Platinum Drugs

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Combating the Drug Resistance of Cisplatin Using a Platinum Prodrug Based Delivery System**

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Platinum-based anticancer drugs are widely used in the clinic for the treatment of a broad spectrum of human malignancies. These drugs are administered to 40–80% of all patients undergoing cancer chemotherapy, either as single agents or in combination with other agents.[1] However, their application is limited by the presence of side effects and drug resistance. Although some tumors are intrinsically resistant to platinum-based drugs, other tumors acquire resistance only after initial treatment.[2] The sensitivity of cells toward platinum-based drugs, such as cisplatin, is dependent on DNA platination because DNA is the ultimate drug target of cisplatin. Tumor cells can acquire cisplatin resistance, that is, can achieve a reduction in the level of DNA platination, through several mechanisms, for example, through reduced drug uptake, through drug deactivation in cells, through DNA repair, and through increased drug efflux.[3]

Several cellular processes can be associated with sensitivity of cells toward cisplatin. The uptake of cisplatin into cells is facilitated by the copper transport protein (Ctr1), which is expressed in low levels in some cisplatin-resistant cells.[4] Metallothionein (MT) is a thiol-rich protein that binds strongly to many heavy-metal ions, including platinum(II). MT plays a role in cellular detoxification by sequestering these heavy-metal compounds, and an increased concentration of this protein in cells is associated with low efficacy of cisplatin.[5] The small peptide glutathione (GSH), which also has high affinity toward cisplatin and is found in increased concentrations in some cisplatin-resistant cells, can play a similar role.[6] Additionally, DNA repair proteins (such as NER) and efflux proteins (such as P-type ATPases) can also reduce the efficacy of cisplatin and contribute to cisplatin resistance.[3,7]

To avoid the problems of resistance associated with the use of cisplatin, several types of nonclassical platinum complexes have been developed, including *trans*-coordinated complexes, polynuclear platinum complexes, and platinum(IV) complexes.[8] These platinum complexes differ from cisplatin in their uptake pathway, their reactivity toward cellular proteins, and their DNA binding modes. Because of these differences, some of these nonclassical platinum complexes, such as *trans-EE*, BBR3464, and satraplatin, have shown promising activity in cisplatin-resistant cells.[3,9] These findings suggest that the design of platinum-based drugs that have different responses to cellular processes is a feasible approach toward circumventing the problems of resistance that affect the use of cisplatin.

Drug delivery systems have drawn particular attention in recent years because they can facilitate the delivery of platinum-based drugs, thus enhancing drug efficacy. A number of drug delivery systems have been developed for the delivery of platinum-based drugs. These systems have been based on polymers,[10] solid lipids,[11] and inorganic nanoparticles; the latter can be further subdivided into magnetic iron oxide,[12] single-walled carbon nanotubes,[13] metallofullerene nanoparticles,[14] gold nanoparticles,[15] nanoscale metal-organic frameworks,[16] and mesoporous silica microparticles.[17] Some of these systems have entered clinical trials.[11a,b] With the conjugation of biologically active molecules, some delivery systems have shown high selectivity in targeting tumor cells.[11b] Although the use of drug-delivery systems has been successful in improving the efficacy of platinum-based drugs, it remains a challenge to develop drug conjugates that combat drug resistances.

We have previously reported that PEGylated gold nanorods (PEG-GNRs) can facilitate the delivery of platinum(IV) prodrugs and significantly enhance the cytotoxicity of these prodrugs in tumor cells.[18] Herein, we report that the use of this drug-delivery system avoids the drug resistance that affects the use of cisplatin. We show that impaired drug uptake that results from the low expression of Ctr1 in the cisplatin-resistant cells A549R can be overcome by using a conjugate of a cisplatin prodrug and PEGylated gold nanorods (Pt-PEG-GNRs conjugate); this conjugate facilitates the delivery of the platinum-based drug into cells through endocytosis. Additionally, the platinum(IV) prodrug is less susceptible to deactivation by the detoxification protein MT and the peptide GSH, which were found in high concentrations in A549R cells. Consequently, the Pt-PEG-GNRs conjugate was highly cytotoxic to tumor cells, especially cisplatin-resistant cells.

