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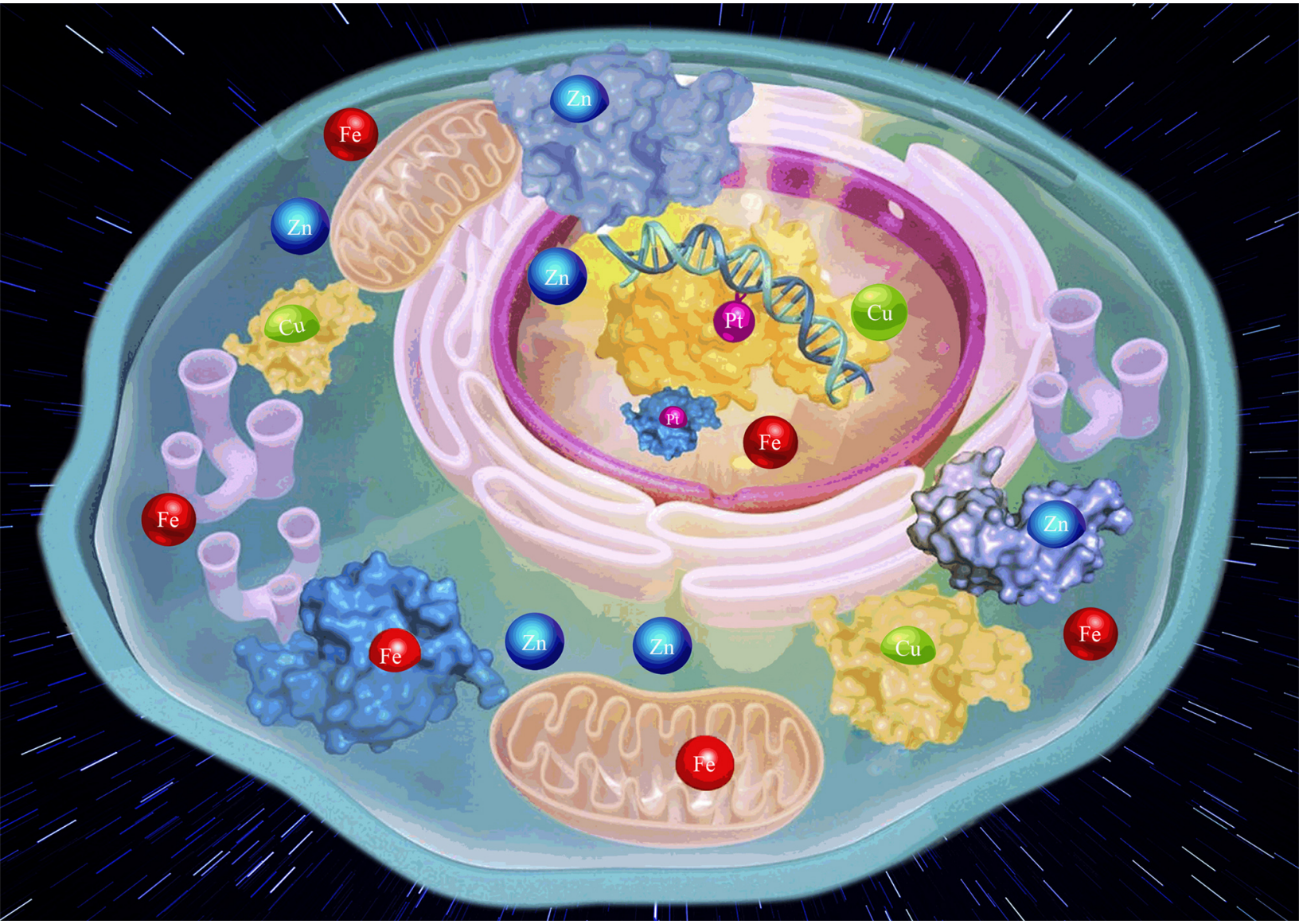

Cover Feature:

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Article

The Disturbance of Anticancer Drug Cisplatin to Cellular Homeostasis of Trace Elements Revealed by ICP-MS and ToF-SIMS

