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Single Amino Acid Change in Metallothionein Metal-Binding Cluster Influences Interaction with Cisplatin

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The issue of tumour cell resistance to anticancer drugs is a major problem in the treatment of this grave disease and it is still not satisfactorily explained. Its base lies in the interaction of a cytostatic with biomolecules synthesized by tumour cells. One of the generally accepted mechanisms of resistance to some metal based cytostatics is the overexpression of metallothionein in tumour cells. In this study, electrochemical profile of interaction between 23 sulphur-rich fragments of the metal-binding protein metallothionein and cisplatin was studied. To evaluate the results, interaction constants were suggested. Here, we found that the maximum increased interaction (more than 100 %) occurred, when conservative aminoacids were substituted for more than one position outside the cysteine cluster. On the contrary, amino acid substitution within the cysteine cluster led to a reduction in interaction constants (up to 10-25% of average). This result clearly indicates that aminoacids outside cysteine binding motif are of high importance for interactions of metallothionein with cisplatin. Based on the results it can be assumed that the substitution of individual aminoacids in the peptide chain of protein markedly influences the interaction with cisplatin, which could be used for designing new types of cytostatics.

Keywords: Aminoacid Sequence; Interaction Study; Metallothionein Fragments; Cisplatin; High Throughput Analysis; Interaction Constants

1. INTRODUCTION

The issue of tumour cell resistance to anticancer drugs is a major problem in the treatment of this grave disease [1] and it is still not satisfactorily explained [2]. Its base lies in the interaction of a cytostatic with biomolecules synthesized by tumour cells [3]. Developing new types of drugs is conditioned by achieving a higher selectivity and lower occurrence of side effects [4]. An example such development are platinum cytostatics, which have been evolving for more than forty years [5]. They are also one of the longest and most widespread used drugs used for systemic therapy of many cancers, but poor response to this treatment may be caused by interactions of platinum based drugs with proteins or with protein complexes with DNA [6]. One of the generally accepted mechanisms of resistance to some cytostatics is the overexpression of metallothionein in tumour cells [7-10].

From the structural point of view, mammalian metallothioneins (MTs) are low molecular mass (from 5 to 7 kDa, Fig. 1) proteins with unique abundance of cysteine residues (more than 30 % from all aminoacids), which directs their metal binding properties. It has been found 250 various structural forms of MTs [11]. Tertiary structure of MTs is divided into two domains, forming cysteine clusters, where the alpha and beta domains can bind to 4 divalent and 3 metal ions, respectively [12,13].