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The Pt-PEG-GNRs conjugate was prepared by tethering the platinum(IV) prodrug, *cis,cis,trans*-[Pt-$(NH_3)_2Cl_2(O_2CCH_2CH_2CO_2H)_2$], to PEGylated gold nanorods (PEG-GNRs) using a previously reported method.[18] The cytotoxicity of cisplatin and the Pt-PEG-GNRs conjugate was assayed using lung cancer cells A549 and cisplatin-resistant cells A549R (Figure 1). The A549R cells clearly

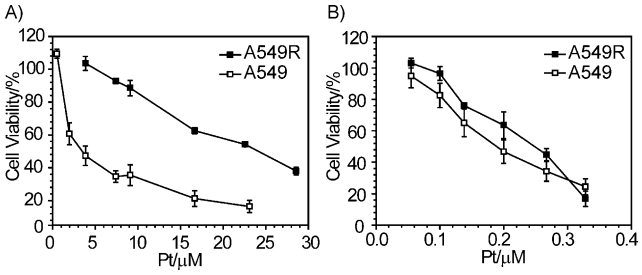

Figure 1. The cytotoxicity assays of platinum complexes. A) Cisplatin. B) Pt-PEG-GNRs conjugate. The A549 cells are denoted by (□) and the A549R cells are denoted by (■). Cells were incubated for 72 h with the platinum compounds.

showed resistance to cisplatin: the IC_{50} value of cisplatin in A549R cells (24.1 μM) was much higher than that in A549 cells (3.4 μM), thus representing a resistant factor (RF) of 7.1. The Pt-PEG-GNRs conjugate was more cytotoxic to both A549 cells ($IC_{50} = 0.19$ μM) and A549R cells ($IC_{50} = 0.24$ μM) than cisplatin (Figure 1B) and the resistant factor was significantly reduced (RF = 1.3). This result indicates that the cytotoxicity of the Pt-PEG-GNRs conjugate is higher than that of cisplatin, especially in the context of cisplatin-resistant A549R cells.

To determine the cause of drug resistance in A549R cells, the level of uptake of the drugs into cells was determined. After incubating the cells with cisplatin for 3 hours, the concentration of platinum(II) in cells was measured using inductively coupled plasma mass spectrometry (ICP-MS). The data showed that only half as much platinum was taken up by the cisplatin-resistant cells A549R relative to that taken up by A549 cells (Figure 2). The drug resistance is clearly related to the difference in drug uptake.

To determine the origin of the low cisplatin uptake in A549R cells relative to that in A549 cells, the level of mRNA

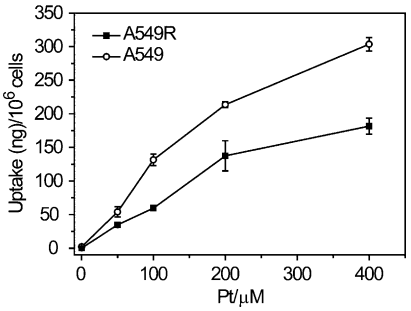

Figure 2. The platinum uptake in A549 and A549R cells. Cells were incubated for 3 h with media containing different concentrations of cisplatin.

that codes for Ctr1 in A549 and A549R cells was measured using real-time polymerase chain reaction (real-time PCR). The mRNA level in A549R cells was approximately 30% of that in A549 cells (Figure 3A). Western blot analysis also indicated that the protein level of Ctr1 in A549R cells was much lower than that in A549 cells (Figure 3B). These observations reveal that the relatively low uptake of cisplatin in A549R cells is due to the low expression of Ctr1 in these cells.

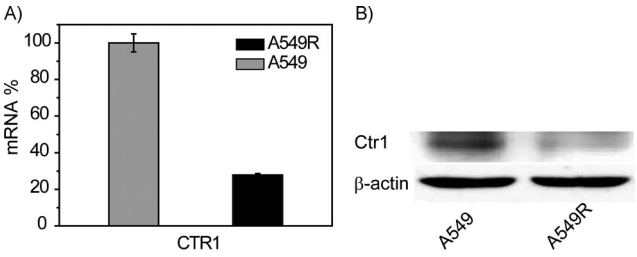

Figure 3. Comparison of Ctr1 in A549 and A549R cells. A) The mRNA level of Ctr1 measured by real-time PCR. B) Western blot analysis of the Ctr1 level.