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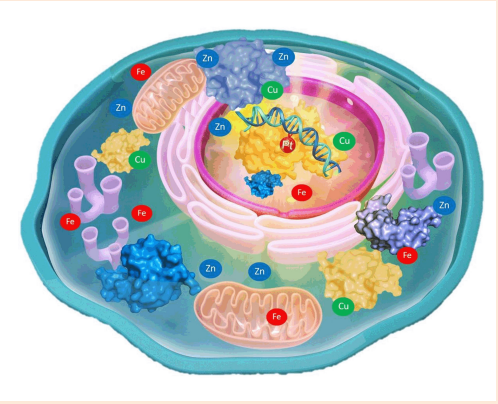
ABSTRACT: Trace elements play important roles in many physiological processes. The disorder in the metabolism and/or homeostasis of the trace elements will cause pathogenic changes, and finally lead to the development of diseases. This present work aimed to investigate the effect of anticancer metallodrug cisplatin on the homeostasis of trace metal elements, iron (Fe), zinc (Zn) and copper (Cu), in both human HEK293 normal cells and A549 lung cancer cells by using inductively coupled plasma mass spectrometry (ICP-MS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS). The levels of trace metal elements in both whole cells and nuclei subjected to cisplatin treatment were measured by ICP-MS, and results showed that accompanying with the increasing concentration of cisplatin, the content of Fe inside whole cells and nuclei of both normal and cancer cells increased, implying that cisplatin may induce ferroptosis, contributing to its anticancer activity. While the level of Cu and Zn in the whole cells increased with increase in cisplatin concentration, the level of Cu and Zn, in particular the latter, in nuclei decreased with increase of cisplatin concentration. These implicate that cisplatin may compete with Zn for binding to metalloproteins in nuclei, leading to efflux of Zn from nucleus. The normalized signal intensity of Fe, Zn, and Cu in single cells detected by ToF-SIMS increased accompanying with increased cisplatin concentration, being in line with that obtained by quantitative ICP-MS on large quantity of cells. These findings provide novel insights into better understanding in the mechanism of action of cisplatin.



INTRODUCTION

Platinum-based drugs, such as cisplatin, carboplatin, and oxaliplatin, are widely used in the treatment of solid tumors, for instance, ovarian, lung, bladder, head, and neck cancers. They are injected intravenously to patients and enter cells through passive diffusion and active transport of membrane proteins.[1, 2] Before they reach the nucleus and interact with the ultimate target, DNA, these drugs are inevitable to interact with proteins inside and outside the cells, such as human serum albumin (HSA), transferrin (Tf), copper transporter 1 (CTR1), metallothionein (MT), etc.[3, 4] For example, after 24 hours of intravenous cisplatin administration, 65-90% of cisplatin is bound to plasma proteins, most of which (50-72%) is attached to serum albumin, and a small amount is linked to transferrin.[5, 6] These proteins are also the main functional proteins for the storage, transport, and uptake of trace metal elements in human.[7]

Trace metal elements, such as iron, zinc, copper, are essential in the human body as they consist of many metalloproteins or enzymes which are involved in vital physiological regulation processes and play crucial roles in maintaining the normal function

of biological systems. The disturbance of cellular homeostasis of these trace metal elements, *i.e.*, deficiency or excess in quantity, or abnormal distribution could cause pathological changes.[8, 9] Fe is one of the most abundant trace metal elements in the human body, and mainly in the form of iron porphyrin complex. Fe deficiency can easily lead to iron deficiency anemia and affect mental and intellectual health,[10] while excessive amounts of Fe is also harmful as it may induce oxidative stress of cells. Some diseases can cause the imbalance of trace elements. For example, tumor would induce disorder of Fe metabolism in major organs, resulting in an increase of Fe level in the liver, kidney, and muscle.[11] Zn is another essential trace element playing a key role in the cell division, DNA synthesis, immunity, and development of organs. It is essential in the composition of structure or as the catalytic center of more than 200 enzymes. One of the most important proteins containing coordinated Zn is superoxide dismutase (SOD), which has been reported to be related to the resistance of platinum drugs.[12-14] Cu is essential for energy production, connective tissue formation, the central nervous system, and melanin synthesis. It has been reported that cisplatin can utilize copper transport proteins like CTR1 and chaperone proteins, for example, antioxidant 1 copper chaperone (Atox1) as transporters to enter the cells and accumulate in organelles, while other copper proteins like ATPase copper transporting alpha (ATP7A) and ATPase copper transporting beta (ATP7B) can pump cisplatin out of cells, which may be relevant to platinum resistance.[15-17]

There are many methods to measure trace metal elements in cells, including HPLC, X-ray fluorescence imaging, transmission electron microscopy (TEM), magnetic resonance imaging (MRI), and mass spectrometry imaging,[18-20] of which each has certain limits. For instance, X-ray fluorescence is harmful to the human body, which requires professional technicians to operate in a specific environment. TEM can obtain subcellular imaging of heavy metal elements such platinum, but it is not sensitive enough to image iron, zinc, copper and platinum simultaneously in cells.[21-23] The emerging mass spectrometry imaging techniques, such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), matrix-assisted laser desorption/ ionization mass spectrometry (MALDI-MS) and desorption electrospray ionization mass spectrometry (DESI-MS) are challenged to meet the requirements of single-cell measurements. In recent years, secondary ion mass spectrometry (SIMS) has attracted wide and increasing attentions especially in biology research. It can detect all elements and their isotopes in the periodic table with many advantages, such as high lateral resolution, low detection limit and the ability of 2D and 3D imaging,[24-27] and has been used to study the distribution of Fe and Cu in cells[28-31] as well as the subcellular distribution of platinum, ruthenium and gold anticancer compounds.[32-35] However, these studies are in lack of more evidence and support from other comparable methods and do not involve the influence to the homeostasis of trace elements by the administration of metallic anticancer drugs.

