THE STUDY OF CISPLATIN EFFECT ON HYDROGEN PEROXIDE AND pH LEVEL IN HELA KYOTO CELL LINE USING GENETICALLY ENCODED SENSORS

UDC 517.21:575:618.146–006.6
Received 13.10.2013

© **A.S. Belova,** Postgraduate, the Department of Biophysics[1];
N.M. Mishina, PhD, Junior Research Worker, the Laboratory of Molecular Technologies[2]; Research Worker, Fluorescence Bioimaging Laboratory, Scientific Research Institute of Biomedical Technologies[3];
A.G. Orlova, PhD, Research Worker, the Laboratory of Biophotonics[4];
E.A. Sergeeva, PhD, Senior Research Worker, the Laboratory of Biophotonics[4];
A.V. Maslennikova, D.Med.Sc., Professor, the Department of Oncology[3];
A.A. Brilkina, PhD, Associated Professor, the Department of Plant Biochemistry and Physiology[1];
N.M. Shakhova, D.Med.Sc., Leading Research Worker, the Laboratory of Biophotonics[4];
V.V. Belousov, PhD, Senior Research Worker, the Laboratory of Molecular Technologies[2]; Senior Research Worker, Fluorescence Bioimaging Laboratory, Scientific Research Institute of Biomedical Technologies[3];
S.A. Lukyanov, D.Bio.Sc., Academician of Russian Academy of Sciences, Vice-Rector on Scientific Work[5]; Head of Fluorescence Bioimaging Laboratory, Scientific Research Institute of Biomedical Technologies[3]

[1]Nizhny Novgorod State University named after N.I. Lobachevsky — National Research University, Gagarin Avenue, 23, Nizhny Novgorod, Russian Federation, 603950;
[2]Shemyakin and Ovchinnickov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Miklukho-Maklaya St., 16/10, Moscow, Russian Federation, 117997;
[3]Nizhny Novgorod State Medical Academy, Minin and Pozharsky Square, 10/1, Nizhny Novgorod, Russian Federation, 603000;
[4]Institute of Applied Physics of Russian Academy of Sciences, Ul'yanova St., 46, Nizhny Novgorod, Russian Federation, 603950;
[5]Pirogov Russian National Research Medical University, Ostrovitianov St., 1, Moscow, Russia Federation, 117997

The aim of the investigation was to study the changes of hydrogen peroxide level and pH level in cytoplasm of cervical cancer cells HeLa Kyoto on cytotoxic exposure using genetically encoded sensors of hydrogen peroxide and pH.

Materials and Methods. In the study we used two cell lines of human cervical cancer HeLa Kyoto containing in cytoplasm a genetically encoded sensor of hydrogen peroxide HyPer2 and a sensor pH HyPer2-C199S. To assess toxic effect of cisplatin on HeLa Kyoto cells we used a standard MTT assay. The changes of pH and hydrogen peroxide (H_2O_2) level were determined using fluorescence microscopy by modification in proportion between fluorescence intensities at sensor's excitation at two wavelengths: 500 and 420 nm (F500/F420). During the experiment the cells were kept in incubator at 37.0°C in carbonate-free and serum-free medium MEM. Cisplatin solution at final concentration corresponding to IC_{50} according to MTT assay was added directly in culture medium. MEM with no cisplatin added was used as a control medium.

Results. Addition of cisplatin resulted in no changes in hydrogen peroxide and pH level in cytoplasm of HeLa Kyoto cells expressing corresponding sensors during the whole period of observation (20 min).

Conclusion. The use of genetically encoded sensors enables to demonstrate cisplatin to have no effect on hydrogen peroxide and pH level in HeLa Kyoto cells.

Key words: cisplatin; genetically encoded sensor; hydrogen peroxide; fluorescence microscopy; carcinoma Hela Kyoto cell line; MTT assay.

For contacts: Belova Anastasiya Sergeevna, phone: +7906-359-88-85; e-mail: belova-as@mail.ru

Chemotherapy (cytotoxic effect on tumor) is one of the main treatment modalities of malignancies. The understanding of molecular mechanisms causing cell death under the effect of various antitumor agents is of great importance for the development of pathogenetically grounded treatment methods.

