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STUDY OF INTERRELATIONS BETWEEN RESISTANCE TO CISPLATIN AND COMPOSITION OF LOW MOLECULAR WEIGHT THIOLS IN MURINE LEUKEMIA L1210 CELL LINES

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Cis-dichlorodiammineplatinum (II), commonly known as cisplatin, is a widely used anticancer drug. However, the therapeutic efficacy of the drug is limited due to various dose-limiting side effects and development of acquired resistance. The resistance of malignant cells to cisplatin may be connected to elevated concentration of cellular thiols. To verify this hypotheses, we compared the concentrations of low weight cellular thiols, metallothionein (MT) and glutathione (reduced, GSH and oxidized, GSSG) and also the metal-binding properties of MT in the two cell lines, known to be resistant (L1210R) or sensitive (L1210S) to cisplatin. It was established that R-cells were characterised by higher concentrations of MTs and GSH, higher ratio of copper in the composition of MTs in comparison to zinc than S-cells. The treatment by cisplatin provoked the decrease of the GSSG concentration only in the S-cells without the changes in GSH concentration. Hence, R-cells insensitivity to cisplatin can be related to higher levels of MTs and GSH. Reported for the first time peculiar metal composition of MTs in R-cells can also give an advance to these cells by particular oxidizing properties of copper-MT. These specific characteristics of thiols in malignant cells could be useful for the indication of cisplatin resistance.

Keywords: cisplatin, resistance, metallothionein, glutathione, copper.

INTRODUCTION

Cis-dichlorodiammineplatinum (II), commonly known as cisplatin, is a widely used anticancer drug [2]. However, the therapeutic efficacy of the drug is limited due to various dose-limiting side effects and development of acquired resistance [4]. In some cases, the changes in low weight cell thiols namely metallothioneins (MTs) and glutathione have been shown to be of importance in the occurrence of these side effects [3]. The involvement of these thiols in the response to cisplatin can be explained by the possibility of their interaction with this electrophilic reagent [15, 33, 36]. Therefore in the cells with the elevated level of MTs and glutathione, the efficacy of cisplatin as a drug may be diminished [15].

MTs are sulfhydryl-rich proteins involved mainly in metal (zinc, copper and cadmium) homeostasis and detoxification [20]. They also can be the oxyradical scavengers [8]. The synthesis of MTs is often induced when organisms are exposed to metals, especially Cd [20, 22]. Many other factors, among them oxidative stress agents, physical stress and other natural phenomena may also induce their synthesis directly or via effect on the homeostasis of essential for MTs metals in the laboratory studies [8, 12, 13].

The relation between the level of MTs expression and carcinogenesis is discussed in a few studies. Over-expression of MTs in intestinal tissue of colorectal cancer patients was shown and considered as a prognostic marker for a poor overall survival. In gastric cancer, however, MT expression in the gastric mucosa is not of prognostic significance [19]. In another studies, the high MTs expression, as detected by immunohistochemistry, predicted a better response rate to chemotherapy whereas tumors lacking or demonstrating low MT expression show a worse prognosis [9].

Reduced glutathione (GSH), an endogenous intracellular thiol-containing three-peptide, plays a crucial role in numerous biochemical reactions, regulation of cellular sulfhydryl status, transport, protection against free radicals and xenobiotics, and detoxification of metals and electrophiles [33]. Therefore it probably can be implicated in the metabolism of cisplatin via its binding causing alteration in the rate of its uptake and elimination. Dalton's lymphoma cells showed an increase in GSH concentration as compared to their normal counterpart and its decrease after *in vivo* cisplatin treatment along with a decrease of glutathione-S-transferase activity [21]. For the other hand, in certain cisplatin-resistant cell lines, GSH is overexpressed [1]. Increased intracellular GSH have been correlated closely with cisplatin resistance in PC12 cells and human ovarian cancer cell lines [24].

With respect to controversially results concerning the relation between the level of cellular low mass thiols and resistance to cisplatin, it was of interest to compare MTs and GSH levels and their characteristics in sensitive and resistant to cisplatin cell lines in the attempt to identify probable utility of these indices as a biomarkers in cisplatin-mediated cancer chemotherapy.

