Romanian Reports in Physics, Vol. 68, No. 4, P. 1545–1553, 2016

INVOLVEMENT OF THE REDOX POTENTIAL IN DEVELOPMENT OF RESISTANCE TO TREATMENT IN BREAST CANCER

D.L. STANCULEANU[1], R. ANGHEL[1], A. LAZESCU[1], D. ZOB[1], I. GRUIA[*2], M.I. GRUIA[1]

[1] "Prof. Dr. Al. Trestioreanu" Institute of Oncology Bucharest, Romania
[2] University of Bucharest, Faculty of Physics, Magurele, Romania
**Corresponding author*: gruia_ion@yahoo.com

Received May 25, 2015

Abstract. This paper aims to identify a potential mechanism of tumour resistance to chemotherapy treatment due to the drug transport**.**

In this study only breast cancer patients were included. Depending on the response to chemotherapy and progression of disease, the patients were divided into two groups: patients with resistance to chemotherapy (group 1) and patients with response to chemotherapy (group 2). Proteins involved in redox reactions (*e.g.*, ceruloplasmin), the thiol (-SH) groups from albumin, and nitrogen oxide (NO) resulted from protein oxidative attack were determined by biochemical assays.

The results show that copper-oxidase activity of ceruloplasmin increases in cancer patients compared to normal values reported in literature (*i.e.*, 80–120 IU). Increased levels of free thiols in patients included in the group 1 are on average higher with 12.4 % as compared to group 2. The serum NO concentration has shown values above the normal range (7–14) μmol/l. We conclude that hypoxic stress proteins and redox potential modifications may induce resistance to chemotherapeutic agents.

Key words: treatment resistance, breast cancer, reactive oxygen species, proteins with sulphur.

1. INTRODUCTION

In Romania breast cancer is a major public health concern the neoplasia having the highest incidence in women. The aim of cancer therapy is to prevent cancer cell proliferation, invasion and metastasis. The resistance to various anticancer drugs is the main cause of treatment failure in 90 % of patients with metastatic breast cancer. Current understanding of main mechanisms of cytostatics action at the molecular level as well as of the genetic changes that induce drug resistance allowed the establishment of new therapeutic strategies where molecular genetics and treatment may act together to increase chemo sensitivity of neoplastic cells and protecting the normal ones. Drug resistance development may be related to factors such as poor absorption, pharmacological activation [1] deficient metabolism and rapid excretion of cytostatics [2], alteration of transport proteins, metastasis in "sanctuaries" as well as cellular factors such as: cytostatic decreased

influx/increased efflux [3] alteration of cellular metabolism (increased degradation, decreased activation), cytoplasmic nuclear inactivation (by glutathione, metallothionein proteins), DNA repair, alteration of cellular targets.

Electron transfer reactions play key roles in diverse processes in chemistry, physics, and biology, ranging from photo-induced reactions [4], electron tunnelling in proteins [5], and electron transport in DNA [6] to the reductive DNA damage [7]. Electron transfer reactions in molecular systems have therefore been the subject of intense experimental and theoretical studies [8].

Reactive oxygen species (ROS) play an important role in cellular signal transduction and are essential in maintaining redox balance in biological systems. The balance between ROS generation and elimination determines the cellular redox state, which is often altered during malignant transformation. It has long been recognized that cancer cells exhibit an increase in ROS generation compared to their normal counterparts, due in part to oncogenic signals, active metabolism, and mitochondrial dysfunction [9].

Studies on breast, prostate, and colorectal cancers have shown that many factors can contribute to chemo resistance, including the individual genetic background as well as epigenetic factors. For example, intracellular cisplatin inactivation by glutathione has been proposed as a mechanism of tumour cisplatin resistance [10]. Glutathione is a cellular antioxidant, preventing damage of important cellular components caused by reactive radicals. It reduces disulfide bonds formed within cytoplasmic proteins to cysteines serving as electron donors. Thus, glutathione may direct cisplatin to target proteins situated relatively away from DNA in the cell [11]. Reversely, the resistance may be circumvented by activating cisplatin with an electron donor to produce reactive radicals that provoke DNA damage and cell death.

This paper aims to identify a potential mechanism of tumour resistance to chemotherapy treatment due to the altered cytostatic transport mediated by proteins. It was proposed a mechanism by which the oxidative stress induces conformational changes of proteins involved in cytostatic drug transport conducting to a poor and inefficient response to chemotherapy.