Cytotoxic effect, at least partially, is based on hypersensibility of tumor cells to oxidative stress [1], and reactive oxygen species (ROS) play a key role in the realization of antitumor agent effect [1–3]. In this view, the most urgent researches are those concerned with the study of oxidative stress nature, the peculiarities of its development in tumors, as well as the possibility to have an effect on tumor oxidative status in order to improve the therapy efficiency.

Until now the participation of some certain ROS forms (generation site, main targets, utilization pathways) in the development of tumor response to different types of antitumor effects have not been yet entirely understood. Hydrogen peroxide (H_2O_2), which is a signal molecule regulating proliferation and migration of both healthy and cancer cells, is of particular interest for understanding the mechanisms of antitumor effects of pharmaceutical agents [4]. Despite the understanding of a key role H_2O_2 plays in cell damage development under the effect of toxic agents, there are some aspects concerning H_2O_2 place of origin, accumulation and degradation in cells, which are not fully understood.

Some researchers along with ROS consider the change in intracellular pH level to be a regulation factor of cell damage. pH level changes have been shown to contribute to cell malignant transformation, growth, invasion, neoangiogenesis, metastasis, as well as the formation of multiple drug resistance [5]. In addition, there is an opinion that intracellular pH level changes in the mechanisms of cell death activation mediate ROS effect [6]. One might assume that both hydrogen peroxide content changes and intracellular pH level changes can play a key role in the processes of cell damage under the effect of cytotoxic agents. A valid answer to this problem can be received if highly sensitive and specific sensors are used in parallel.

The aim of the investigation was to study the changes of hydrogen peroxide level and pH level in cytoplasm of cervical cancer cells HeLa Kyoto on cytotoxic exposure using genetically encoded sensors of hydrogen peroxide and pH.

Materials and Methods. In the study we used two cell lines of human cervical cancer HeLa Kyoto transfected by genetically encoded cytoplasmic sensor of hydrogen peroxide — HyPer2 and pH — HyPer-2C199S (HeLa Kyoto–HyPer2 and HeLa Kyoto–HyPer2-C199S). HyPer is a chimeric protein consisting of two domains: one — sensitive to H_2O_2 (regulatory domain of transcription factor OxyR of Escherichia coli) and another – fluorescent (so called circularly permuted yellow fluorescent protein — cpYFP) [7]. HyPer2 sensor was produced by point mutation of A406V (alanine is replaced by valine in location 406) in HyPer protein structure that corresponds to A233V replacement in wild type OxyR [8]. HyPer has two excitation peaks (420 and 500 nm) and one emission peak at 516 nm (Fig. 1). When H_2O_2 level increases, in cells fluorescence intensity decreases proportionally at excitation in the range 400–450 nm and intensity increases at excitation in the range 450–510 nm that is recorded as the change of a corresponding ratio, i.e. HyPer is a ratiometric sensor [7]. Using HyPer sensor there was obtain the information on hydrogen peroxide role in different biological processes including data on the change of its intracellular concentration under the effect of epidermal growth factor (EGF) [9].

HyPer2-C199S sensor was produced by point mutation of C199S in HyPer2 sensor structure. The substitution of cysteine for serine in location 199 prevents the formation of disulphide bonds in protein structure in its oxidation. HyPer2-C199S is not sensitive to H_2O_2 content change. The change of this sensor conformation is determined by pH level changes only [10].

At the first stage of the investigation we studied the peculiarities of cisplatin toxic effect in relation to cell lines HeLa Kyoto–HyPer2 and HeLa Kyoto–HyPer2-C199S aiming to reveal the agent concentration causing the death of a certain percentage of cells. The study was carried out using a standard MTT assay based on the capability of mitochondrial dehydrogenases in viable cells to convert water-soluble 3-(4,5-dimethylthiazole-2-il)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in formazan, which crystallizes inside a cell. The measurement of formazan concentration in the solution after its interaction with dimethylsulphoxide (DMSO) enables to determine the number of viable cells and in cytotoxic studies — assess specific cell death induced by a certain agent [11].

