INTERACTION OF PLATINUM-BASED CYTOSTATICS AND PLATINUM NANOPARTICLES WITH METALLOTHIONEIN - POTENCIAL SOURCE OF THE ANTITUMOR DRUG RESISTENCE

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Abstract

Platinum-based cytostatic represent a unique class of DNA-damaging antitumor agents and they are one the most frequently used drugs in oncology. Problem is that some kind of cancer is resistant against this type of cytostatics. This resistance can be potentially caused by metalloproteins such as metallothioneins. MTs belong to the group of intracellular cystine-rich, metal-binding proteins and hold a number of functions in body. One of them is detoxification of heavy metals. This ability of MTs can cause a decreased therapeutic effect of platinum-based cytostatics. In this work, the interaction between two isoforms of MTs (MT3 and MT2a) and several types of platinum cytostatics (oxaliplatin, carboplatin and cisplatin) as well as platinum nanoparticles (size of 10 and 40 nm) was examined by fluorimetric analysis using a fluorescence zinc indicator (Fluozin-3). Both, stationary fluorescence spectrometry and capillary electrophoresis with laser-induced fluorescence detection (ex - 488 nm, em - 530 nm) was used in the study.

Keywords: Platinum, nanoparticle, cytostatics, fluorescence, metalloprotein

1. INTRODUCTION

Platinum-based cytostatics play an important role in the treatment testicular, ovarian, lung, bladder, head, neck and cervical cancer [1], [2]. In this work three platinum-based cytostatics (cisplatin, carboplatin and oxaliplatin) that have found application in clinical practice, will be observed. The effect of these drugs is not fully understood but assumed that it is based on interaction of platinum with DNA and formation of DNA adducts with subsequent triggering the apoptosis leading to cell death [3]. These cytostatic have some disadvantages, which includes the undesirable secondary effects, that are caused by that the effect of cytotoxic agents is not limited to cancer cells but also affects healthy tissue with a high frequency of cell division. Another big problem is that some types of tumours report a resistance against them. This resistance is probably caused by metallothioneins (MTs) [4].

MTs are a group of intracellular metal-binding and cysteine rich proteins, which consist from two domains (α, β). These proteins are present in almost all forms of life. Nowadays, it is known that mammals express at least four isoforms of MT (MT1-MT4) that are consist of 61-68 amino acids, and among them, 20 are cysteines [5]. Between individual isoforms are differences that are caused mainly from post-translational modifications, small changes in primary structure, or speed of degradation. Differences are also in their presence in individual organs. Meanwhile MT1 and MT2 are present almost in all types of soft tissues, MT3 is expressed mostly in brain tissue, but also in kidneys, heart and reproductive organs and the MT4 gene was detected in some epithelial cells. MTs are participating to maintaining homeostasis of zinc in tissues and detoxification of organism against heavy metals such as cadmium or lead. Presumably these proteins are binding platinum to their structure and make the interaction with DNA of cell impossible [6].

Promising material for future cancer therapy are platinum nanoparticles. There is a theory that PtNPs release Pt^{2+} as a result of weakening of strength of the Pt-Pt bond in acid environment of carcinoma cells. Released Pt^{2+} create complex Pt-DNA and causing time-dependent apoptosis [7]. However, the mechanism of cytotoxicity has never been proved. Use of PtNPs seems to be an appropriate solution for the problem of resistance of some cells against platinum-based cytostatics [8].

2. MATERIALS AND METHODS

2.1. Chemical

All chemical used for prepare platinum nanoparticles and all chemicals used for electrochemical detection were purchased from Sigma-Aldrich (USA) in ACS purity. N-[2-(2-{2-[bis(carboxy-methyl)amino]-5-methoxyphenoxy}ethoxy)-4-(2,7-difluoro-6-hydroxy-3-oxo-3H-xanthen-9-yl)phenyl]glycine (FluoZin-3) was purchased from Labeling and detection (USA). HEPES was obtained from Merck KGaA (Germany). Both proteins (MT2, MT3), all metals (zinc, platinum and lead) and cisplatin were purchased from Sigma-Aldrich (USA). Carboplatin and Oxalplatin were purchased from Teva (CZ). Chelex 100 Resin was from BIO-RAD (USA).