The present study aimed to combine the time of flight secondary ion mass spectrometry (ToF-SIMS) and ICP-MS to assess the effect of cisplatin administration to the homeostasis of trace elements (Fe, Zn, and Cu) in both cancer cells and normal cells for comparison. This may give us a new sight into the action mechanism of cisplatin, and will also provide a new platform for the research on the metabolic balance of biological trace elements at the single-cell level.

EXPERIMENTAL

Materials. The adenocarcinoma human alveolar basal epithelial cell line A549 and the human embryonic kidney cell line HEK293 were obtained from the Center for Cell Resource of Peking Union Medical College Hospital (Beijing, China). Nuclear extraction kit was bought from BestBio (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin-EDTA, and penicillin/streptomycin were obtained from Gibco (Thermo Fisher Scientific, USA). Cisplatin was obtained from Beijing Ouhe Technology Co., Ltd. (AR, Beijing, China), PBS buffer was purchased from Solarbio for cell culture (Beijing, China), ammonium acetate was obtained from J&K Scientific Ltd. (Beijing, China) and used as supplied. The Fe, Cu, Zn standard solutions were from Guobiao (Beijing) Testing & Certification Co., Ltd. (Beijing, China) and HNO_3 (TraceMetal Grade) for trace metal ICP-MS analysis was obtained from Thermo Fisher Scientific. The deionized water used in the experiments was prepared by a Milli-Q system (Millipore, Milford, MA).

Cell culture and drug treatment. A549 cells and HEK293 cells were cultured in 90% DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 ºC in a humidified incubator with 5% CO_2 overnight to adhere. Cisplatin was dissolved in deionized water to prepare 1.0 mM stock solution and further diluted with the culture medium to the desired concentration before being added to the culture media.

Sample preparations for ICP-MS. A549 or HEK293 cells with a density of 1×10^5 were cultured for 24 h in each dish and then incubated in culture medium with 0 μM, 20 μM and 50 μM cisplatin for another 24 h. After rinsing with PBS, all dishes of cells were collected and counted by a cell counter (Luna™, Logos Biosystems, South Korea). Then, the cells were divided into two groups for the whole cell and nucleus analysis, respectively. The first group of the cells was washed with deionized water and collected by centrifugation. The nucleus of the second group of cells was extracted following the protocols of nuclear extraction kit. The cells and nucleus extracts were stored at −80 °C, until analysis.

The above cells and nucleus extracts were digested by 2 mL 50% HNO_3 at 200 °C three times and evaporated to dryness. The residues were resolved in 2 mL 1% nitric acid and injected to