HeLa Kyoto cells were seeded into a 96-well plate: 3000 cells into each well. The wells were then added 200 μl of Eagle's MEM medium (PanEco, Russia) containing 2 mmol glutamine and 10% FBS (HyClone, USA) and incubated in CO_2-incubator for 24 h (37.0°C, 5% CO_2). 24 h later the initial medium was changed by

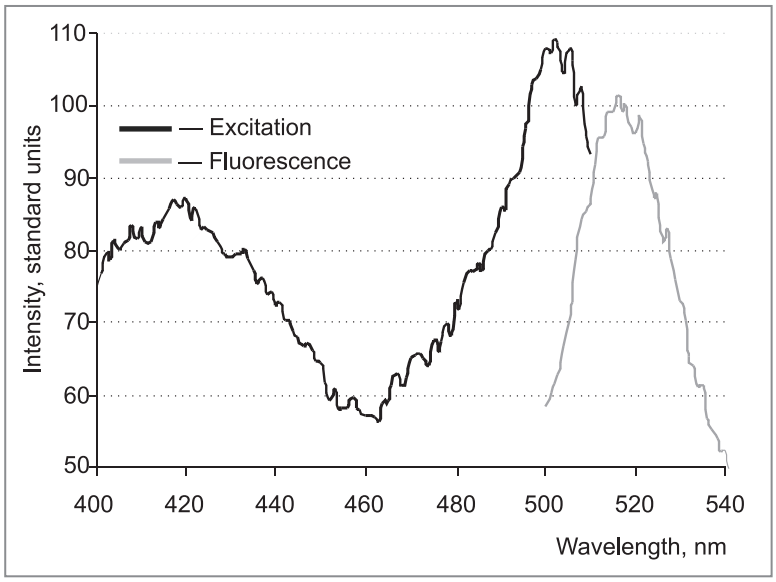

Fig. 1. Excitation and fluorescence spectra of HyPer sensor (www.evrogen.com)

Eagle's MEM medium with glutamine and 10% FBS containing cisplatin. We varied cisplatin concentrations from 0 to 15 μg/ml. A day later we changed the medium for medium with MTT in amounts of 10% of the medium volume by 200 μl into each well. The plate was placed in CO_2-incubator for 4 h, after that the medium was removed and 100 μl of DMSO was added into each well for 40 min while stirring continuously. Optical density of the obtained formazan solution in DMSO was measured on plate spectrophotometer Synergy MX (BioTek, USA) at wavelength of 570 nm. The staining intensity in wells with cells without cisplatin was taken for 100% survival rate. The dependence diagram of the number of survived cells against cisplatin concentration was averaged according to four experimental findings. To study the cell response to cisplatin effect we chose cisplatin concentration, at which 50% of cells lose their viability within 24 h (IC_{50}).