MATERIALS AND METHODS

Chemicals. 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), GSH, bovine serum albumin, cytochrome *c*, insulin, phenylmethylsulfonyl fluoride, β -mercaptoethanol, RPMI 1640 medium, fetal bovine serum, EDTA, DEAE-cellulose, and Sephadex G-75 were purchased from Sigma. All other chemicals were of analytical grade.

Cells collection and cisplatin treatment. Mouse lymphocytic leukemic cells of L1210 line, sensitive and resistant to cisplatin action, were obtained from National Collection of Cell Lines at the Institute of Experimental Pathology, Oncology and Radiobiology, Kyiv, Ukraine. Both cell types were cultured in RPMI 1640 medium (Sigma Chem. Co, USA) supplemented with 10% fetal bovine serum (Sigma Chem. Co, USA). Cells were maintained under standard cell culture conditions at 37 °C with 5% CO₂ in air at 100% humidity.

Cells were cultured in 96-well plate at 2×10^5 per well. Cisplatin (Ebewe, Austria) was added to the culture medium at concentration of 10 μ g/ml, and cells were incubated for 24 h at 37 °C. Yield and viability of cells before and after the cells treatment with cisplatin were assessed by trypan blue exclusion method. Only cells with >95% viability were used for the experiments.

All procedures of preparing cells lysates were carried out at 4 °C.

Metallothioneins assay. MTs were eluted by the size-exclusion chromatography on Sephadex G-75 from thermostable solution of cells [31]. For the obtaining of thermostable solution, the cellular suspension which contained 0.5×10^6 cells was homogenised in ice-cold 10 mM Tris-HCl buffer, pH 8.0, containing 20 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulphonyl fluoride for the inhibition of proteolysis in a Teflon/glass homogenizer [7]. The homogenate was centrifuged at $10\,000 \times g$ for 45 min at 4°C . After that the supernatant was incubated under the 85°C for 5 min and subsequently centrifuged at $10\,000 \times g$ for 45 min at 4°C . The obtained supernatant was subjected to chromatography on a Sephadex G-75 column (1.5×50 cm) equilibrated with the same buffer at a flow rate $0.33 \text{ ml} \times \text{min}^{-1}$. Fractions (5 ml) were collected and analysed for absorbance at 280 and 254 nm (D_{280} and D_{254}). Column calibration was achieved by applying a mixture of the following standards: serum albumin (67.0 kDa), cytochrome c (12.3 kDa), insulin (5.8 kDa). The fractions of MTs containing peak (total 10 ml) were pooled. The ultraviolet (UV) absorption spectra were measured. The samples were also subjected to metals determination.

The MTs from L1210R cells were consequently applied to ion-exchange chromatography (IOC) on a column (1.5×50 cm), packed with DEAE-cellulose. After removing of non-bound proteins (in the volume of buffer 70 ml) elution was carried out in gradient mode with 0–1.0 M NaCl in a 10 mM Tris-HCl (pH 8.0) containing 10 mM 2-mercaptoethanol and 1 mM 2-isopropanol at a flow rate of $0.5 \text{ ml} \times \text{min}^{-1}$. The fractions of each peak (15 ml) with high absorbance at 254 nm were pooled for the UV-spectra and the metals determination.

Metals and metallothioneins level determination. To determine the concentration of copper and zinc, pooled MTs fractions were dried for 24 h at 105°C , and then digested with 5 ml HNO_3 for 3 h at 105°C under pressure, using an acid-cleaned Teflon bomb until digestion was complete. The content of Cu and Zn was evaluated by the atomic absorption spectrophotometry against certified standards on the spectrophotometer C-115, („Lomo”, Russia). The reliability of these measurements towards selected elements was assessed by analyzing ERM-CE 278 certified reference material, and the recoveries of metals were between 90% and 110%. Metals concentration was expressed as $\text{nmol} \times \text{mg}^{-1}$ of proteins.

The MTs concentration was estimated by metal summation [25].