2.2. Electrochemical detection

MT was determination by differential pulse voltammetry (DPV) The measurement was performed with 663 VA Computrace instrument (Metrohm, Switzerland), using a standard cell with three electrodes (working electrode - hanging mercury drop electrode with a drop area of 0.4 mm^2, the reference electrode - Ag/AgCl/3M KCl electrode and auxiliary electrode - glassy carbon electrode). For data processing 663 VA Computrace software from Metrohm CH was employed. For deoxygenation of analyzed samples was used purging with argon (99.999%). The Brdicka supporting electrolyte containing 1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonia buffer (NH3(aq) + NH4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement by differential pulse voltammetry were as follows: initial potential of −0.7 V, end potential −1.8 V, deoxygenating with argon 90 s, deposition 120 s, time interval 0.2 s, step potential 1.95 mV, modulation amplitude 25 mV, and modulation time 0.57 s. For electrochemical measurement the volume of injected sample was 10 µL and volume of measurement cell 2 mL (10 µL of sample + 1990 µL ammonium buffer). All measurements were carried out at temperature 6 ± 1 °C [9].

2.3. Capillary electrophoresis (CE)

Samples were analyzed by CE (Agilent technologies, Waldbronn, Germany) with laser-induced fluorescence detector (ZetaLIF, Picometrics, Toulouse, France) with solid-state laser (λem = 488 nm) as an excitation source. Fused silica capillary with internal diameter of 75 µm and with the total length of 64.5 cm and effective length of 56 cm was used. The sample was introduced hydrodynamically by 50 mbar for 5 s and separation voltage of 25 kV was applied, 80 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer pH 7.4 with 1.5 µM FluoZin-3 was used as an electrolyte. Prior to the analysis, the capillary was washed for 240 s by NaOH, 240 s by 80 mM HEPES pH 7.4 and then filled for 150 seconds by 80 mM HEPES pH 7.4 with 1.5 µM FluoZin-3. Concentrations of both isoforms of MTs were 100 nM and concentration of platinum-based cytostaics and cadmium were 1 uM.

2.4. Preparation of platinum nanoparticles

Platinum nanoparticles were prepared by dissolving $PtCl_4$ (0.034 g) in acidic water (5 mL) with 16 µL of 37% HCl. The solution of $PtCl_4$ (5 mL) was added, with stirring, to another solution of 0.135 g PVP in water (45 mL). The mixture was stirred for 1 hour at temperature 25 °C. After the addition of $NaBH_4$ (50 mg) the final color of the solution became black. The mixture was stirred overnight [2].

2.5. Sample preparation

For comparison of interaction between both MT isoforms 5 µl 60 nM protein in MiliQ water was used. 3 µM Fluozin-3 (Fluozin-3 was prepared in 100 mM HEPES, pH 7.4) was added followed by 5 µl of 800 nM metal ion in MiliQ water. Reaction of MT with metal (e.g. Pt^{2+}, Pb^{2+} and other) led to release of zinc. Released zinc was detected by Fluozin-3 (Zn^{2+} selective fluorescent indicator). Fluorescence of FluoZin-3 and Zn^{2+} was measured at 25 °C by an Plate reader Infinite® 200 PRO-Tecan (Switzerland) using a NanoQuantPlate (Tecan).

3. RESULTS AND DISSCUSION

3.1. Confirmation of actual protein concentration

In this study, the series of experiments concentrated for finding differences in binding affinity of two isoforms of MT to various platinum species (ions, Pt-based cytostatics, as well as Pt nanoparticles) has been done. These experiments were based on interaction of FluoZin-3 and zinc realized from the protein. FluoZin-3 is used very broadly in life sciences as intra- and extracellular fluorescent chelator selective for Zn^{2+} ions at physiological concentrations. By this method is possible to prepare calibration curve of zinc (**Figure 1A**). Linear dependence in Zn concentration on fluorescent signal of FluoZin-3 was observed exhibiting the coefficient of determination R^2 = 0.9976.