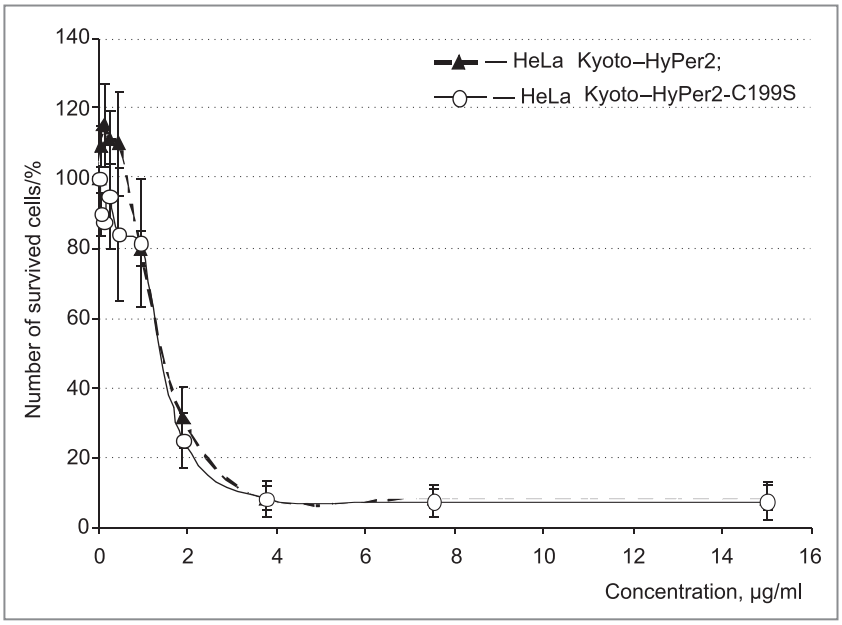

Fig. 2. Cisplatin concentration effect on cell viability of cells of HeLa Kyoto–HyPer2 and HeLa Kyoto–HyPer2-C199S cell lines

At the second stage of the study we analyzed the response of sensors to cisplatin addition. We used fluorescent inverted microscope Leica DMI6000 (Leica Microsystems GmbH, Germany). Fluorescence was stimulated by mercury-halogen lamp interchangeably using filters of 427/10 nm and 504/12 nm. Fluorescence was recorded using 542/27-nm filter. Fluorescent images were recorded every 30 s for 20 min. Hydrogen peroxide and pH level changes were determined by the change of F500/F420 ratio of fluorescent useful signals from cell cytoplasm at corresponding excitation wavelengths [12]. F500/F420 ratio graph for each sensor variant was averaged according to fluorescence level of 5 cells.

The cells were plated (on Petri dish with glass bottom) and contained in CO_2-incubator at 37.0°C under the atmosphere containing 5% CO_2. The cells were cultured in DMEM medium containing 2 mmol glutamine and 10% FBS. 24 h later the plate was placed in a micro-incubator at 37.0°C. Cisplatin solution in medium was added directly to the plate with carbonate-free serum-free solution MEM (Sigma-Aldrich, USA). 0.1 ml of medium containing cisplatin at final concentration corresponding to IC_{50} according to MTT-test was added to 1.4 ml of medium in the plate. MEM of the same volume without cisplatin was used as control.

The findings were presented as mean and standard deviations. The data were processed using EMBL ImageJ program and license software Excel 5.0.

Results. According to MTT-test there we plotted a curve demonstrating the dependence of HeLa Kyoto–HyPer2 and HeLa Kyoto–HyPer2-C199S cell viability on cisplatin concentration in medium (Fig. 2). For further study of H_2O_2 content changes in cells we chose cisplatin concentration — 2.937 μg/ml causing viability loss of 50% of cells.

According to ratiometric monitoring of HyPer2 fluorescence, addition of cisplatin in the indicated concentration caused no changes of F500/F420 ratio suggesting no H_2O_2 level changes in cytoplasm of HeLa Kyoto–HyPer2 cells compared to control (Fig. 3). Insignificant growth of F500/F420 ratio immediately after the addition was observed in both cases: when MEM was added, and after cisplatin addition.

In HeLa Kyoto–HyPer2-C199S cells we found no changes of intensity ratio on two fluorescence peaks regardless of MEM or cisplatin addition. It indicates non-change of intracellular pH level under cytotoxic agent effect.

Discussion. Currently, the role of oxidative stress in initiation and realization of cell death induced by cytotoxic agents based on platinum is being studied in detail using a complex of methods [1]. The most researchers describe the concentration changes of the whole ROS pool, while the data on hydrogen peroxide role in cell response to the contact with cisplatin is incomplete and controversy. The authors of the study [13] have demonstrated a temporary increase of hydrogen peroxide production level when cisplatin is added to prostate cancer cells. Other authors in their work [14] have shown hydrogen peroxide to take part in initiation of cell death by necrosis mechanism but not by apoptosis mechanism.

There are facts that the change of intracellular H_2O_2 and pH levels are linked and play a key role in cell death processes. In particular, there was shown that the shift of balance between such ROS forms as hydrogen peroxide and superoxide anion-radical towards H_2O_2 (hydrogen peroxide concentration increase or superoxide anion-radical concentration decrease) leads to the activation of apoptotic pathway components mediated by cytoplasmic pH decrease [6]. In addition, cisplatin was found to cause rapid (within 5–15 min) pH decrease in the range of 0.1 unit in colon cancer cells [15].