Determination of glutathione concentration. Reduced (GSH) and total glutathione (GSH + oxidized glutathione, GSSG) in GSH equivalents, were determined in the protein free cells suspension using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) by the method of Sedlak and Lindsay [28]. The cellular suspension was homogenized (1/5 w/v) in 0.1 M phosphate buffer containing 100 mM KCl, 1 mM EDTA, pH 7.4). After the removing of pellet by centrifugation the thiols were determined.

For the determination of GSH, 100 μl of the protein-free supernatant was added to 3.9 ml of 0.4 M Tris-EDTA buffer (1.0 ml, pH 8.9) followed by 100 μl of Ellman's reagent (10 mM DTNB in methanol).

The determination of total glutathione in the protein free supernatant was carrying out using sodium borohydride as reducing agent [14]. To 0.5 ml of protein free supernatant 0.5 ml of a 2% freshly prepared aqueous sodium borohydride was added and solution was incubated for 30 min at 40°C with occasional shaking. After reduction the excess of sodium borohydride was stopped by adding 0.3 ml 1 N HCl and mixing for 2 min followed by addition of 1 ml acetone and mixing for 3 min. The pH was brought to about pH 7.5–8 by adding 1 ml 1 M Tris-HCl buffer pH 8.5 containing 0.5 mg/ml EDTA followed by addition of 0.1 ml of Ellman's reagent.

After 30 min of incubation at room temperature, the solutions were read at 410 nm and the sulfhydryl content was calculated using a molar extinction coefficient of 1.36×10^4 for GSH and 1.43×10^4 for total glutathione (GSH+2GSSG). The redox-index (RI) of glutathione as the ratio of content GSH/(GSH+2GSSG) was calculated.

The protein's content in cellular suspension was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Statistical analysis. MTs chromatographic analyses were carried out in triplicate. All measurements were expressed as means \pm SD of two repeat measuring for 4 samples analysed. The comparative analysis was run by applying the Fisher's test.

RESULTS

Metallothioneins analysis. Gel-filtration of the thermostable solution from both L1210S and L1210R cells lines revealed two protein fractions (Fig. 1). For L1210R cells, the ratio D_{254}/D_{280} of high molecular weight and low molecular weight fractions were 1.35 and 1.98. For L1210S-cells, these parameters were 1.35 and 1.67, correspondently. The low molecular weight fraction was identified as MT-containing fraction based upon its spectral features (comparative high density ratio D_{254}/D_{280}), thermostability, and low molecular weight [20]. This peak was more abundant in the R-cells in comparison with S-cells. The ion-exchange chromatography demonstrated the comprising of MT-containing fraction from two peaks that is the typical feature of animals' MTs [20]. These peaks were designated as MT-1 and MT-2 (Fig. 2). More abundant elution of MT-2 than of MT-1, the typical feature for mammals' MTs [5], was observed. All eluted MTs had the similarity of UV-spectra (Fig. 3) with high level of absorption in the middle UV, indicating the presence of characteristic metals-thiolate clusters [20]. The concentration of MTs in R-cells ($1.45 \pm 0.12 \mu\text{g} \times \text{mg}^{-1}$ proteins) was almost 1.5 higher than in S-cells ($0.95 \pm 0.08 \mu\text{g} \times \text{mg}^{-1}$ proteins). The evaluation of the composition of metals in MTs (Fig. 4) showed that Zn:Cu concentration ratio was lower in R-cells than in S-cells due to higher level of Cu in the composition of MTs.

The level of GSH and GSSG. R-cells were characterised by higher concentration of GSH and GSSG but comparable value of RI of glutathione in comparison with S-cells