2. MATERIALS AND METHODS

2.1. CLINICAL STUDY

In this study, 36 patients with metastatic breast cancer resistant to chemotherapy (group 1) and 43 breast cancer patients without chemotherapy resistance (group 2) were included. We monitored the clinical parameters such as: age at onset, histopathological diagnosis, immunohistochemistry time of resistance appearance, and oxidative stress biochemical parameters. In this study protocol were included: patients with histologically and immunohistochemically confirmed

metastatic breast cancer, patients with signed consent to collect data and biological samples, patients older than 18 years of age, the ECOG 0-2 IP (Eastern Cooperative Oncology Group-Zubrod and Karnofsky scales) performance status, breast cancer patients that required and received chemotherapy (either neoadjuvant, adjuvant for metastatic phase according to current treatment guidelines), patients with measurable disease progression by RECIST (Response Evaluation Criteria in Solid Tumours). Patients with a history of other cancers in the last 5 years except for *in situ* cervical carcinoma, basal cell carcinoma, the patients with early stage cutaneous breast cancer (who received surgery only), as well as patients under hormone therapy, and radiotherapy were excluded from the study.

2.2. BIOCHEMICAL ASSAYS

In order to determine biochemical parameters of oxidative stress, biological samples (5 ml venous blood) were collected from patients in both groups (group 1 without resistance to the treatment, group 2 with the development of resistance mechanisms). The same redox potential parameters at the protein level for both groups were determined. It is believed that either the free thiol groups produce chemical compounds which are able to counteract DNA binding or conformational changes induce alteration of drug cytoplasmic transport mechanisms. For this purpose it was determined: 1) the level of *ceruloplasmin oxidase activity* using the Ravin method based on its reaction with p-phenylene diamine in acetic acid buffer [12], 2) the *albumin thiol* level using a method based on spectrophotometric absorbance of the complex formed with Elmann reagent [13], and 3) the *nitrogen oxide level* determined by Griess reaction [14].

2.3. STATISTICAL ANALYSIS

All experiments were performed in triplicate and the data are representative for the observed results. Data are presented as the mean ± SD. Independent Student's t-tests were used to compare continuous variables between the two groups. Data were analyzed using JMP (statistical software). P-value < 0.05 was considered statistically significant.

3. RESULTS

3.1. CERULOPLASMIN ACTIVITY

Higher values of ceruloplasmin oxidase activity were found in the patient group 2 that developed resistance to chemotherapy (Table 1).

Table 1

Ceruloplasmin activity. Normal values are between of 80 and 120 IU standardised by Ravin spectro-photometric method

Normal median values (average values expressed in IU)	Group 1	Group 2
100 ± 12	132 ± 17 IU	152 ± 15 IU

By this technique, we measured the intensity of copper oxidase activity of the protein. This technique demonstrates the enzymatic activity of the protein and, consequently, its capacity to use copper ions from its active catalytic centre to be mobilized in oxidation-reduction reactions. It is well known in biochemistry that the total quantitative expression of one protein is sometime different than its enzymatic activity.

As seen from Fig. 1, the oxidase activity of ceruloplasmin increases in cancer patients compared to normal median values (100 %) from the literature [12], with percentages between 32 and 52 %.

These increases are primarily associated with protein overproduction induced by tumour growth. The excessive synthesis of this protein with antioxidant activity is also due to the redox cellular status. It is well known that tumour tissue induces reactive radical species, but in the same time it synthesizes an increased amount of protein with antioxidant action to counteract redox reactions which locally can be devastating for cells [15].

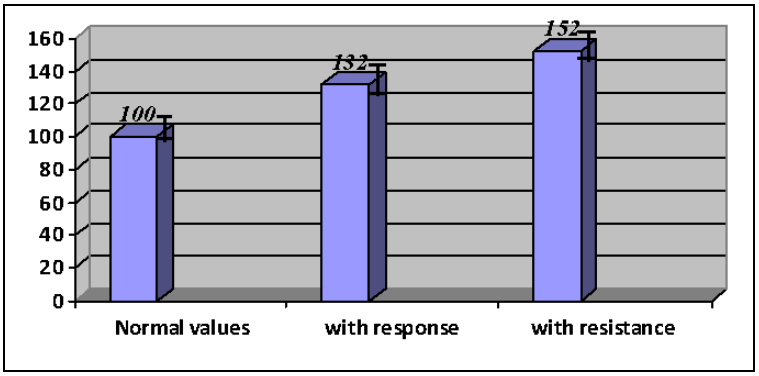

Fig. 1 – The ceruloplasmin activity distribution in patients from the group 1 and group 2.