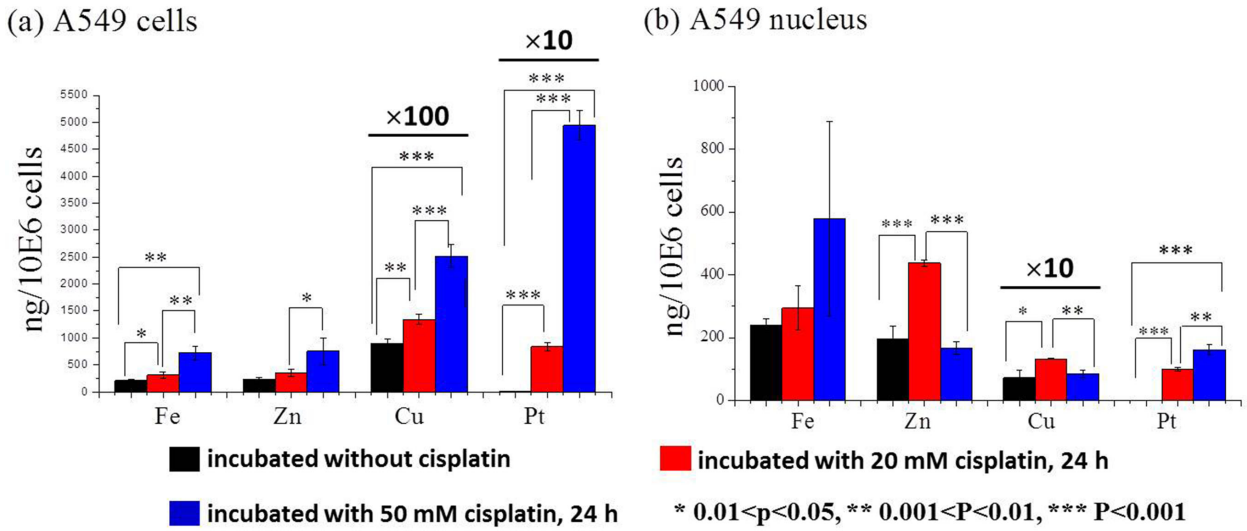


Fig. 1 Quantitative analysis of trace elements in A549 cells by ICP-MS. Since the concentrations of some elements were quite low, for clarity the detected concentration values are magnified 10 times or 100 times larger and marked as ×10 or ×100, respectively.

inductively coupled plasma mass spectrometry (ICP-MS 7700, Agilent, USA) to determine the concentration of Fe, Zn, Cu, and Pt. The ICP-MS data was calibrated with a certified standard solution for each element, respectively. The level of various elements in cells or nucleus were calculated and reported in nanogram per 10^6 cells (ng/10^6 cells). All samples were prepared and detected in triplicate to get the average and standard derivation.

Sample preparations for ToF-SIMS. A549 cells and HEK293 cells were cultured on silicon wafers at a density of 1×10^4 cells/cm^2 for 16 – 24 h in a culture dish for cell attachment, respectively. The silicon wafers had been cleaned by sonication in methanol, dichloromethane, acetone, and ethanol and deionized water in sequence before use. Culture medium containing 0 μM, 20 μM or 50 μM cisplatin was added, respectively. After incubation of 24 h, the cell-adhered silicon wafers were removed from media and washed three times by PBS and 150 mM ammonium acetate, respectively. Then, the silicon wafers were merged into the liquid nitrogen for quick freezing and transferred intermediately into a LGJ-12 lyophilizer (Beijing Songyuanhuaxing Technology Develop Co., Ltd, China) at a low temperature between −65 °C and −80 °C for freezing-drying overnight.

ToF-SIMS measurements. Measurement of trace element level in single cells was performed using an IONTOF TOF-SIMS 5 spectrometer (ION-TOF GmbH, Münster, Germany) equipped with a liquid metal primary ion source. Before analysis, a 10 kV Ar_{1700}^+ cluster (5.9 nA) was used as a sputter gun to etch an area of 200 × 200 μm^2 for three times to remove the cell membrane and other impurities on the cell surface. A 30 keV Bi_3^+ primary ion beam was used to scan an area of 100 × 100 μm^2 at the center of the crater with a frame of 256 × 256 pixels. A low energy electron flood gun was used for charge compensation.

The IONTOF software (SurfaceLab, version 6.8) was used for SIMS data collection and analysis. The spectra were calibrated using CH^+, CH_3^+, $C_2H_3^+$, $C_2H_5^+$, Si_5^+, Si_6^+ peaks in positive mode. The region of interest (ROI) of cells was created by the threshold of $C_5H_{15}NO_4P^+$ image. Raw data were pretreated by normalization of ion signal intensity to the intensity of total ions.

RESULTS AND DISCUSSION

To investigate the effect of cisplatin on the metabolism and distribution of trace metal elements in cells, ICP-MS was firstly applied to quantitatively analyze the trace metal elements in A549 cancer cells and HEK293 normal cells before and after cisplatin treatment. Since the levels of Fe, Cu and Zn are significantly different in cells, to guarantee the accuracy of the data we measured separately these three elements by ICP-MS using different numbers of cells so as to fit the linear range of the standard working curves. The results for A549 and HEK293 cells are shown in Fig. 1 and Fig. 2, respectively, which show that the concentrations of all three trace metal elements in A549 cancer cells were higher than those in HEK293 cells under cisplatin-free conditions, perhaps due to the demand for more nutrition consumptions of cancer cells.

After incubating with 20 μM cisplatin for 24 h, the concentrations of Fe, Zn, and Cu in A549 cells increased both in the whole cells and in nuclei (Fig. 1). Further increasing the concentration of cisplatin to 50 μM, the whole cell level of all three elements elevated more obviously, accompanied with significant amount of Pt detected. However, in the nuclei, distinct variations appeared. The concentration of Fe was elevated as that of Pt, whereas the Zn and Cu levels decreased. This means that low concentration of cisplatin induced increasing cellular uptake of all three elements in cancer cells, but the high concentration of