Given that the Pt-PEG-GNRs conjugate is highly cytotoxic to cisplatin-resistant cells, the level of cellular uptake of the Pt-PEG-GNRs conjugate was determined. The platinum content in cells was determined using ICP-MS after the cells were incubated with cisplatin or the Pt-PEG-GNRs conjugate (0.2 μM, this concentration is close to the IC_{50} values of these compounds in A549R cells) for 3 hours. The data shows that the platinum uptake derived from the Pt-PEG-GNRs conjugate (0.49 ng Pt/10^6 cells) was about 4.7 times higher than that derived from cisplatin (0.105 ng Pt/10^6 cells). These data show that the Pt-PEG-GNRs conjugate has significantly higher drug uptake than cisplatin, thus suggesting that it is not subject to the resistance that is associated with a low expression of Ctr1.

The cellular uptake of the Pt-PEG-GNRs conjugate was analyzed by transmission electron microscopy (TEM). After incubating the A549R cells with 0.2 μM of the Pt-PEG-GNRs conjugate for a period of 3 hours, the nanorod particles were found in the endosome (Figure 4A). This endocytosis manifested itself in the formation of a newly formed endosome containing the Pt-PEG-GNRs conjugate (see the Supporting Information, Figure S1). Dark-field imaging further confirmed the presence of the Pt-PEG-GNRs conjugate in A549R cells (see the Supporting Information, Figure S2). These data represent direct evidence that the enhanced uptake of the platinum-based drug is achieved by endocytosis facilitated by their conjugation with PEG-GNRs. In this case, a low level of Ctr1 in the cells does not impair the uptake of the platinum-based drug.

It has been reported that glutathione and metallothionein can deactivate platinum-based drugs in the cytoplasm, thus leading to a reduction in DNA platination and contributing to the lowering of a cell's sensitivity to cisplatin.[3] Although a relatively high concentration of GSH has been observed in some cisplatin-resistant cells, a previous systematic study has

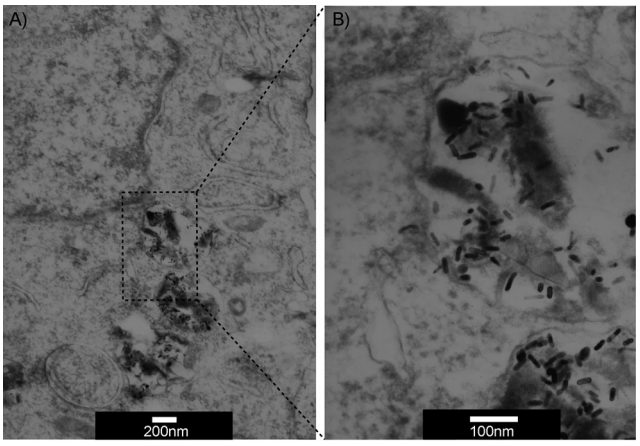

Figure 4. TEM image of endocytosis of the Pt-PEG-GNRs conjugate in A549R cells. A) Pt-PEG-GNRs conjugates trapped within endosome (scale bar:200 nm). B) Enlarged area from (A; scale bar:100 nm).

shown that a correlation between GSH concentration and drug sensitivity is not certain.[5,19] We found that the GSH concentration in A549R cells (52.76 nmol per 10^6 cells) was approximately 1.5-fold higher than that found in A549 cells (Figure S3, 36.72 nmol per 10^6 cells). To verify whether there was a correlation between the GSH concentration and drug resistance, A549R cells were treated with the GSH suppression agent D,L-buthionine-(*S*,*R*)-sulfoximine (BSO).[3] The assay showed that the drug sensitivity was enhanced upon treatment of the cells with BSO (Table 1), thus confirming a correlation between the concentration of GSH and cisplatin resistance in A549R cells.

Table 1: Cisplatin sensitivity assay on A549 and A549R cell lines with and without depletion of GSH and MT.

Cell Lines	A549[a]	A549R[a]	A549R + BSO[b]	A549R + PPG[c]
IC_{50} [μM]	8.3	36.8	20.7	24.2

[a] IC_{50} values in this assay are higher than those of the assay described in Figure 1 because cells were grown for an additional 24 h before adding cisplatin so that they could be comparable to those experiments in which GSH and MT are depleted. [b] Cells were treated with BSO (100 μM) for 24 h to deplete GSH concentration prior to adding cisplatin. [c] Cells were treated with PPG (100 μM) for 24 h to deplete MT prior to adding cisplatin.