3.2. DETERMINATION OF ALBUMIN THIOLS

The action of ROS to the structural or enzymatic proteins causes the protein denaturation. The most common ROS interactions occur with protein thiol groups

to form tiil radicals which, in turn, can suffer dimerization and oxidation, forming disulfides or sulfonic acid derivatives.

The oxidative degradation inhibits the enzymatic activity by modifying enzyme active catalytic site (also by oxidative degradation of aromatic rings in the structure of component amino acids).

The data show an increase in serum thiol levels for patients with responsive mammary tumours with values of about 5.36 % higher than normal and 24.39% higher than normal for resistant group, suggesting an excess production of ROS that act on albumin and sulphur containing proteins with antioxidant role, too.

These results suggest that the albumin may be involved in transport mechanisms of cytostatics and, once linked to anti-tumour drugs, it will not be so easily damaged by redox reactions.

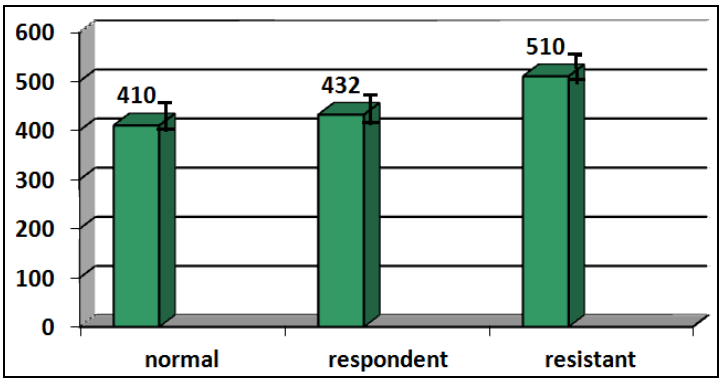

Fig. 2 – The level of serum albumin thiols. The normal value is from literature [16, 17].

Increased levels of free thiols in the patients with developed resistance to chemotherapy is up to 24.39 % higher than normal range (Fig. 2), although there are individual fluctuations up to 33 %. This increase is consistent with the data from the literature [16, 17] and confirms the antioxidant role of proteins containing sulphur. Secreted by the tumour in larger quantity they participate in antioxidant defences in order to restore the cellular oxidative stress balance [18].

The total thiol species detected in the serum can be indicators of development of resistance to treatment. There are two possible mechanisms of induced drug resistance:

- The anthracyclines and cytostatic drug classes metabolize local production of reactive species inductors of cytotoxicity. This mechanism is prevented by excessive production of proteins that contains sulphur and the antioxidant activity of natural defences systems.

• A transport mechanism involving platinum or metal compounds and other metal based compounds which preferentially bind sulphur electrons and not those of nitrogen from DNA where formation of adducts induces cellular toxicity.

3.3. DETERMINATION OF THE NITROGEN FREE RADICALS

Nitric oxide (NO) is a molecule that acts either as an oxidizing or reducing agent, depending on the target molecules. NO are small radical molecules exhibiting low solubility in water and high solubility in lipids. These physical properties allow its direct diffusion through biological membranes and explain its rapid diffusion into cells or into the bloodstream where it can mediate cytotoxic functions. NO dissolves in the blood and binds irreversibly to hemoglobin which becomes toxic.

Cytoprotective and cytotoxic effects of NO are important and contrasting, their balance being determined by local NO concentration within the given structure.

Elevated levels of NO in tissues are predictive of a poor survival in patients with breast or other cancers [19]. To prove this statement some experimental models were undertaken. The models involve murine xenografts or cell cultures and mimic the specific microenvironment of aggressive tumours, which includes inflammation factors (cytokines), lack of nutrients and hypoxia, as well as metabolic pathway of production of NO in an aggressive tumour phenotype [20]. Evidence for a link of breast cancer progression with production of these radical species, highlighting their involvement in progression and metastasis of malignant lesions, was previously found [21].

The results on the NO level in serum are presented in the Table 2.