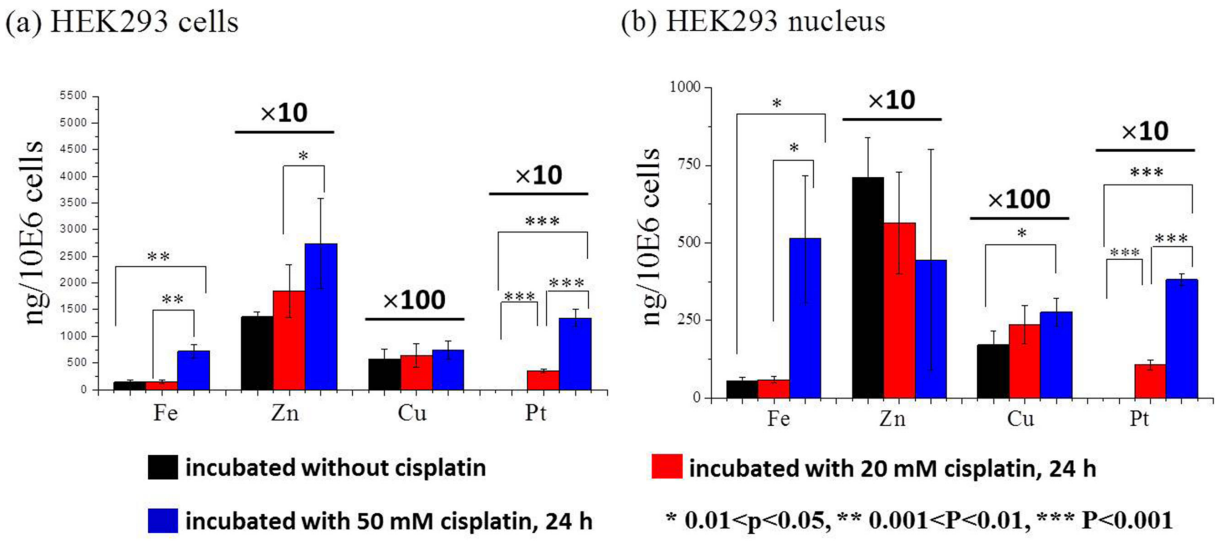


Fig. 2 Quantitative analysis of trace elements in HEK293 cells by ICP-MS. Since the concentrations of some elements were quite low, for clarity the detected concentration values are magnified 10 times or 100 times larger and marked as ×10 or ×100, respectively.

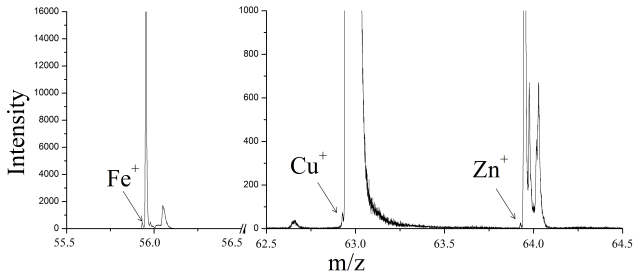


Fig. 3 The typical mass spectrum of naked iron, zinc and copper obtained by ToF-SIMS.

cisplatin caused more Zn and Cu elements to escape from the nuclei and accumulate in the cytoplasm. In contrast, Fe level in nuclei still increased with the elevated Pt concentration.

Similar elevation of all these three elements at the whole cell level was also observed in HEK293 normal cells when incubated with increased cisplatin concentration (Fig. 2a). The zinc elevation is the most significant among the three elements in the whole cell, however, its level in the nucleus changed oppositely compared to that in the whole cell (Fig. 2b). This indicates that zinc ions may be pumped out from the nucleus to cytoplasm due to the increased Pt amount into the nucleus where the final target of cisplatin, genomic DNA, localized. A gradually slow increase was observed for the Cu level accompanied with the elevated Pt concentration both in the whole cell and in the nucleus. The iron level was nearly unchanged after 20 μM cisplatin administration, but showed about a 3-fold increase when the cisplatin concentration increased to 50 μM. Similar variation was also observed for the Fe level in the nucleus (Fig. 2b).

Next, ToF-SIMS was applied to analyze the cellular uptake and distribution of the trace elements in single cells. ToF-SIMS is a very sensitive surface analysis technique. To get more accurate MS data, we chose to use a spectrometry mode with high mass resolution but relatively low image resolution to image the three elements in cells. In this case, all naked Fe, Zn, and Cu ions peaks could be well discriminated from background and interfering signals, though their signal intensity were quite low (Fig. 3). With a spatial resolution of 200 – 300 nm, ToF-SIMS image is able to visualize the subcellular distribution of chemicals.[26, 35-40] However, the low content of trace elements and complex components in cells made it difficult to obtain high quality of images which can discriminate the trace element located in cytoplasm and nuclei of the single cells as shown in Fig. 4 and Fig. 5. With regard to this, and taking the heterogeneity of cells into account, we normalized the ion counts of three elements to the count of total ions for each single cell obtained by ToF-SIMS analysis so as to compare exactly the levels of Fe^+, Cu^+, and Zn^+ ions in single cells with and without cisplatin treatment. The results were shown in Fig. 6 for A549 cells and Fig. 7 for HEK293 cells, respectively.