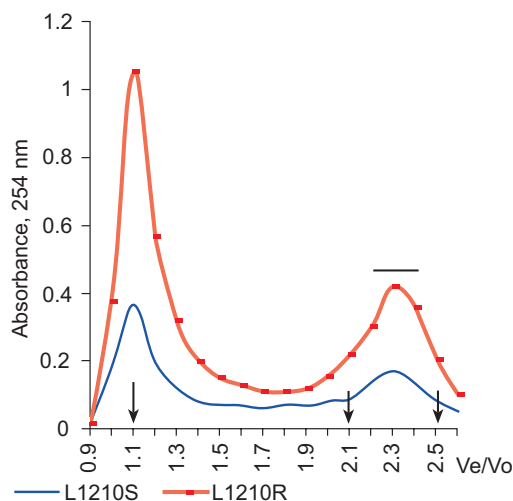


Fig. 1. Elution profile on Sephadex G-75 of the thermostable extract from cells in 0.01 Tris-HCl buffer, pH 8.0 at a flow rate $0.33 \text{ ml} \times \text{min}^{-1}$. Arrows highlight the elution volume of markers: 67.0 kDa, 12.3 kDa, 5.8 kDa. S–S-cells; R–R-cells. MTs-containing fraction which indicated by the bar, was pooled for ion-exchange chromatography. Under the peaks the density ratio D_{254}/D_{280} is designated

Рис. 1. Профіль елюції термостабільного екстракту клітин на сефадексі G-75 в 0,01 трис-НСІ буфері, рН 8,0, швидкість елюції 0,33 мл/хв. Стрілками вказано об'єм елюції маркерів: 67,0 кДа, 12,3 кДа, 5,8 кДа. S–S-клітини, R–R-клітини. Металотіонеїн-вмісні фракції, вказані рискою, були об'єднані для іонно-обмінної хроматографії

(Table). After treatment with cisplatin, the level of GSH in treated cells was the same as in the untreated cells of an appropriate line. However, the decrease of GSSG level in comparison with untreated cells was observed only in S-cells. Therefore among treated cells, the RI of glutathione was higher in S-cells.

Fig. 2. Chromatography profile of R-cells metallothioneins on DEAE-cellulose (1.5×50 cm) in gradient mode with 0–1.0 M NaCl generated from 500 ml of 10 mM Tris HCl (pH 8.0) and 500 ml of 10 mM Tris HCl (pH 8.0) containing 1 M NaCl at a flow rate of 0.5 ml×min⁻¹. Elution profile is shown by the solid line, a gradient of elution buffer – by the dotted line

Рис. 2. Профіль елюції металотіонеїнів R-клітин на ДЕАЕ-целюлозі (1,5×50 см) у градієнті 0–1,0 М NaCl, отриманих змішуванням 500 мл 10 мМ трис-НСl (рН 8,0) і 500 мл 1 М NaCl в 10 мМ трис-НСl (рН 8,0), швидкість потоку 0,5 мл/хв. Профіль елюції зображений суцільною лінією, градієнт елюювання – пунктирною лінією

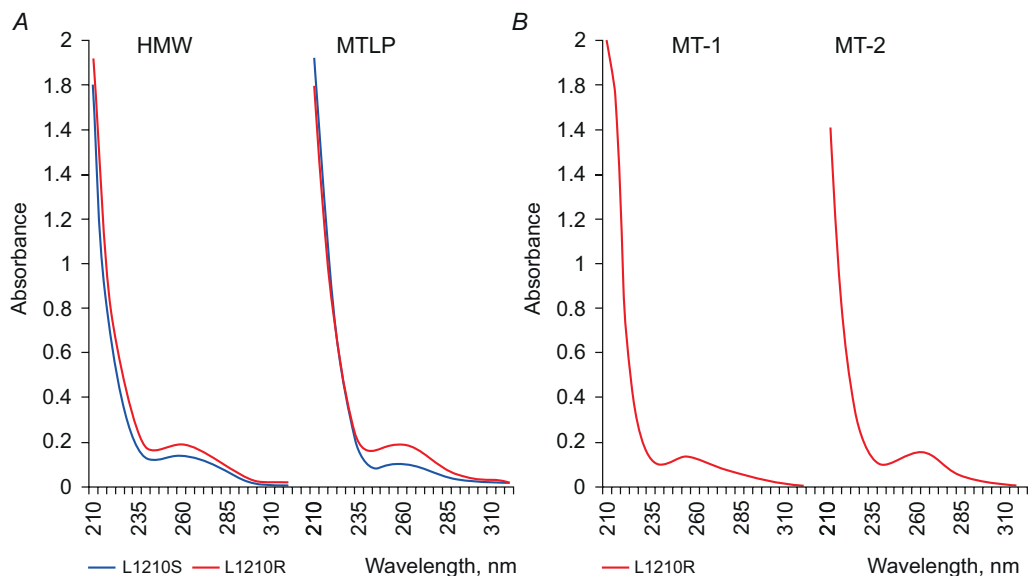
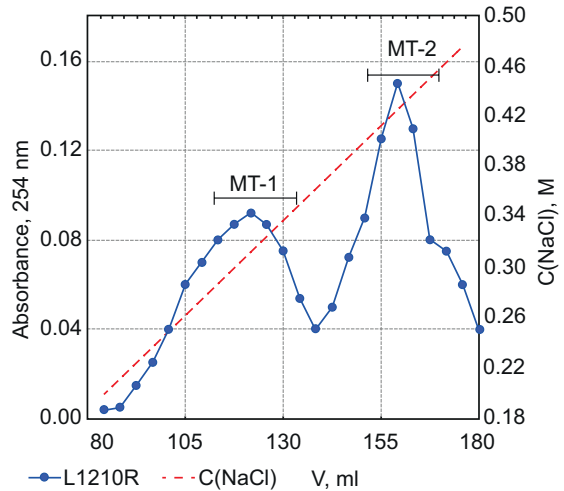