A relatively high level of MT was also detected in the cisplatin-resistant cells A549R. The level of the mRNA that codes for the proteins, MT1G and MT1X, which belong to the MT family, was measured using real-time PCR. The result showed that the level of mRNA that codes for MT1G and MT1X in A549R cells was approximately twice as high as that in A549 cells (Figure 5A). Western blot analysis confirmed the relatively high expression of MT in A549R cells (Figure 5B). To verify whether there was a correlation between MT level and drug resistance, A549R cells were treated with the MT suppression agent D,L-propargylglycine (PPG).[20] The MTT assay showed that upon treatment of the cells with PPG, the sensitivity of cisplatin was increased

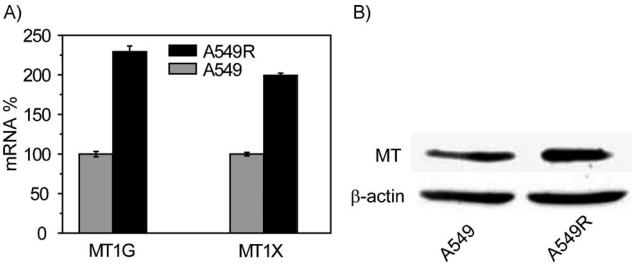

Figure 5. Comparison of the MT level in A549 and A549R cells. A) The mRNA level of MT1G and MT1X measured by real-time PCR. B) Western blot analysis of the MT level.

(Table 1), thus confirming a correlation between the level of MT and cisplatin resistance in A549R cells.

The rates of the reactions of cisplatin and the platinum(IV) prodrug (*cis*,*cis*,*trans*-[$Pt(NH_3)_2Cl_2$ ($O_2CCH_2CH_2CO_2H)_2$]) with metallothionein and glutathione were investigated. The reactions were monitored by measuring the UV absorption at 260 nm for the GSH reaction[21] and at 285 nm for the MT reaction;[22] these absorbance values are associated with the Pt–S bonds that are formed upon coordination of the drug to GSH or MT. The data showed that the platinum(IV) prodrug is considerably less reactive than cisplatin, in both the reaction with GSH and the reaction with MT (Figure 6).

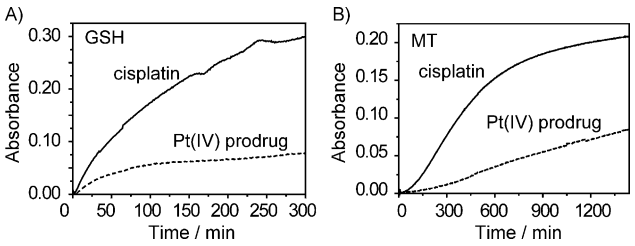

Figure 6. Time course of reactions of platinum complexes with GSH or MT at 37°C, pH 7.0. A) Reaction of GSH (16 mM) with cisplatin (32 μM, solid line) and the platinum(IV) prodrug (*c*,*c*,*t*-[$Pt(NH_3)_2Cl_2(O_2CCH_2CH_2CO_2H)_2$]; 32 μM, dashed line). B) Reaction of MT (10 μM) with cisplatin (20 μM, solid line) and the platinum(IV) prodrug (20 μM, dashed line). Reactions were monitored by observing the increase in the absorption at 260 nm for the GSH reaction (A) and at 285 nm for the MT reaction (B).

The amount of platinum bound to MT was measured by ICP-MS. (Figure 7A) When MT was treated with the platinum(IV) prodrug, much less MT-bound platinum was detected relative to when MT was treated with cisplatin, thus representing direct evidence that the platinum(IV) prodrug is more inert than cisplatin in the context of their interaction with MT; this result is in agreement with the kinetic study. The platination of MT results in the release of zinc from the protein; the quantity of zinc released can be measured by using the dye 4-(2-pyridylazo) resorcinol (PAR). The resulting complex between the dye and zinc, [$Zn(PAR)_2$], exhibits maximum absorption at 500 nm (see the Supporting Information, Figure S4). The reactions between MT and the platinum complexes in the presence of PAR were monitored by UV/Vis spectroscopy (Figure 7B). During the first 8 hours of reaction, only half as much Zn^{II} is released from the