The normalized intensities of the three trace metal elements in both A549 (Fig. 6) and HEK293 (Fig. 7) whole cells were increased along with the elevated cisplatin concentration from 0 to 50 μM, showing a similar tendency to those observed by ICP-MS analysis of large number of cells. When sampling for ICP-MS and SIMS analysis, only live cells were collected, and dead cells were washed away. Therefore, the results obtained by ICP-MS analysis of large number of cells and those by ToF-SIMS measurement of limited single cells were consistent, which both indicated that with the increase of cisplatin concentration, the level of trace elements in the surviving A549 cells and HEK293 cells was increasing.

It has been reported that tumors may induce disorder of trace elements in tissues or organs. Iron content, especially the ferritin

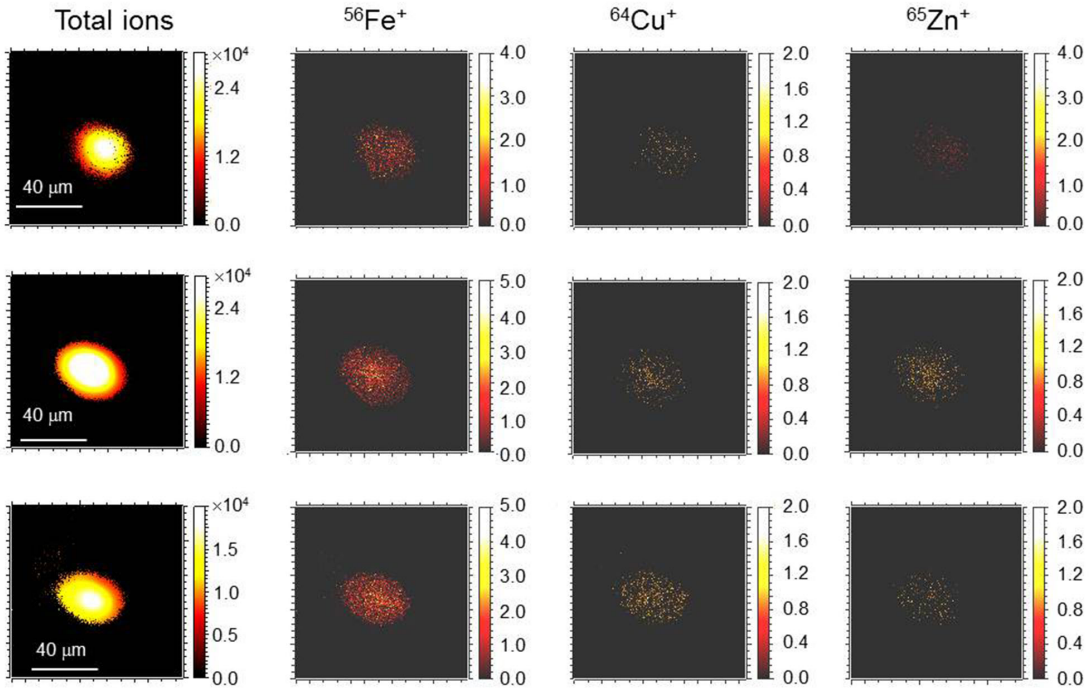


Fig. 4 Representative ToF-images of A549 cells without exposure to cisplatin (top panel), and treated with 20 μM (middle panel) or 50 μM (bottom panel) cisplatin for 24 h.