Fig 3. UV-spectra of the thermostable proteins from S and R-cells (A) and of MT-1 and MT-2 isoforms from R-cells (B)

Рис 3. УФ-спектри термостабільних білків S- і R-клітин (A) та ізоформ MT-1 і MT-2 R-клітин (B)

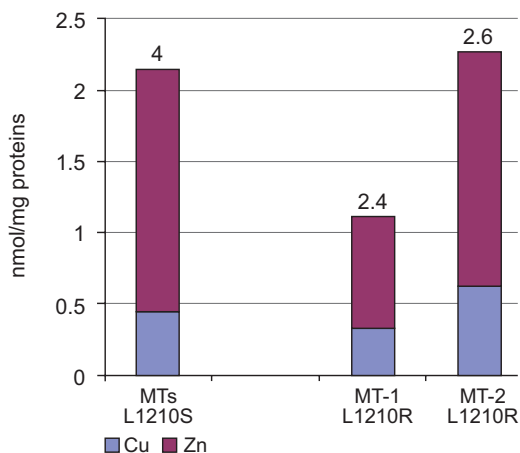


Fig. 4. The metals' distribution in the metallothioneins of S- and R-cells, nmol×mg⁻¹ of proteins: numbers above the columns are the Zn:Cu ratio

Рис. 4. Розподіл металів у металотіонеїнах S- та R-клітин, нмоль/мг білка: цифри над стовпчиками вказують відношення Zn:Cu у металотіонеїнах

Content of low molecular weight thiols in L1210 cell lines Вміст низькомолекулярних тіолів у клітинах ліній L1210

Parameters	Untreated cells		Cis-Pt treated cells	
	L1210S	L1210R	L1210S	L1210R
GSH, nmol×mg ⁻¹ proteins	55.1±2.2	86.2±4.1 ^a	57.2±1,3	82.1±7.2 ^a
GSSG, nmol×mg ⁻¹ proteins	64.3±5.4	84.2±16.1 ^a	16.2±3.2 ^b	63.3±11.1 ^a
RI GSH	0.46±0.03	0.50±0.07	0.77±0.09 ^b	0.56±0,08 ^a

Comments: ^a – the values for two cell sublines differ significantly; ^b – the values for treated and untreated cells differ significantly, always $P > 0.05$). ND – not determined.

Примітки: ^a – значення для двох клітинних субліній вірогідно відрізняються; ^b – значення для оброблених та необроблених клітин вірогідно відрізняються, $P < 0,05$). ND – показник не визначали.