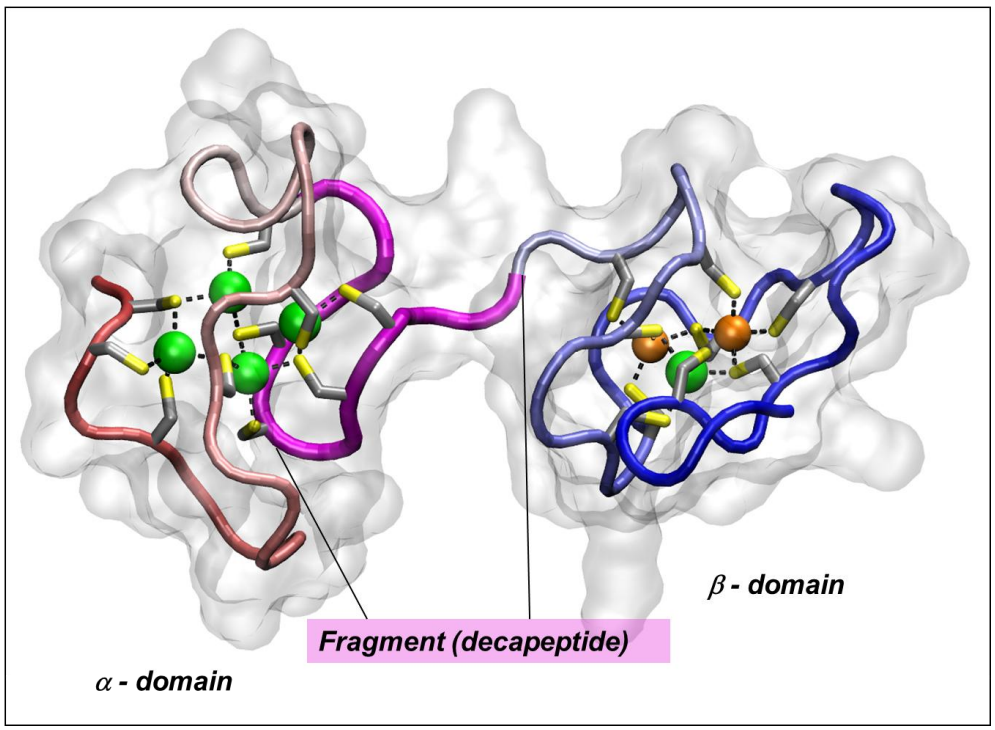

Figure 1. The structure of the protein metallothionein, α – metallothionein domain containing four cadmium atoms (green), β – metallothionein domain containing two zinc atoms (orange) and one atom of cadmium. The position of fragment of metallothionein, which was synthesized for subsequent in vitro interaction studies, is marked by pink. (Source: www.expasy.org).

Due to high abundance of cysteines in the MT structure, several sections of the chain, which contribute significantly to the interaction with the metal ions, can be described. For the purposes of our

in vitro interaction study, metallothionein protein fragments occurring in the metal binding cluster of various vertebrates were selected (Fig. 1). We evaluated redox parameters of the selected fragments of metallothionein (FMT) and studied the effects of various physical and chemical conditions on their interactions with cisplatin due to elucidation of resistance formation in tumour cells against this drug. Particularly, we aimed our attention at i) temperature (10, 15, 25, 35 and 45 °C), ii) ratio of cisplatin (100 μM) and FMT (50, 100 and 150 μM), and iii) time of interaction (1, 2, 3, 4, 5, 6, 7 and 8 hours).

2. EXPERIMENTAL PART

2.1 Bioinformatics

The data source was the internet proteomic database Expasy (www.expasy.org). For data processing software Matlab version 7.9.0 (The MathWorks, Inc., Natik, MA, USA) was used. Alignment was performed by using the conservative sections of the global multiple sequence alignment using the BLOSUM50 substitution matrix. To better assess the similarity of the sequences, distribution was weighted on the number of "characters". Data were processed using MICROSOFT EXCEL® (Microsoft, Prague, Czech Republic) and STATISTICA.CZ Version 8.0 (StatSoft CR s.r.o. Prague, Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless noted otherwise.

2.2 Chemicals and pH measurement

Standards of fragments of metallothionein (FMTs) were synthesized by Clonestar (Clonestar s.r.o., Brno, Czech Republic). Other chemicals were purchased from Sigma-Aldrich (St.Lois, MO, USA) in ACS purity unless noted otherwise. Stock standard solutions of FMTs (1 mg/ml) was prepared with ACS water (Sigma-Aldrich) and stored in dark at -20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (WTW GmbH, Weilheim, Germany), controlled by software MultiLab Pilot (Weilheim). Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified using Millipore RG (Millipore Corp., Billerica, MA, USA, 18 MΩ) – MiliQ water.

2.3 Interaction conditions

Complexes of FMTs with cisplatin were prepared in the following molar ratios cisplatin 100 μM : FMT 50, 100 and 150 μM in the presence of phosphate buffer pH 7.5 (20 mM). Incubation of the complexes was performed in a total volume of 400 μl of the mixture, which was continuously vortexing during incubation in a heating block at 400 rpm.

2.4 FIA-ED system

FIA-ED was consisted of one chromatographic pump and electrochemical detector. Sample (20 µl) was injected by autosampler (Model 542, ESA, Sunnyvale, CA USA). Electrochemical detector Coulochem III (ESA, Sunnyvale, CA USA) was connected directly to the autosampler. For electrochemical detection the electrochemical cell model 5040 (ESA, Sunnyvale, CA USA) was used. This cell is equipped by planar electrode from glassy carbon. Mobile phase was phosphate buffer pH 7.5 (20 mM). Flow rate of mobile phase was 1 ml/min.