Table 2

Serum NO level values. The normal values are from literature [14]

Average normal values	Group 1	Group 2
7.15 ± 2.09 μmol/l	24.23 ± 3.18 μmol/l	43.78 ± 2.94 μmol/l

The detection limit of the technique used (*i.e.*, Griess reaction) is about 1.5 μmol/l. The data present suggestively the increasing level of nitrogen oxides in cancer patients compared to the normal group. The marked increase of this oxide in patient blood exhibits chemotherapy resistance.

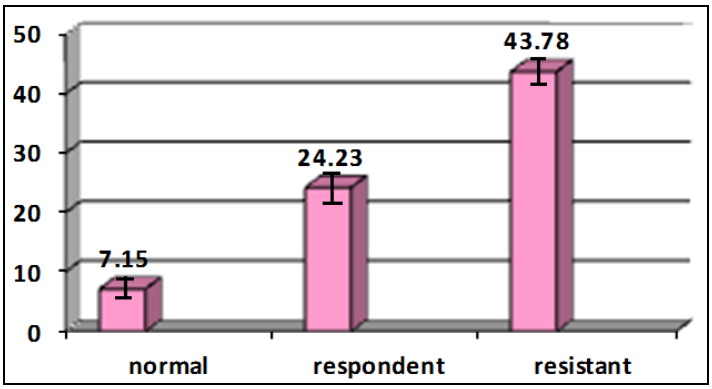

Fig. 3 – The level of serum nitric oxides measured by Griess reaction.
The ordinate numbers are given in μmol/l. The normal values are from literature [14].

The results show elevated values above those in normal serum in both groups of patients involved in the study. These results are explained by involvement of reactive nitrogen species in the reaction sequence that accompanies malignant transformation as well as in the cascade of reactions resulting in resistance mechanisms [22]. Thus, production of reactive nitrogen species in cancer patients is associated with installation of oxidative stress. The specific values are about three times higher (group 1) and six times higher (group 2) than those in the normal group (Fig. 3). The literature data confirm the cause-effect relationship between the presence of transformed malignant tissue and intense oxidative metabolism [23–25].

4. CONCLUSIONS

Current knowledge regarding the regulating role of cisplatin for expression of copper transporter proteins is emphasizing the importance of these proteins in the specific cytostatic drug transport. Therefore, the determination of serum levels of cooper-oxidase activity of ceruloplasmin is a very important step. Its serum values indicate an increase of up to 32–52 % as compared to normal ones. Immobilization of copper in transporting protein may be a potential competitive mechanism to cytostatic transport.

Measurements of serum total thiols from sulphur protein degradation were performed. The data obtained reveal an increase of serum thiol levels in patients with breast tumours that respond well to treatment with values of about 5.36 % higher, suggesting an excess production of reactive radical species that act on proteins albumin containing sulphur.

An increased level of free thiols in patients in the group that has developed resistance to chemotherapy is up to 24.39 % higher than the normal values. This increase is consistent with data from the literature. Secreted by tumour, larger

amounts of small proteins which contain sulphur participate in antioxidant defences in order to restore the cellular oxidative stress balance.

Hypoxic stress proteins may induce resistance to chemotherapeutic agents. Once these drugs with antitumour activity are bound to these transport molecules, they will not be so easily damaged by reactive oxygen species resulting from redox reactions.

The high levels of nitrogen oxides are predictive of patient poor survival in breast cancer and other cancers. The serum concentration of NO showed elevated values in both investigated groups.

The identification of small molecules involved in the cascade of events associated with malignant transformation may be useful for monitoring tumour treatment effectiveness.