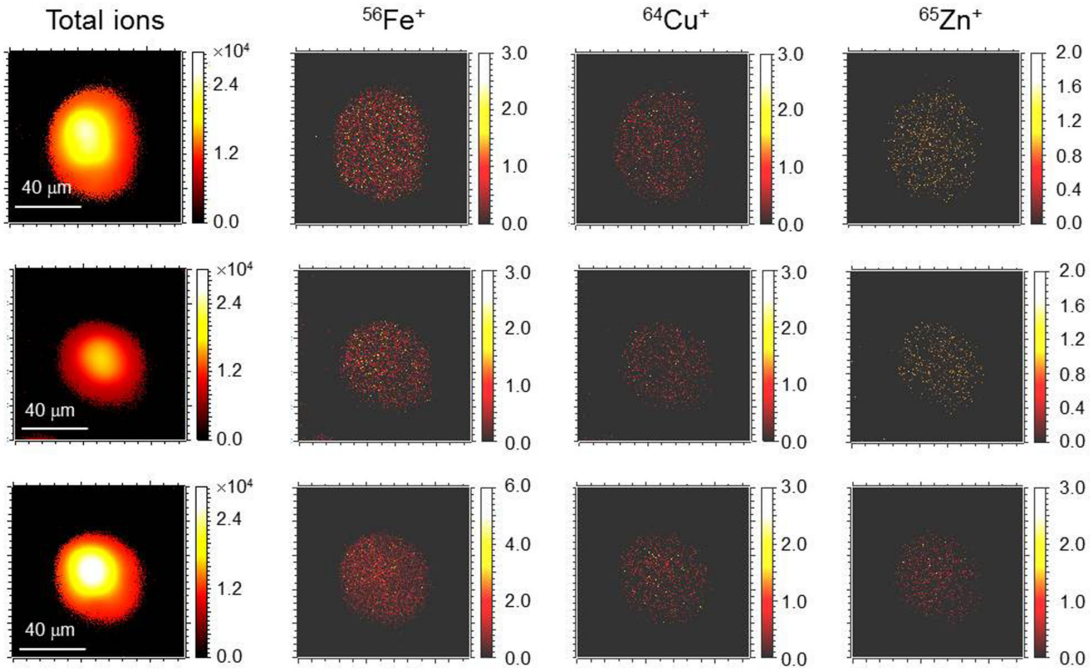


Fig. 5 Representative ToF-images of HEK293 cells without exposure to cisplatin (top panel), and treated with 20 μM (middle panel) or 50 μM (bottom panel) cisplatin for 24 h.

content, drastically extended in the liver, lung, and muscle of mice after tumor implantation.[11] Several studies compared the trace elements levels in cancerous tissues, para-carcinoma tissues and control normal tissues, and showed that the concentration of iron in cancerous tissues of the kidney was higher than that in para-carcinoma tissues,[41] and the concentration of zinc in cancerous tissues of urinary bladder was also higher than that in normal tissue.[42] In serum of bladder cancer sufferers the concentration of copper was increased, but the concentration of iron and zinc were decreased as compared to those in healthy persons. The level of trace elements varied in distinct organs and remarkably changed in the tissues of tumor lesions which may be related to the causes

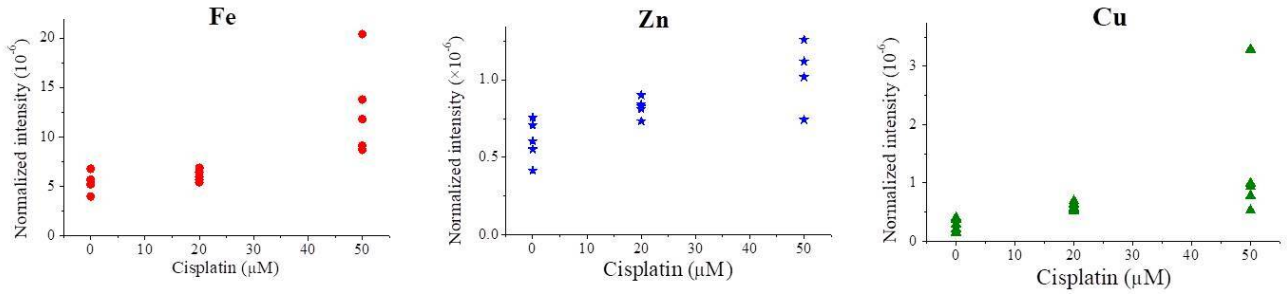


Fig. 6 Normalized intensity of trace elements in A549 cells examined by ToF-SIMS.

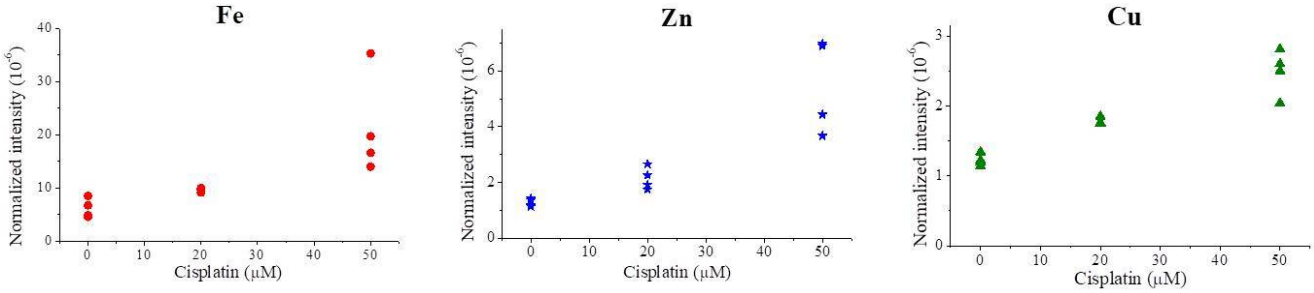


Fig. 7 Normalized intensity of trace elements in HEK293 cells examined by ToF-SIMS.