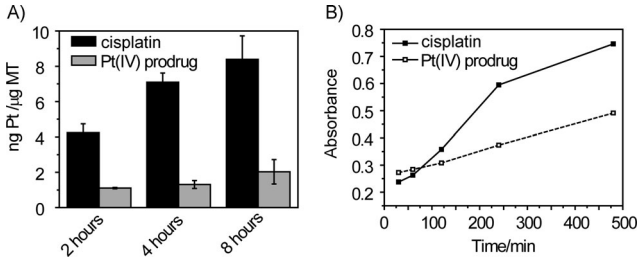

Figure 7. Time-dependent platination of MT by cisplatin and the platinum(IV) prodrug. A) The amount of platinum bound to MT after different reaction times using cisplatin (black bars) and the platinum(IV) prodrug (grey bars). The platinated MT was purified with an ÄKTA purifier equipped with a HiTrap desalting column, and the platinum content was measured by ICP-MS. B) The amount of Zn^{II} released by the platination of MT was recorded for different reaction times. The reactions between MT (10 μM) and both cisplatin (20 μM, solid line) and the platinum(IV) prodrug (20 μM, dashed line), in the presence of PAR (0.1 mM), were monitored by observing the absorption at 500 nm, which is a measure of the amount of $Zn(PAR)_2$ formed. The control experiment confirmed that no reaction occurred between MT and PAR in the absence of platinum complexes.

reaction of MT with the platinum(IV) prodrug than that released from the reaction with cisplatin; this result is consistent with the data obtained from the platinum-binding experiments. These observations imply that the platinum(IV) prodrug is more inert than cisplatin in the context of their interaction with MT and GSH.

The relationship between GSH concentration and DNA platination was also examined because DNA is the ultimate target of platinum drugs. Calf thymus DNA was treated with the platinum compounds in the presence of GSH at 37 °C. After a 12 hour reaction time, the platinum complexes that were not bound to DNA were removed using gel-filtration chromatography on an ÄKTA purifier. The amount of platinum bound to DNA was measured by ICP-MS (see the Supporting Information, Figure S5). The platination of DNA in the presence of cisplatin decreased upon increasing the concentration of GSH, presumably because the cisplatin is deactivated through its binding to GSH. Conversely, the platination of DNA in the presence of the platinum(IV) prodrug increased upon increasing the concentration of GSH from 0–1000 μM. This result supports the hypothesis that GSH plays different roles in the platination of DNA in the presence of cisplatin and platinum(IV) prodrugs.

During the 30 years in which cisplatin has been used in the clinic, the high incidence of drug resistance has been the main limitation in its application in cancer chemotherapy.[23] Studies have shown that drug resistance can develop because of changes in the various cellular processes that cisplatin is involved in, such as drug uptake and deactivation, as well as changes in DNA repair. Human copper transporter (Ctr1) is involved in the cellular uptake of cisplatin, and relatively low Ctr1 expression levels are found in some cisplatin-resistant cells.[4a,24] It was previously observed that Ctr1 expression in cultured human ovarian carcinoma cells is down regulated in the presence of cisplatin: a decrease in Ctr1 concentration was detected within 1 minute of exposure of these cells to cisplatin.[25] The reduction in the expression of Ctr1 is considered to be associated with acquired resistance to platinum-based drugs.[4b]

Herein, relatively low levels of Ctr1 was detected in cisplatin-resistant cells A549R. This low level of Ctr1 causes A549R cells to have approximately half as much cellular uptake of cisplatin relative to that of A549 cells. The Pt-PEG-GNRs conjugate facilitates the delivery of platinum drugs into cells through endocytosis, thus avoiding its reliance on Ctr1 for cellular uptake. Consequently, the uptake of the platinum drug, in the form of a Pt-PEG-GNRs conjugate, into A549R cells is approximately 4.7 times higher than the uptake of cisplatin; this additional pathway also means that it avoids the problem of drug resistance caused by low levels of Ctr1.