REFERENCES

1. L. Miron, I. Miron, *Principiile chimioterapiei cancerului*, Chimioterapia cancerului, ed. Kolos 2005, pp. 3-73,.
2. *Drug absorption, distribution and elimination; pharmacokinetics*; http://www.columbia.edu/itc/gsas/g9600/2004/GrazianoReadings/Drugabs.pdf Retrieved from the Columbia University. worldwide web 24 May 2011.
3. G. J. R. Zoman, M. J. Fleus, M. R. van Lensden *et al.*, *The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump*, Proc. Natl. Acad. of Sci. of USA **91**, 8822-8826 (1994).
4. R. A. Marcus, *Electron transfer reactions in chemistry: Theory and experiment* (Nobel Lecture), Rev. Mod. Phys. **65**, 599-610 (1993).
5. H. B. Gray, J. R. Winkler, *Electron transfer in proteins*, Ann. Rev. Biochem. **65**, 537-561 (1996).
6. C. Wan, T. Fiebig, O. Schiemann, J. K. Barton, A. H. Zewail, *Femtosecond direct observation of charge transfer between bases in DNA*, Proc. Natl. Acad. Sci. USA **97**, 14052-14055 (2000).
7. C. R. Wang, J. Nguyen, Q. B. Lu, *Bond breaks of nucleotides by dissociative electron transfer of nonequilibrium prehydrated electrons: A new molecular mechanism for reductive DNA damage,* J. Am. Chem. Soc. **131**, 11320-11322, 2009.
8. Q. B. Lu, *Effects of ultrashort-lived prehydrated electrons in radiation biology and their applications for radiotherapy of cancer*, Mutat. Res. Rev. **704**, 190-199 (2010).
9. D. Trachootham, J. Alexandre, and P. Huang, *Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?*, Nature Reviews Drug Discovery **8**, *7*, 579-591 (2009).
10. M. M. Gottesman, *Mechanisms of cancer drug resistance*, Annual Review of Medicine **53**, 615-627 (2002).
11. C. Hwang, A. J. Sinskey, and H. F. Lodish, *Oxidized redox state of glutathione in the endoplasmic reticulum*, Science (New York) **257**, 1496-1502 (1992).
12. H. A. Ravin, S. Nomoto, *Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity*, Clin. Chem. **16**, 903-910 (1970).
13. A. Elmann, A. Telerman, S. Mordechay, H. Erlank, M. Rindner, R. Ofir, Y. Kashman, *3,5,4'-Trihydroxy-6,7,3'-trimethoxyflavone protects astrocytes against oxidative stress via interference with cell signaling and by reducing the levels of intracellular reactive oxygen species*, Neurochemistry International **78**, 67-75 (2014).
14. D. Giustarini, R. Rossi, A. Milzani, I. Dalle-Donne, *Nitrite and nitrate measurement by Griess reagent in human plasma: evaluation of interferences and standardization*, Methods Enzymol. **440**, 361-380 (2008).

15. H. H. W. Chen, M. Tien Kuo, *Overcoming Platinum Drug Resistance with Copper-lowering Agents*, Anticancer Res. **33**, *10*, 4157-4161 (2013).
16. D. M. Guttmann, C Koumenis, *The heat shock proteins as targets for radiosensitization and chemosensitization in cancer*, Cancer Biol. Ther. **12**, *12*, 1023-1031 (2011).
17. J. Lin, I-Min Lee *et al*., *Plasma homocysteine and cysteine and risk of breast cancer in women*, Cancer Res. **70**, *6*, 2397-2405 (2010).
18. H. Zahreddine, K. L. B. Borden, *Mechanisms and insights into drug resistance in cancer*, Frontiers in Pharmacol. **14**, *4*, 28-35 (2013).
19. L. C. Jadeski, K .O. Hum, C. Chakraborty, P. K. Lala, *Nitric oxide promotes murine mammary tumour growth and metastasis by stimulating tumour cell migration, invasiveness and angiogenesis*, Int. J. Cancer. **86**, 30-39 (2000).
20 J. L. Heinecke, L. A. Ridnour, R. Y. Cheng *et al*., *Tumor microenvironment-based feed-forward regulation of NOS_2 in breast cancer progression*. Proc. Natl. Acad. Sci. USA. **111**, 6323-6328 (2014).
21. M. Kelm, *Nitric oxide metabolism and breakdown*. Biochim. Biophys. Acta **1411**, 273-289 (1999).
22. M. Kartalou, J. M. Essigmann, *Mechanisms of resistance to cisplatin*. Mutat. Res. **478**, *1*, 23-43 (2001).
23. A. Angelskaya, I. Gruia *et al*., *Manifestations of linear dichroism changes in cancer biotissues*, Rom. Rep. Phys. **65**, *3*, 1052-1062 (2013).
24. L. C. Ciobotaru, I. Gruia, *Study of the carbon atoms production in methanol/ethanol – nitrogen flowing post-discharge plasma*, Rom. J. Phys. **60**, *9-10*, 1536-1549 (2015).
25. L. Gales *et al*., *In Vivo Study of BPA (boron10-phenilalanine) Use in Boron Neutron Capture Radiotherapy as an Alternative for Hepatic Cancer Treatment*, Rom. J. Phys. **60**, *3-4*, 521-527 (2015).