In this study with genetically encoded sensors we demonstrated the contact with cisplatin in IC_{50} concentration to cause no increase of hydrogen peroxide level in cytoplasm of cells of HeLa Kyoto–HyPer2 cell line.

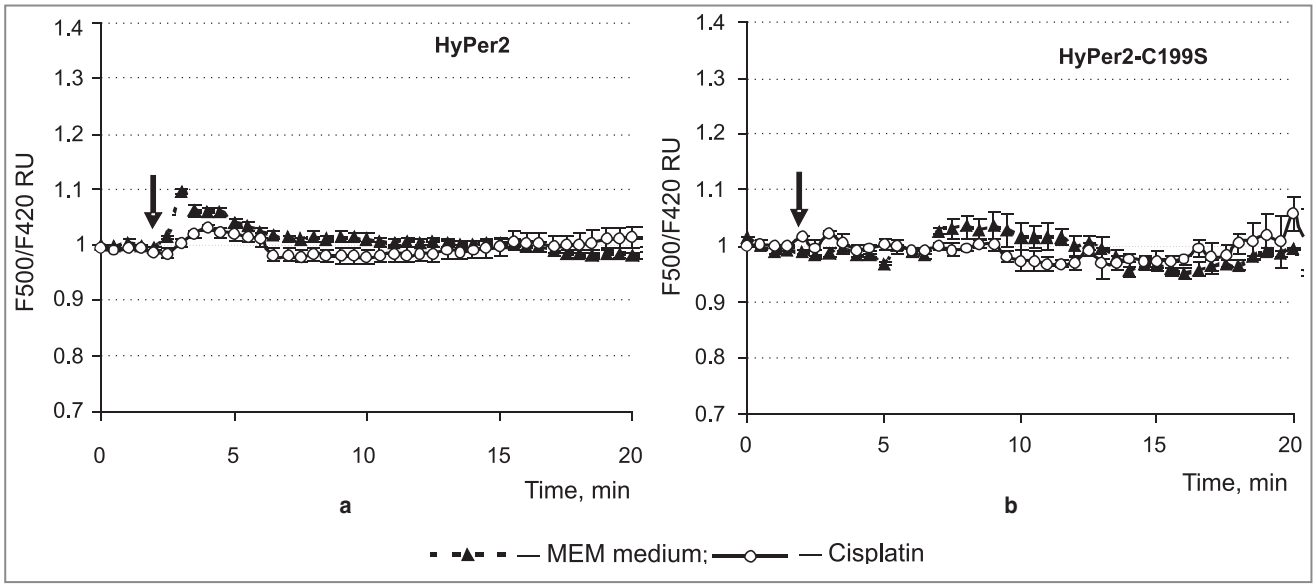

Fig. 3. H_2O_2 level in cytoplasm of HeLa Kyoto–HyPer2 cells (*a*) and pH level in HeLa Kyoto–HyPer2-C199S cell cytoplasm (*b*) after cisplatin addition (IC_{50}) and MEM with no cisplatin added. The arrow indicates the time cisplatin was added

The findings correspond to the data of the study [16], the authors of which did not find the increase of reactive oxygen species forms in osteosarcoma cells SOSN2. Moreover, we recorded no changes of cytoplasm pH in cell culture HeLa Kyoto–HyPer2-C199S. It provides the necessity for further studies of the mechanisms of cisplatin-induced death of HeLa Kyoto cells depending on cisplatin concentration.

Conclusion. The use of genetically encoded sensors enables to demonstrate cisplatin to have no effect on hydrogen peroxide and pH level in HeLa Kyoto cells.

Study Funding. The work was supported by the grants within the frame of RAS Presidium program "Fundamental sciences to medicine", the project of Russian Foundation for Basic Research 13-04-97165, the project of Ministry of Education and science of the Russian Federation No.8147, the project of Ministry of Education and science of the Russian Federation No.8269, the project of Ministry of Education and science of the Russian Federation No. 11.G 34. 31.0017.