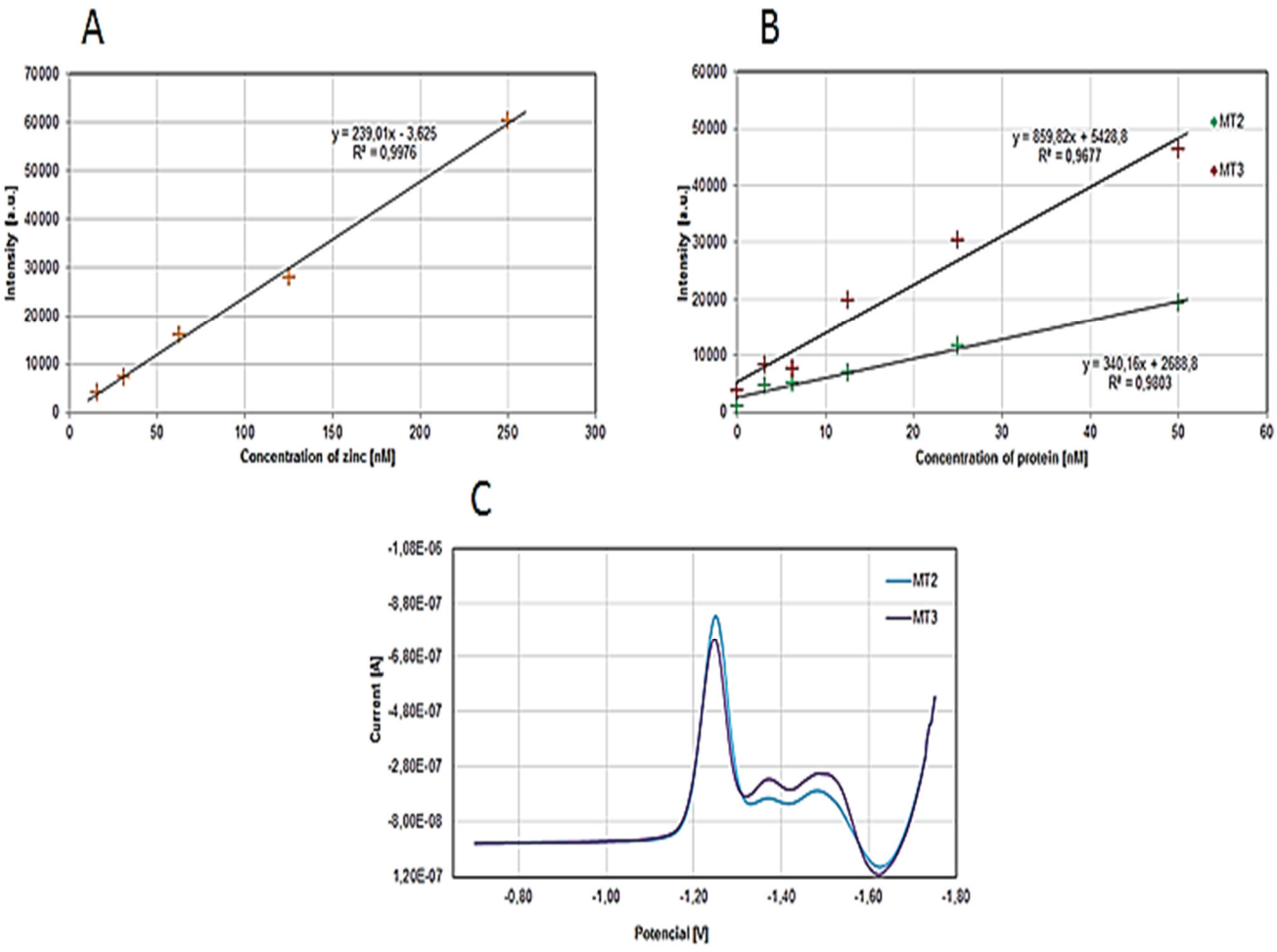

Figure 1 A) calibration curve of zinc, B) calibration curves of MT2A and MT3, C) Verification of protein concentration performed by electrochemistry

Afterward, this method was used for detection of zinc released from MT. Each MT contains seven atoms of zinc in its structure. This zinc can be released by interaction with another metal ion with higher affinity (e.g. Pt^{2+}, Pb^{2+} etc.). In this experiment, Pb^{2+} was used for release of zinc from MT. Released zinc was detected by interaction with Fluozin-3. By this interaction, it is possible to prepare calibration curves of both MT isoforms (**Figure 1B**). From calibration curves is noticeable that MT3 is giving higher zinc signal compared to MT2. It is possible that this difference is caused by distinct primary structure or different function in body. While MT2 is present almost in all types of soft tissues, MT3 is occurring predominantly in brain. It is probable that MT3 plays a special role in brain zinc metabolism and therefore it is expected that it has to be able to release zinc flexibly upon certain stimulus. These results are in agreement with previous study of Sweden scientists [10].