DISCUSSION

Both MTs and GSH are composed of approximately one-third of cysteine. Both can bind metal ions and other electrophiles, both have antioxidant activity and are induced by oxidant stressors and metals [20, 33]. MTs can protect cells from apoptosis induced by oxidative stress and metals. Shimoda et al. [29] revealed a strong linear negative correlation between basal MT levels and etoposide-induced apoptosis in the human tumor cell lines PLC/PRF/5, H460, and HepG2 ($r = -0.991$). Therefore, MTs may provide a strategy for altering cellular resistance to chemotherapeutic compounds. Notably high MTs level was observed in resistant cells BM1R2 [35]. The enhanced expression of MTs was also reported in cisplatin resistant cells of the MTII subtype [34]. On the other hand, Reeves and Saari [26] showed that cisplatin treatment increased kidney MTs in rats even further, despite elevated MTs gave no protection from the toxic effects of the drug. Farnworth et al. [11] showed that the resistance of L1210-PDD cells to cisplatin was not associated with cross-resistance to group IIb metals, whereas their sensitivity to cadmium did reflect the relative inability of the cells to synthesize MTs. Hence, the diagnostic potential of cellular low weight thiols as a cancer marker is contradictory [3].

In the present study, the significantly higher concentrations of MTs and GSH in the R-cells in comparison with S-cells were demonstrated. Moreover, despite common features of MTs, the elevated Cu concentration in the R-cells indicates the antioxidant preferences of MTs in the R-lines. It is known that in the adult mammalian cells, the main

MTs function is devoted to supplying of Zn storage, therefore the mechanisms of Cu homeostasis are connected to other proteins [16, 27]. However, MTs of invertebrate animals and calf of vertebrate animals are more related to Cu-dependent functions and demonstrated more high reactivity [7, 10, 30].

Intracellular GSH content was found to be higher in cisplatin-resistant cells than sensitive variant [6, 24]. Our data confirmed these observations concerning mouse lymphocytic leukemic L1210-cells. It was about 30% higher in the resistant cells, both in treated and untreated in comparison to sensitive cells. But in controversy with these data no significant differences were detectable between sensitivity and resistance to cisplatin L1210 cell lines regarding glutathione level in other reports [17, 23]. The other remarkable difference between two lines was the higher level of RI of glutathione in R-cells in comparison with S-cells. The reduction of GSSG in treated S-cells may indicate the prevention of oxidising of GSH as the result of its conjugation to cisplatin and inability to compensate the elevated utilisation of GSH in usual way [1].

Boubakari et al. [4] did not find correlations between concentrations of intracellular GSH/GSSG in 14 human cancer cell lines growing *in vitro* and the cell growth inhibitory activities of four Pt-complexes, particularly cisplatin despite the increased intracellular levels of GSH in cancer cells have been implicated in the development of acquired resistance to platinum antitumor agents [4]. Yasuno et al. [35] characterizing a cisplatin-resistant human neuroblastoma cell line, BM1R2, show that glutathione-S-transferase as well as MT could exert crucial roles on this resistance. These data confirm that the GSH-dependent resistance in cancer cells is devoted to GSH related enzyme, glutathione-S-transferase, that represents an integral part of the detoxification system protecting cells by catalyzing the S-conjugation between the thiol group of GSH and the electrophilic moiety of toxic substrates including cisplatin [21]. Due to a decreased level of GSSG in the treated with cisplatin S-cells, these data may reflect the intensification of GSH exploring in this reaction for the detoxification of cisplatin whereas in R-cells other mechanisms are probably involved in this process.

Common mechanisms of resistance including the participation of both thiols were observed in A2780 acquired cisplatin and ZD0473R (resistant) lines? as well as reduced drug transport and DNA platination, enhanced DNA repair, activated Cu, Zn-SOD [2, 32]. These data agree with the reflecting of the same regularity for difference in MTs and GSH level between sensitive and resistant cells in present study. In opposite, Hrubisko et al. [18] was found positive correlation between resistance to cisplatin and GSH level in L1210 but not MTs.

Taken together, the observed differences of cells lines allow considering a successful exploitation of the usual molecular mechanisms of the metal detoxification with the participation of low molecular weight thiols against cisplatin effect in L1210-cells. L1210 R-cells were characterised by higher levels of MTs and GSH and high ratio of copper in MTs composition. These patterns suggest that enhanced synthesis of low molecular weight thiols could be involved in the mechanism by which mouse lymphocytic leukemic L1210-cells are resistant to cisplatin cytotoxicity. The expediency of the evaluation of MTs and GSH to determine probable efficacy of the utilization of cisplatin as drugs has been established.