Conflict of Interests. The authors have no conflict of interest to disclose.

References

1. Conklin K.A. Chemotherapy-associated oxidative stress: impact on chemotherapeutic effectiveness. *Integrative Cancer Therapies* 2004; 3(4): 294–300.

2. Manda G., Nechifor M.T., Neagu T.-M. Reactive oxygen species. Cancer and anti-cancer therapies. *Current Chemical Biology* 2009; 3: 342–366.

3. Florea A.-M., Bьsselberg D. Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. *Cancers* 2011; 3: 1351–1371.

4. Szatrowski T.P., Nathan C.F. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 1991; 51: 794–798.

5. Harguindey S., Orive G., Pedraz J.L., Paradiso A., Reshkin S.J. The role of pH dynamics and the Na^+/H^+ antiporter in the etiopathogenesis and treatment of cancer. Two faces of the same coin — one single nature. *Biochim Biophys Acta* 2005; 1756(1): 1–24.

6. Pervaiz S., Clement M.V. A permissive apoptotic environment: function of a decrease in intracellular superoxide anion and cytosolic acidification. *Biochem Biophys Res Commun* 2002; 290(4): 1145–1150.

7. Belousov V.V., Fradkov A.F., Lukyanov K.A., Staroverov D.B., Shakhbazov K.S., Terskikh A.V., Lukyanov S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat Methods* 2006; 3(4): 281–286.

8. Markvicheva K.N. *Issledovanie signal'nykh funktsiy peroksida vodoroda v protsesse fagotsitoza*. Avtoref. dis. … kand. biol. nauk [The study of hydrogen peroxide signal functions in phafocytosis. Abstract for Dissertation for the degree of Candidate of Biological Sciences]. Moscow; 2010.

9. Mishina N.M., Tyurin-Kuzmin P.A., Markvicheva K.N., et al. Does cellular hydrogen peroxide diffuse or act locally? *Antioxid Redox Signal* 2011 Jan 1; 14(1): 1–7.

10. Poburko D., Santo-Domingo J., Demaurex N. Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. *The Journal of Biological Chemistry* 2011; 286(13): 11672–11684.

11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65(1–2): 55–63.

12. Mishina N.M., Markvicheva K.N., Bilan D.S., Matlashov M.E., Shirmanova M.V., Liebl D., Schultz C., Lukyanov S., Belousov V.V. Visualization of intracellular hydrogen peroxide with HyPer, a genetically encoded fluorescent probe. *Methods Enzymol* 2013; 526: 45–59.

13. Tomohiro I., Riyako T., Keitaro K., Keita N., Takashi D., Masashi1 A., Yasuyuki T., Masafumi I., Yoshinori N. Cisplatin induces production of reactive oxygen species via NADPH oxidase activation in human prostate cancer cells. *Free Radical Research* 2011; 45 (9): 1033–1039.

14. Baek S.M., Kwon C.H., Kim J.H., Woo J.S., Jung J.S., Kim Y.K. Differential roles of hydrogen peroxide and hydroxyl radical in cisplatin-induced cell death in renal proximal tubular epithelial cells. *J Lab Clin Med* 2003; 142(3): 178–186.

15. Rebillard A., Tekpli X., Meurette O., Sergent O., LeMoigne-Muller G., Vernhet L., Gorria M., Chevanne M., Christmann M., Kaina B., Counillon L., Gulbins E., Lagadic-Gossmann D., and Dimanche-Boitre M.-T. Cisplatin-induced apoptosis involves membrane fluidification via inhibition of NHE1 in human colon cancer cells. *Cancer Res* 2007; 15(67): 7865–7874.

16. Katsuda H., Yamashita M., Katsura H., Yu J., Waki Y., Nagata N., Sai Y., Miyamoto K. Protecting cisplatin-induced nephrotoxicity with cimetidine does not affect antitumor activity. *Biol Pharm Bull* 2010; 33(11): 1867–1871.