of cancer occurrence.[43] In our study, we found the levels of all three trace elements in A549 cancer cells were higher than those in HEK293 cells, in agreement with proviously reports.[41-43] Trace element levels have been reported be associated with the sensitivity to drugs, curative effect, and toxicity.[44, 45] A previous study reported that cancer cells with acquired resistance to cisplatin exhibited substantial alterations in the intracellular trace elements content and in the levels of regulatory proteins involved in the cellular uptake, storage and efflux of these elements.[46] Hence, modulating trace elements in the diet[44] or adding metal chelators to synergize with chemotherapy for cancer treatments[47] may promote the activity, reduce the toxicity of antitumor drugs,and even improve the prognosis.[48] On the other hand, determining the variations of trace element level in cells treated with anticancer drugs will be helpful to better understand the mechanism of action, and to guide and improve their clinical applications.

Iron is one of the most abundant trace elements in cells, and its overload is thought to be one of biomarkers of ferroptosis which has been recently defined to be an iron-dependent form of programmed cell death.[49, 50] Cancer cells usually contain high concentration of glutathione (GSH) which scavenges reactive oxygen species (ROS), inhibiting ferroptosis and lead to resistant to cisplatin.[51] Therefore, Erastin and GPX4 inhibitors were applied to induce ferroptosis of cisplatin-resistant cancer cells.[50, 51] Here, we demonstrated that the iron level of A549 cancer cells increased with the increase of cisplatin concentration, suggesting that cisplatin may trigger ferroptosis by depleting intracellular GSH[51] *via* coordination with GSH.[52]

On the other hand, cisplatin could induce excessive ROS generation and give rise to fatal lesions inducing cell deaths.[53] It has been reported that the antioxidant protein, human ferritin (H-Ferritin, FHC) is underscored by varied mechanisms leading to its transcriptional and post-transcriptional up-regulation in response to oxidative stimuli.[54, 55] This could cause an increase of iron or iron-related proteins in cells, being consistent with our experimental results in some aspects.

In the clinic, cisplatin chemotherapy has also been observed to increase iron and zinc levels in the blood, which may also be related to oxidative damage or toxic side effects.[56, 57] Cisplatin could enter the tumor cells *via* competing with copper to bind to the major copper transporter CTR1, and that ATP7A and ATP7B contributed to the influx and efflux of cisplatin, respectively. Consequently, the level of intercellular and intracellular copper might affect the intake of cisplatin, and then influence its efficacy and side effects.[58] Many scholars had reported that elevated CTR1 expression could be induced by Cu chelation, resulting in reduced intracellular Cu and thereby lowered cisplatin resistance.[59-61] In contrast, the overexpression of hCTR1 was demonstrated to increase cellular uptake of copper in breast cancer cells.[62] In our studies, the treatment of cisplatin did increase the concentration of cellular Cu, probably due to up-regulation of the CTR1 transporter by cisplatin stimulating.

The similar circumstance was also occurring for Zn uptake subjected to cisplatin treatment. It has been reported that cisplatin

treatment could significantly induce expression of THAP domain-containing protein 5 (THAP5), which is a human zinc finger protein and mainly locate at nuclei.[63] The presence of more zinc also up-regulated the metallothioneins expression which may increase cisplatin-resistance.[64] In our previous study, cisplatin was showed to occupy two histidine sites on serum albumin, replacing Zn ions binding to serum albumin.[65] This implicated the ability of Pt to compete with Zn in other Zn-containing proteins, for example, metallothioneins.[66] Although the level of Cu and Zn were elevated in whole cells, the subcellular distribution of them, especially Zn, was disturbed to some extent by cisplatin. Zn level in nuclei significantly reduced with increase in cisplatin concentration, implying that cisplatin may displace Zn in Zn-containing protein in nuclei, leading to release of Zn from nuclei. Taken together, our results revealed that the changes of level and distribution of trace elements subjected to cisplatin administration are more likely related to the function and location of metalloproteins.

CONCLUSIONS

In the present work, by using ICP-MS and ToF-SIMS analysis, we obtained the statistical results of the levels of three trace elements, Fe, Cu and Zn, in large quantity of cells and in the single cells, respectively. We demonstrated that the administration of cisplatin increased the level of the trace elements in the whole cell, but reduce the level of Cu and Zn, in particular the latter, in nuclei with increase in cisplatin concentration. The interactions of cisplatin with various cellular metalloproteins may be involved in this progress. These findings provide new experimental basis for further understanding the mechanism of anticancer metallodrug cisplatin. Moreover, we highlight the power of the combination of ICP-MS and ToF-SIMS for studying the influence of other drugs on the level and cellular distribution of trace elements in cells.

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Notes

The authors declare no competing financial interest.

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