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ВЗАЄМОЗВ'ЯЗОК МІЖ РЕЗИСТЕНТНІСТЮ ДО ЦИСПЛАТИНУ ТА НИЗЬКИМ РІВНЕМ НИЗЬКОМОЛЕКУЛЯРНИХ ТІОЛІВ У КЛІТИННИХ ЛІНІЯХ МИШАЧОЇ ЛЕЙКЕМІЇ L1210

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Цис-дихлородіамінплатина (II), вживана фармакологічна назва – цисплатин, є поширеним протипухлинним засобом. Проте його терапевтична дієвість обмежена через різні дозолімітувальні побічні ефекти і розвиток набутої резистентності. Резистентність пухлинних клітин до цисплатину може бути спричинена підвищеною концентрацією клітинних тіолів. Для з'ясування чинності цієї гіпотези ми порівняли концентрації низькомолекулярних клітинних тіолів металотіонеїну (MT) і глутатіону

(у відновленій (GSH) і окисненій (GSSG) формах), а також металозв'язувальні властивості МТ у двох клітинних лініях, відомих як резистентна (L1210R) та чутлива (L1210S) до цисплатину. Було встановлено, що R-клітини характеризуються вищою концентрацією МТ і GSH, вищою часткою купруму в складі МТ порівняно з цинком, ніж S-клітини. Обробка цисплатином лише у S-клітинах викликала зменшення концентрації GSSG без змін у концентрації GSH. Отже, нечутливість R-клітин до цисплатину може бути пов'язана з високим рівнем МТ і GSH. Особливості складу металів у R-клітинах, які були відзначені вперше, також можуть забезпечити певні переваги для цих клітин з огляду на особливу здатність купрум-МТ до окиснення. Встановлені специфічні характеристики тиолів у пухлинних клітинах можуть бути корисними в індикації резистентності до цисплатину.

Ключові слова: цисплатин, резистентність, металотіонеїн, глутатіон, купрум.

ВЗАИМОСВЯЗЬ МЕЖДУ РЕЗИСТЕНТНОСТЬЮ К ЦИСПЛАТИНУ И НИЗКИМ УРОВНЕМ НИЗКОМОЛЕКУЛЯРНЫХ ТИОЛОВ В КЛЕТОЧНЫХ ЛИНИЯХ МЫШИНОЙ ЛЕЙКЕМИИ L1210

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Цис-дихлородиаминоплатина (II), используемое фармакологическое название – цисплатин, является распространенным противоопухолевым средством. Однако его терапевтическое действие ограничено из-за различных дозоограничивающих побочных эффектов и развития приобретенной резистентности. Резистентность опухолевых клеток к цисплатину может быть вызвана повышенной концентрацией клеточных тиолов. Для выяснения справедливости этой гипотезы мы сравнили концентрации низкомолекулярных клеточных тиолов металлотионеина (МТ) и глутатиона (в восстановленной (GSH) и окисленной (GSSG) формах), а также металлосвязывающие свойства МТ в двух клеточных линиях, известных как резистентная (L1210R) и чувствительная (L1210S) к цисплатину. Было установлено, что R-клетки характеризуются более высокой концентрацией МТ и GSH, более высокой долей купрума в составе МТ по сравнению с цинком, чем S-клетки. Обработка цисплатином вызвала уменьшение концентрации GSSG без изменения в концентрации GSH только в S-клетках. Таким образом, нечувствительность R-клеток к цисплатину может быть связана с высоким уровнем МТ и GSH. Особенности состава металлов в R-клетках, отмеченные впервые, также могут обеспечить определенные преимущества для этих клеток, исходя из усиленной способности купрум-МТ к окислению. Установленные специфические характеристики тиолов в опухолевых клетках могут быть полезны в индикации резистентности к цисплатину.

Ключевые слова: цисплатин, резистентность, металлотионеин, глутатин, купрум.

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