Verification of concentrations of both isoforms of MT was carried out by DPV. As can be seen in the (**Figure 1C**) the concentration of both isoforms detected in the stock solutions was same (6.5 μM).

3.2. Study of reaction between MTs and platinum based cytostatics using fluorescence spectrometry and capillary electrophoresis

In next part of this study, the reaction between both isoforms of MTs and different types of platinum-based cytostatics (cisplatin, carboplatin, oxaliplatin) was examined by using two methods - fluorescence spectrometry (**Figure 2A**) and CE with laser-induced fluorescence detection (**Figure 2B**). The reaction of platinum-based cytostatics with both isoforms of MT led to the release of zinc that was detected by FluoZin-3. As can be observed in both cases, the amount of released zinc was higher in case of MT3. Furthermore, it was observed that metal ions reacted with MT more intensively than platinum-based cytostatics. It is possible that it is caused by larger size of platinum drugs in the comparison with ions. From acquired data is seen that carboplatin the highest intensity of interaction from studied platinum-based cytostatics with MT reports. Carboplatin is second-generation of platinum drugs that produce less severe side-effect than cisplatin [11]. Although carboplatin forms the same bio-functional adducts as cisplatin, their formation is different and it is much slower. It was found that cisplatin-based chemotherapy is slightly superior to carboplatin-based chemotherapy [12]. So it is possible that lower effectivity of carboplatin is caused by easer interaction with MT resulting in inactivation of the drug.

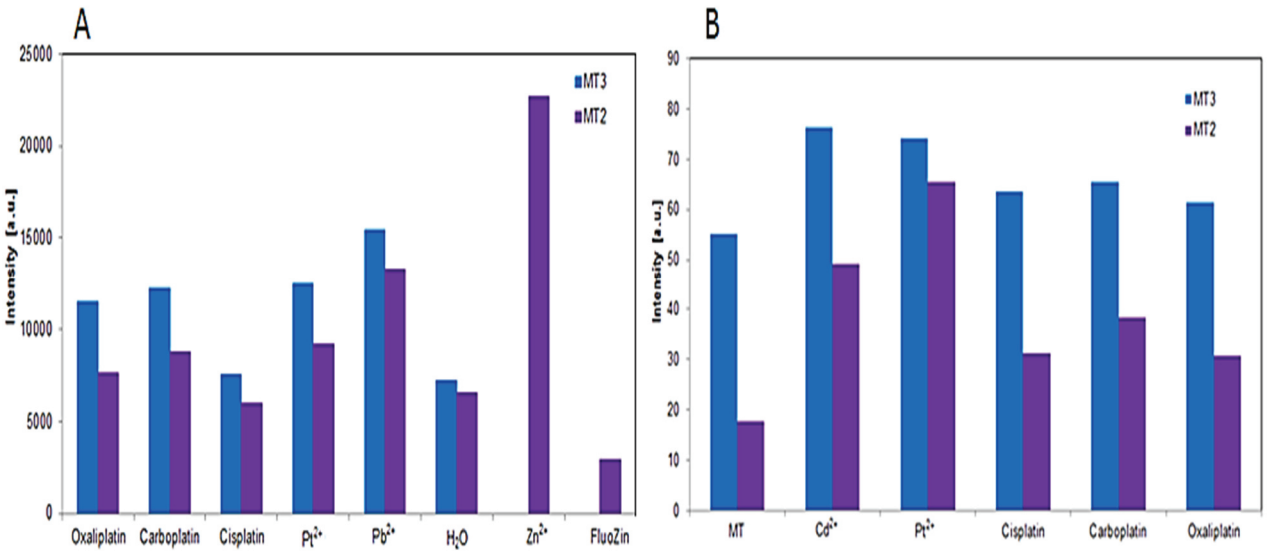

Figure 2 Interaction between platinum-based cytostatic and lead with both isoforms of metallothionein using fluorescence spectrometry (A) and CE (B)

