMTU; (d) after exposure to 16.6 μM cisplatin and 15 mM DMTU (24 h of incubation); viable cells are located in the left lower quadrant; cells undergoing early apoptosis are in the right lower quadrant, and dead cells are in the left and right upper quadrants.

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sensor down to 300 a.u. Addition of DMTU with cisplatin simultaneously caused a decrease in the median fluorescence intensity of the sensor HyPer2 to 235 a.u., however, for SypHer2 this decrease was not observed (453 a.u.). In both cases, the shape of the fluorescence peak for cells with the addition of DMTU is similar to that for control cells.

In populations of cells passing the stage of early apoptosis (Figure 4 (e), (f)), the addition of DMTU caused a decrease in the median fluorescence intensity in both lines. The addition of cisplatin to HeLa Kyoto–HyPer2 cells caused an almost double increase in the median value, from 170 to 331 a.u., while the addition of the same drug to HeLa Kyoto–SypHer2 cells resulted in a significant decrease in the median fluorescence intensity of the sensor from 422 to 294 a.u. Simultaneous incubation with DMTU and cisplatin resulted in a decrease in the median fluorescence intensity below the median values for cells treated with cisplatin only. This effect was observed both in the HeLa Kyoto–HyPer2 cell line (160 a.u.), and in the HeLa Kyoto–SypHer2 cell line (198 a.u).

It should be noted that for all cell probes that underwent simultaneous incubation with DMTU and cisplatin the peaks have similar shape with those for cell probes treated with DMTU only.

Figure 3. Distribution of HeLa Kyoto–SypHer2 cells over fluorescence intensity of 7-AAD and PE Annexin V:
(a) without addition of cisplatin and DMTU; (b) after exposure to 16.6 μM cisplatin; (c) after exposure to 15 mM DMTU; (d) after exposure to 16.6 μM cisplatin and 15 mM DMTU (24 h of incubation); viable cells are located in the left lower quadrant; cells undergoing early apoptosis are in the right lower quadrant, and dead cells are in the left and right upper quadrants.
Figure 4. Histograms of the fluorescence of HeLa Kyoto–HyPer2 (a), (c), (e) and HeLa Kyoto–SypHer2 (b), (d), (f) under the action of cisplatin and/or DMTU, plotted for different cell populations: (a), (b) all cells; (c), (d) viable cells; (e), (f) cells in the early apoptosis stage. Division to cell populations is based on the cells distribution of over the fluorescence intensity of 7-AAD and PE Annexin V shown in Figures 2, 3. M1 is the area of determination of the sensors fluorescence intensity median value.
Discussion

Analysis of diagrams of the staining of HeLa Kyoto–HyPer2 and HeLa Kyoto–SypHer2 cells with trypan blue (see Figure 1), indicated that the reactions of both cell lines to the addition of cisplatin and/or DMTU are similar: incubation for 24 h with 16.6 μM cisplatin leads to the death of about 50% of cells, and this effect can be neutralized by adding 15 mM DMTU to the incubation medium. The ROS scavenger DMTU added to cisplatin significantly increases the survival rate of the drug-treated cells, indicating that an increase in the level of intracellular ROS mediates cisplatin-induced death of tumor cells. Addition of DMTU only did not cause any significant changes in cells viability.

The distribution of cells of both lines according to 7-AAD and PE Annexin V fluorescence intensity (see Figures 2, 3) also indicated the similarity of their responses to cisplatin, proving that the differences in the structure of the sensors did not affect significantly the functional state of the cells. It should be noted that in both cell lines, the majority of the dying cells initially moved from the quadrant of the diagram typical for living cells to the quadrant typical for early apoptotic cells, and only then migrate to the region of dead cells. This indicates that the cisplatin-induced death realized rather by the apoptotic pathway than by necrosis. Similar results, the initiation of apoptosis in tumor cells under the treatment with cisplatin, were previously demonstrated in a number of studies [4, 21, 26]. There are also papers reporting treatment with cisplatin led to necrosis or apoptosis and necrosis simultaneously in the same population of tumor cells [27, 28]. There is an assumption that, depending on the level of cell damage caused by cisplatin, necrosis can take place either directly or as a result of an incomplete or defective apoptotic program [29–32]. Similar to experiments with trypan blue staining, in the experiments with flow cytometry a significant increase in the number of viable cells during simultaneous incubation with DMTU and cisplatin was observed. Since the addition of DMTU resulted in a significant decrease in the percentage of dead cells and did not increase the percentage of early apoptotic cells, we can deduce that DMTU protects the protective role for cells treated with cisplatin. The results are consistent with works of other researches showing the ability of DMTU to reduce the accumulation of singlet oxygen, hydroxyl radical [17], H₂O₂ [18], and block the activation of p53, leading to weakening of p53 upregulated modulator of apoptosis alpha (PUMA-α) production and suppression of the mitochondrial pathway of apoptosis during cisplatin treatment [16, 21].

The use of a specific sensor HyPer2 allowed to evaluate the contribution of H₂O₂ among a number of other ROS in the process of tumor cell death upon exposure to cisplatin.

The increase in the HyPer2 fluorescence intensity of cisplatin-treated cells as compared to control cells and the absence of such an increase in the experiments with cells with SypHer2 (see Figure 4) noted in the histograms indicated a significant increase in the amount of H₂O₂ in both viable and apoptotic cells. In a number of studies, it has been shown that the accumulation of intracellular H₂O₂ contributes significantly to enhanced apoptosis, although other mechanisms of its initiation cannot be excluded [4, 5, 33].

For example, H₂O₂, like other forms of ROS being important components of cellular signaling, can also cause DNA damage [33]. It should also be mentioned that the incubation of HeLa Kyoto–SypHer2 cells with cisplatin did not increase the fluorescence of the sensor, even leading to its decrease. Since the SypHer2 sensor is sensitive to changes in pH, like HyPer2, being insensitive to H₂O₂ level changes, unlike HyPer2, their reactions are multidirectional: the apparent increase in fluorescence of H₂O₂ sensitive sensor has been partially weakened by intracellular pH changes. Addition of DMTU to cisplatin-treated cells led to a decrease in the level of H₂O₂ down to the values close to those in the control, and an increase of pH within the cell to the control values. It should be noted that since DMTU scavenge mainly the hydroxyl radical, an indirect reaction can be observed caused by the removal of the hydrogen peroxide precursor.

Conclusion

In our study, the participation of H₂O₂ in the response of tumor cells HeLa Kyoto to cytotoxic effects of cisplatin has been demonstrated. The removal of H₂O₂ caused by the presence of ROS scavenger DMTU leads to a significant increase in the viability of cisplatin-treated cells leading to viability similar to that for cells that were not exposed to the drug. The cisplatin-induced death is realized mainly due to the activation of the apoptotic pathway initiated by an increase in the amount of ROS, including H₂O₂.

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