3. RESULTS AND DISCUSSION

3.1 Metallothionein fragments selection

Mathematical comparison of different metallothioneins from vertebrates' sequences of length 60 or 61 amino acids was done according to their amino acid sequence of the primary structure. The complete amino acid sequences of a total of 145 metallothioneins were primarily aligned according to conservative sections. There was generated sequence logo representing conservative positions in individual sequences from the aligned sequences (Fig. 2). Ten amino acid long clusters (in position 31-40 or 41) with the highest cysteine content per number of amino acids within the decapeptide with high conservatism that was related to FMT peptide 2, which was the most conservative, i.e. the sum of occurrence of individual amino acids expressed by percent was the highest, were then determined, as it is shown on percentage conservatism: 1 - K (98.62 %), 2 - free (93.79 %), 3 - S (97.93 %), 4 - C (100 %), 5 - C (99.31 %), 6 - S (63.45 %), 7 - C (100 %), 8 - C (99.31 %), 9 - P (100 %), 10 - S (51.72), 11 - G (93.79). Variability positions of 2 and 10 less distant from cysteine residues were < 94 %. Based on these presumptions, we selected fragments that were different at least by just one amino acid. From 145 FMTs, which can be found in nature, twenty three ones fit to this. For each of these 23 sequences, a short fragment within the range from 31 to 40 (41) amino acids was selected. For these 23 fragments, a degree of conservatism of the sequence was estimated. *Pj* is the percentage of degree of conservatism of sequence *j*. It indicates the degree of sequence similarity expressed in relation to the preservation of genetic information of each sequence position. Degree of conservatism calculates as it follows:

$$P_j = \frac{1}{k}\sum_{i=1}^{k}\frac{f_{ij}}{n}\cdot 100, \quad \forall j = 1 \ldots n$$

where *k* is the length of the sequence, *i* is the marker of position in the chain sequence, *j* is the sequence indicator and *n* is the number of sequences. Parameter f_{ij} is the frequency of s_{ij} character on the *i-th* position in the aligned sequences, i.e. a number of times the character located in the *j-th* sequence in the *i-th* position occurs at the same position in all sequences.