3.3. Study of reaction between MTs and PtNPs

In the last part of this study, the reaction between both isoforms of MTs and two types of platinum nanoparticles of different size (40 and 10 nm) was studied and compared. As can be seen in (**Figure 3**), the nanoparticles with smaller size (10 nm) interact with both isoforms of MTs with higher intensity than the bigger ones (40 nm). These results are in agreement with other studies [2]. Higher reactivity of smaller nanoparticles maybe connected with their higher ratio of surface to volume. There is a possibility that smaller PtNPs have higher tendency to decay and release bigger amount of platinum ions than the bigger ones because they have higher ratio of surface area to volume. The bigger amount of released platinum ions interacts with MT and it is the reason of higher of interaction. It is probable that PtNPs would create in the cell a source of ions which would constantly attack DNA and cause of apoptosis in a long-term horizon. Therefore, it may be advantageous in comparison with conventional platinum-based cytostatics. However, confirmation of this theory would require further research especially *in vivo.*

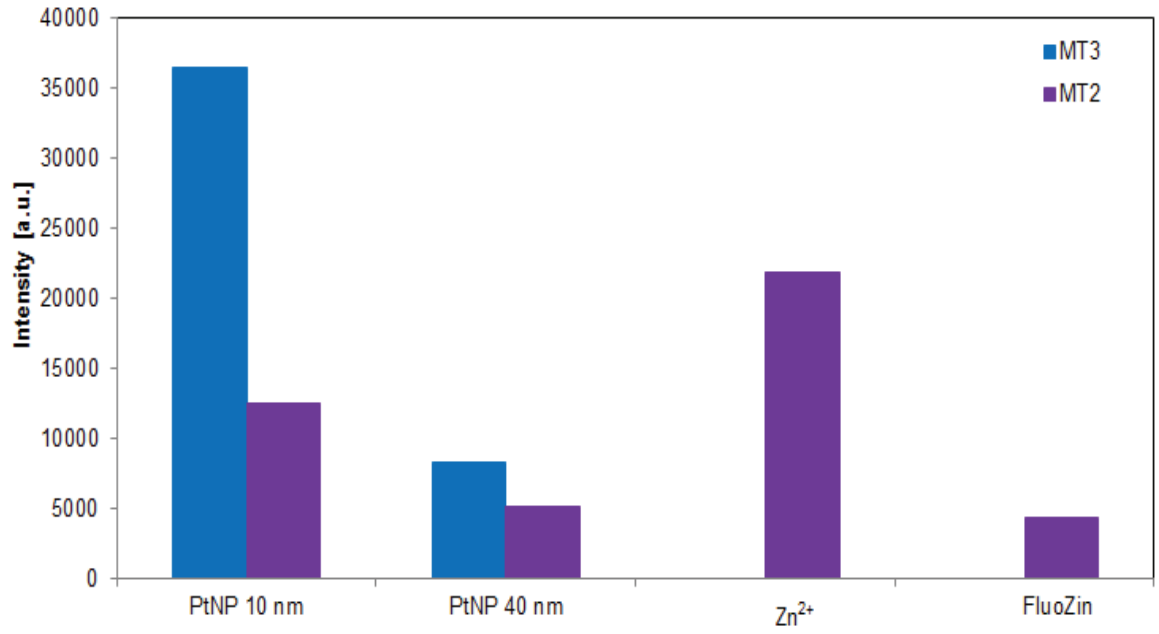

Figure 3 Comparison of interaction between MT isoforms with PtNPs of different size (10 and 40 nm)

4. CONCLUSION

It was established that the interaction of MT3 isoform with either platinum-based cytostatics or PtNPs caused the release of higher amount of zinc ions in comparison to isoform MT2. The difference of affinity of zinc of both isoforms of MTs is probably caused to the different role in organism.

Also differences between the particular cytostatics (cisplatin, carboplatin, and oxaliplatin) in the ability of zinc release were observed. From acquired data is seen that the highest ability to release zinc ions was observed in case of carboplatin. Although the carboplatin create the same adduct as cisplatin the formation of bio-functional adduct is different and in the case of carboplatin is much slower. The different of creation of bio-functional adducts maybe the cause of easier interaction of carboplatin with MT.

Next, the interaction between two types of nanoparticles with different size (10 and 40 nm) with both of isoforms of MT was investigated. It was found that smaller PtNPs react with both isoforms of MT with higher intensity than the bigger ones. This phenomenon is probably caused by easier of released of platinum ions from smaller platinum nanoparticles due to high ratio of surface area to volume of smaller particles.

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