The efficiency of DNA platination determines the drug efficacy, because DNA is the ultimate drug target of platinum-based drugs. However, most cellular platinum does not reach its target DNA in the nucleus because platinum(II) reacts faster with thiol-rich molecules (mainly GSH and MT) in the cytoplasm than with DNA. GSH is the most abundant thiol-containing molecule in cells (0.5–10 mM). MT is composed of 61 or 62 amino acids and, of these, 20 or 21 are cysteine.[26] The overexpression of MT in human ovarian cancer cells led to a sevenfold increase in cisplatin resistence.[3] The reactions of cisplatin with GSH and MT lead to a reduction in DNA platination, thus reducing the drug efficacy. In addition, the products of the reaction of GSH with cisplatin are readily excreted by a glutathione S-conjugated export pump.[6] A relatively high GSH concentration was detected in some cisplatin-resistant cells and other cells did not exhibit a clear correlation between drug resistance and GSH concentration.[5]

Herein, relatively high concentrations of both GSH and MT were detected in A549R cells. Furthermore, lowering of the concentration of either GSH or MT led to an enhancement in the drug efficacy of cisplatin in A549R cells, thus suggesting that there is a correlation between drug resistance and the concentration of GSH and MT in the cells that were used in this work. The reactions of the platinum(IV) prodrug with GSH and MT are much slower than the corresponding reactions of cisplatin. This result suggests that the platinum(IV) prodrug that is delivered in the form of PEG-GNRs is more resistant to cellular deactivation.

Because of its enhanced drug uptake and its decreased propensity toward cellular deactivation, the Pt-PEG-GNRs conjugate avoids the corresponding types of resistance. There are still other effects that may contribute to cisplatin resistance, including enhanced DNA repair and drug efflux. The Pt-PEG-GNRs conjugate is more cytotoxic to cisplatin-resistant cells A549R than normal cancer cells A549, although the associated resistant factor (1.3) is much lower than the corresponding factor associated with cisplatin (7.1). Drug resistance might be further reduced by the incorporation of more functional groups into the conjugates, which could be more effective for cisplatin-resistant tumors.

There is broad consensus that a new generation of platinum-based antitumor drugs, which circumvent the problem of drug resistance and decrease side effects, are needed. Several new platinum-containing compounds, such as picoplatin and satraplatin, have entered clinical trials, although

they do not have significant advantages over currently used platinum drugs.[23] On the other hand, the conjugation of platinum compounds to drug delivery agents represents a novel approach that has attracted great attention in recent years.[27] There is hope that these conjugates will have increased efficacy and cause less side effects relative to current drugs. Lipid-encapsulated cisplatin causes less side effects than cisplatin, and has entered phase III clinical trials.[28] The use of nanosized carriers increases drug efficacy in many different ways. These delivery systems exhibit an enhanced permeability and retention (EPR) effect, that is, they selectively accumulate in cancer tissue.[11b] Additionally, a large variety of nanovehicle materials can be used as biocompatible carriers that convey special therapeutic properties that allows the drug to be used in photothermal therapy[29] and tumor targeting through pH sensing.[30] Drug delivery systems can be further conjugated with other functional molecules, for example, folic acid or antibodies, which can be tethered to the nanovehicles to facilitate targeting of cancer cells.[10,31] The encapsulation of siRNA within the conjugate formed between the drug and the drug delivery agent, can lead to the interference of certain cell functions to enhance drug activity.[17] Herein, we have demonstrated that the use of drug delivery agents can also combat drug resistance, which is a more pressing challenge than drug efficacy in the case of platinum-based drugs.

In conclusion, we have reported that a platinum(IV) prodrug conjugated with a PEGylated GNRs delivery agent is not affected by the types of cellular resistance that affect other platinum-based drugs. We have shown that cisplatin resistance is associated with low expression of Ctr1 and high concentrations of GSH and MT in the cisplatin-resistant cells. The uptake of the Pt-PEG-GNRs conjugate into cells is not affected by low expression levels of Ctr1. The endocytosis of the Pt-PEG-GNRs conjugate causes it to have a higher cellular uptake into cisplatin-resistant cancer cells than nonconjugated platinum drugs. The platinum(IV) prodrug that is delivered in the form of the Pt-PEG-GNRs conjugate is more inert than cisplatin in its interaction with the drug deactivation agents glutathione and metallothionein, which are found in high concentrations in the cisplatin-resistant cells A549R. Therefore, this Pt-PEG-GNRs conjugate can overcome cellular resistance associated with deactivation. Our investigations have also contributed to the understanding of cisplatin resistance, which should aid in the design of delivery systems geared toward overcoming drug resistance.

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