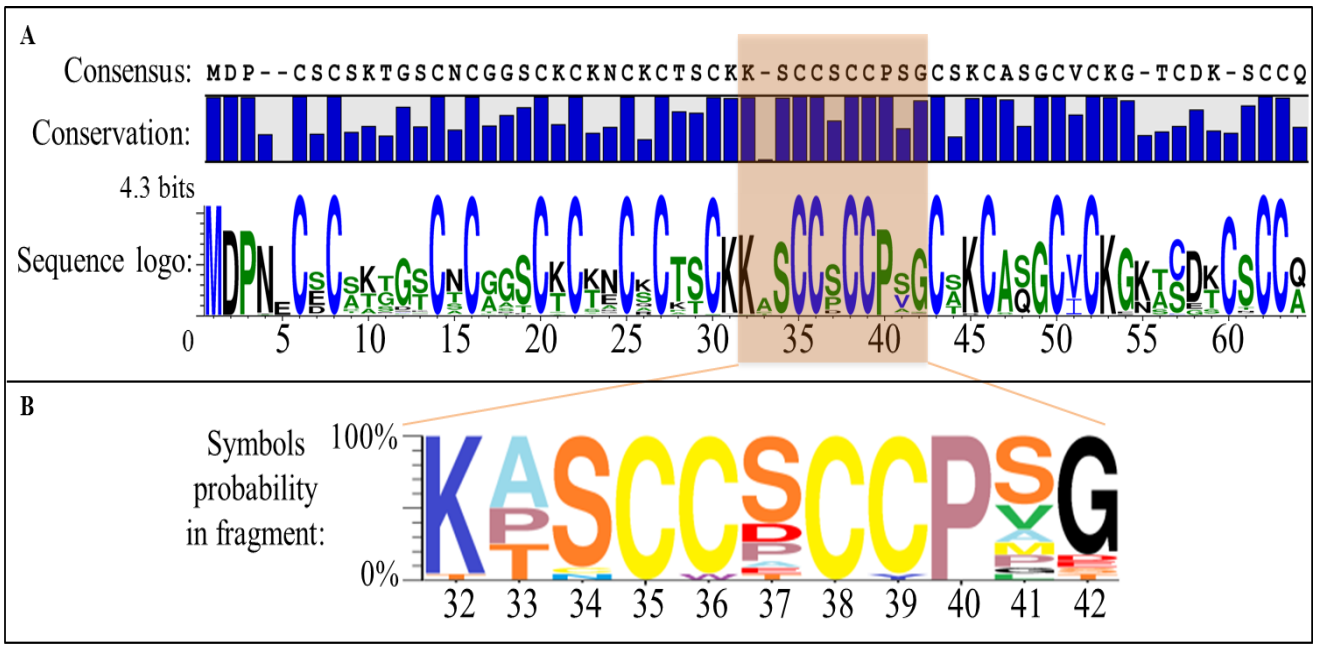

Figure 2. (**A**) Sequence logo assessed for 145 sequences of vertebrate MTs with a length of 60-61 amino acids. (**B**) Modified sequence logo of fragment in positions 32-42 representing the quantity of point mutations single-nucleotide polymorphism of 23 selected sequences.

3.2 Interaction experiment

Samples were incubated for 1, 2, 3, 4, 5, 6, 7 and 8 hours at 10, 15, 25, 35 and 45 °C at a concentration of 100 µM FMT and 50, 100 and 200 µM cisplatin. For each combination from hydrodynamic voltammogram (HDV) was obtained in the potential range from 100 to 1200 mV (n = 3, RSD <15 %). Based on the results obtained, equimolar ratio of 100 µM FMT and 100 µM cisplatin was chosen as the best under 1 h long interaction at 45 °C.

3.3 Data interpretation

Based on the obtained HDVs, regression equations of the dependences measured from the test mixtures (S_{mix}), and platinum itself (S_{Pt}) and fragments themselves (S_{FMT}) were determined. Each mixture dependence was subtracted from platinum and corresponded fragment and gave the value of which presents only the resulting change of the signal due to interaction (S_{int}), which is expressed according to equation No. 1.

Equation No. 1: $S_{int} = (S_{mix} - S_{FMT}) - S_{Pt}$

To determine the influence of various factors on the interactions as ("a 1 – 5", 10 - 45 °C respectively; "b 1 – 8", 1 – 8 hours respectively; "c 1 – 3", 50 - 200 µM respectively), graphical evaluation was done according to $X = S_{(a)}$ versus $Y = \Sigma_{S(a1+a2+a3\ldots)}$, where $S_{(a)} = \Sigma S/p_{parameter}$, for each studied fragment (Figs. 3 and 4).

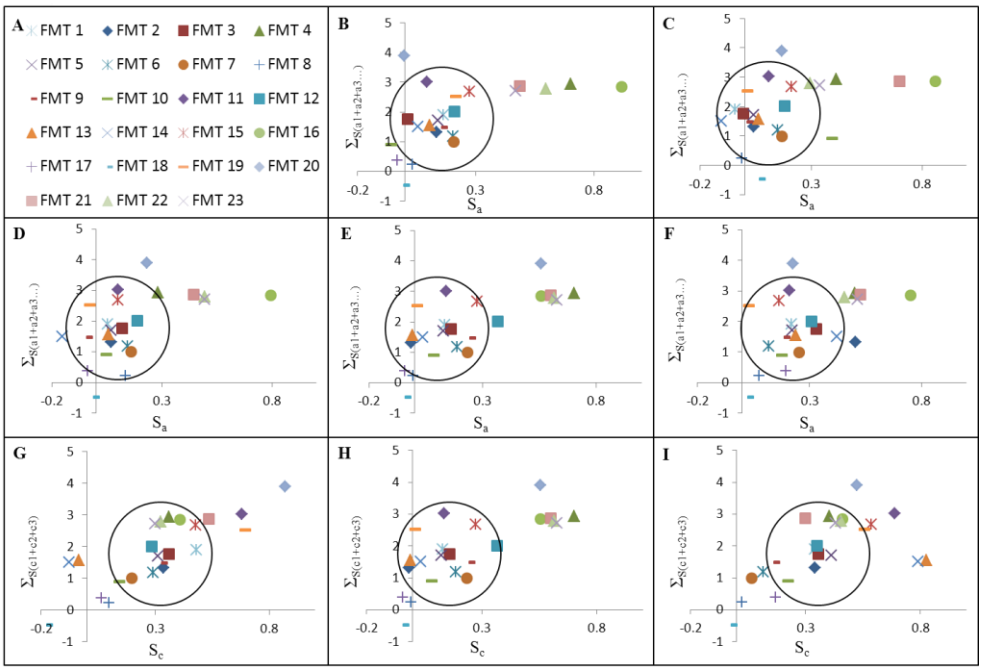

Figure 3. (**A**) Measured fragments of metallothionein. Graphical presentation of slopes affected by certain tested physical and chemical factors as (**B**) Temperature 10 °C; (**C**) Temperature 15 °C; (**D**) Temperature 25 °C; (**E**) Temperature 35 °C; (**F**) Temperature 45 °C; (**G**) Concentration 50 µM; (**H**) Concentration 100 µM; (**I**) Concentration 200 µM.

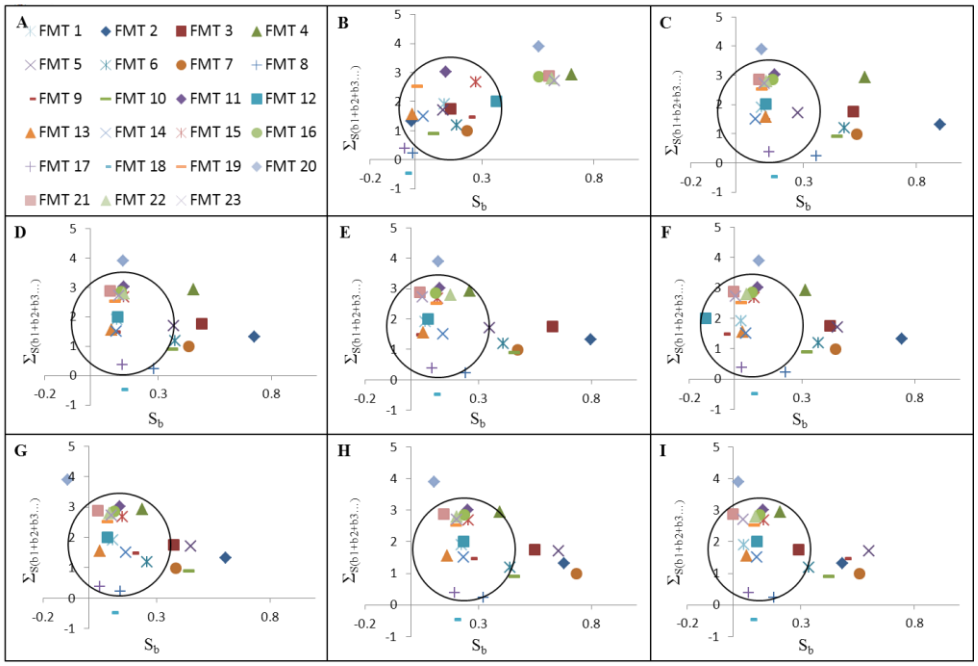

Figure 4. (**A**) Measured fragments of metallothionein. Graphical presentation of slopes affected by certain tested physical and chemical factors as (**B**) Time of interaction 1 h; (**C**) Time of interaction 2 h; (**D**) Time of interaction 3 h; (**E**) Time of interaction 4 h; (**F**) Time of interaction 5 h; (**G**) Time of interaction 6 h; (**H**) Time of interaction 7 h; (**I**) Time of interaction 8 h.

For the selection of only those conditions, under which there were recorded significant increased interaction, a graph with a radius obtained from the relationship Σ_{max}*0.3= 1.17 for y-axis was constructed for the median interval. On the x-axis, the value of the average median interval was $S_{(a)}$=0.16. Points located within the median interval were excluded as insignificant (2× RSD, reliability under 90). On the contrary, values outside the range of median values were found to have significant interactions associated with a diversity of structures of studied FMTs. The values of deviations on x-axis from the edge of the median were further summed on the amount that was interpreted as the total effect of temperature, concentration and interaction time and therefore gave us the interaction constant called IC_{FMT} according to equation No. 2. All fragments except FMT 1 had higher degree of the interaction, i.e. positive IC_{FMT}.

Equation No. 2: $IC_{FMT}= (S_a+S_b+S_c)$

3.4 Influence of aminoacid change on interaction constant

Due to high conservatism of FMT 2, all interactions were associated to this fragment as a percentage change (Fig. 5A). The highest values of IC_{FMT} were found for FMTs 18 and 20, where a marked change in non-neighbouring amino acids directly with cysteines could be observed. At FMT 18, there was found P in position 10 and at FMT 20, position 10 was occupied by V and, moreover, strongly conservative position 1 contained T instead of K. IC_{FMT} values of both mentioned fragments were for more than 100 % higher compared to other studied fragments. On the other hand, FMTs 2, 4, 16 had lower ability to interact with cisplatin despite the fact that there were still conservative amino acids S, S and P next to cysteines. For FMT 4 only there were conservative cysteine clusters surrounded by P instead of S, but the end of peptide was distinguished by substitution of N-terminal D for G, resulting in an overall increase in IC_{FMT} form more than 14 %. It was also observed the increase of IC_{FMT} of FMT 16 for 6 %, which was caused only by substitution of M for S in position No. 10.

Overall, at least conservative peptide FMT 21 of all tested peptides showed the lowest similarity to other tested FMTs. This was caused by replacing a conservative S with N next to the first cysteine cluster in position 3, followed by substitution of C for Y inside the second cysteine cluster in position 8 and finally by the substitution of S for V in position 10, resulting in an overall reduction of IC_{FMT} for more than 10 %. Other reported decreased levels of IC_{FMT} in the order of 25-37% were observed in FMTs 7, 8, 22 and 23. Structure of FMTs 7 and 8 was identical to FMT 2 with the exception of the aminoacid substation in position 6, which was neighboured to both cysteine clusters. Compared to the conservative sequence FMT 2, a mismatch of S for T in position 6 in the case of FMT 8 resulted in the reduction of IC_{FMT} for more than 20 % and a mismatch of S for A in position 6 in the case of FMT 7 caused a decrease in IC_{FMT} for more than 30 %. FMT 22 had aminoacids surrounding the cysteine cluster in positions 3, 6 and 9 as S, P and P, which caused a reduction of IC_{FMT} for more than 25 %. FMT 23 showed at least conservative arrangement (69.92%). In position 2, there were done substitutions of free for A, and of W for C in position 5, and of S for D in position 6, which resulted in the reduction of IC_{FMT} for more than 25 %.

The most significant reduction in the value of IC_{FMT} in the order of 50-100% was observed in all other studied FMTs as 17, 14, 3, 10, 13, 6, 5, 11, 19, 9, 12, 15 and 1, which were majority found within the median range. At FMTs 17, 6, 9 and 1, there was substitution in position 2 for D, E, D and in position 2 for P. The combination of location of P decreased IC_{FMT} mostly. At FMTs 14, 3, 13, 11, 19, 12 and 15, the substitution in position 10 for V, A, M, V, A, G or L was done. In addition to these changes, C was in position 3 at FMT 4, and M in position 10 at FMT 13.

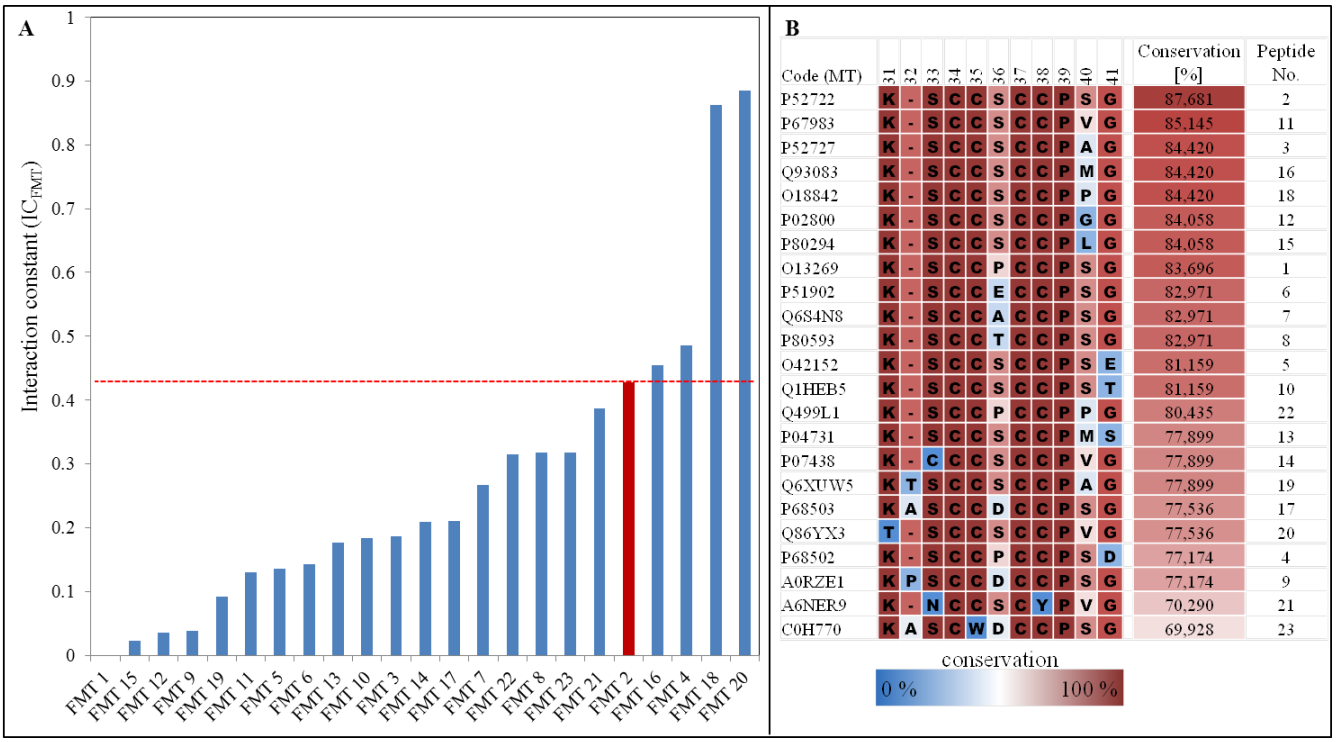

Figure 5. (**A**) Values of IC_{FMT} for all 23 studied metallothionein fragments resulting from the calculation based on the tested parameters (temperature, molar concentration, time of interaction). Value of the most conservative IC_{FMT} 2 (0.43) is highlighted in red. (**B**) Table where you can see the original MT code, amino acid sequence of FMT, which indicates the percentage of similarity in the conservative group and serial number of the peptide. Amino acids significantly affecting the level of interaction are highlighted in blue.

The insertion of these sulphur-containing amino acids at these positions resulted in the reduction of IC_{FMT} for more than 50 %. Moreover, FMT 19 had also changed aminoacid in position 2 to T, which resulted in the decrease in IC_{FMT} for more than 75 %. Compared to similar FMT 20, where K was in front of T and V instead of A in position 10, this was one of the most significant effects characterized by an overall decrease in IC_{FMT} for more than 190 %. The highest effect characterized by a total difference of 201 %, however, was inverse substitution of P in positions 6 and 10 at FMTs 18 and 1. At FMTs 10 and 5, substitution for T and E in position 11 resulted in decreased IC_{FMT} for more than 50 %.

The obtained results show that the greatest influence on the interaction of cisplatin with FMT have aminoacid changes in positions 1 and 10, which are distant by more than one position from the cysteine cluster, where quite significantly affects the overall location of the interaction of aminoacids P, T and V at FMTs 20 and 18. In comparison with these amino acids, aminoacid changes in P and D in positions 6 and 11 at FMT 4 and followed by the M substitution in position 10 at FMT 16 also influenced the interaction with cisplatin but much lower compared to P, T and V.

4. CONCLUSIONS

Metallothionein has been previously studied by electrochemical methods, which are utilizable for this purpose due to high content of cysteine in its structure. In terms of complex formation at the level of aminoacids it is advisable to study only a fragment of this protein [14]. As part of monitoring the effect of histidine, which is not too frequent, on the redox changes in the sequence of MT with the view of the possibility of increasing the coordination of metals was similar to our work. In that study, the authors used a different methodological approach (NMR and ICP-MS) [15], where NMR provides structural information and ICP-MS provides information on the quantity of the elements of the interest. Compared to this multi instrumental approach, our method combines structural and quantitative information. The suitability of electrochemical methods for the study of complex MT with metal has been demonstrated using cathodic stripping voltammetry [16], square wave voltammetry [17] or cyclic voltammetry [18-22]. In addition to these studies, we used FIA-ED method, which was chosen thanks to the experience from the previous studies of interactions between the thiol group of peptide and cisplatin [23,24]. In this study, it is shown in detail that the substitution of individual aminoacids in the peptide chain of protein markedly influence the interaction with cisplatin.

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