Metallofullerene nanoparticles circumvent tumor resistance to cisplatin by reactivating endocytosis

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Cisplatin is a chemotherapeutic drug commonly used in clinics. However, acquired resistance confines its application in chemotherapy. To overcome the acquired resistance to cisplatin, it is reasoned, based on our previous findings of mediation of cellular responses by [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} nanoparticles, that [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} may reverse tumor resistance to cisplatin by reactivating the impaired endocytosis of cisplatin-resistant human prostate cancer (CP-r) cells. Here we report that exposure of the CP-r PC-3-luc cells to cisplatin in the presence of nontoxic [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} not only decreased the number of surviving CP-r cells but also inhibited growth of the CP-r tumors in athymic nude mice as measured by both optical and MRI. Labeling the CP-r PC-3 cells with transferrin, an endocytotic marker, demonstrated that pretreatment of the CP-r PC-3-luc cells with [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} enhanced intracellular accumulation of cisplatin and formation of cisplatin-DNA adducts by restoring the defective endocytosis of the CP-r cancer cells. The results suggest that [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} nanoparticles overcome tumor resistance to cisplatin by increasing its intracellular accumulation through the mechanism of restoring defective endocytosis. The technology can be extended to other challenges related to multidrug resistance often found in cancer treatments.

As a major chemotherapeutic agent for tumor treatment, cisplatin remains a cornerstone of the present-day chemotherapy regimes against not only epithelial malignancies but also a number of metastatic and advanced malignancies (1, 2). However, because of high toxicity and easy development of drug resistance, successful treatment with cisplatin often is limited (3, 4). Following the discovery of ATP-binding cassette (ABC) transporters and their roles in drug resistance in various types of tumors (5), much research has been done to explore the relationship between ABC transporter activity and specific chemotherapeutics, including cisplatin. Because no ABC transporter has been identified for “pumping” cisplatin out of cisplatin-resistant human prostate cancer (CP-r) cells (6–8), it would be difficult to sensitize CP-r cells by using any known strategy that targets resistant cancer cells by inhibiting multidrug resistance (MDR)–associated proteins on plasma membrane of the CP-r cells. Diffusion has been considered as a pathway for cisplatin to penetrate plasma membrane. Recently, studies have indicated that cisplatin entered cells by endocytosis and other mechanisms (9–12).

To increase susceptibility of cancer cells to cisplatin, i.e., to reverse drug resistance, many efforts have been made through chemical modification, gene therapy, vector delivery, and other means (2, 9, 13). Combination of traditional chemotherapy with nanotechnology may provide a promising alternative for novel cancer treatments. The use of nanoparticles to sensitize tumor cells to cisplatin in vitro and in vivo has been described recently (14–16). In these studies, cisplatin-encapsulated nanoparticles were used to control release of cisplatin into the CP-r cells (15), and the effects were cell-line specific (16). Nanoparticles have potential for a wide range of biomedical and biotechnological applications. Fullerene molecules, the third form of pure carbon in addition to the diamond and graphite forms, have attracted much attention to their biomedical applications (17). It has been demonstrated that fullerenes can generate singlet oxygen and suppress tumor growth without damage to normal skin of the mice exposed to visible light (18). We have found that metallofullerene nanoparticles penetrate plasma membrane of tumor cells and result, to some degree, in shrinkage of solid tumors in vivo (19, 20). As a proof of concept, we have demonstrated that the metallofullerene nanoparticles, formulated as [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+}, are able to effectively inhibit proliferation of solid tumors and to decrease the activities of those enzymes responsible for catalyzing the production of reactive oxygen species in vivo (20–22). In addition, [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} nanoparticles did not show significant side effects in vivo. In the present study, we showed that multihydroxylated metallofullerene nanoparticles ([Gd@C\textsubscript{82}–(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+}) could reactivate the defective endocytosis of cisplatin in the CP-r cells and cause accumulation of intracellular cisplatin in the CP-r cells. Consequently, tumor resistance to cisplatin was circumvented by treatment with a combination of [Gd@C\textsubscript{82}–(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} with cisplatin both in vitro and in vivo.

Results and Discussion

Physical Properties and Characterization of [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} nanoparticles. Metallofullerenes with gadolinium (Gd) have been demonstrated to be capable of effectively enhancing magnetic resonance image contrast (23). [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+}, synthesized by our group, has been verified as a new generation of highly efficient contrast agents for magnetic resonance imaging (MRI) (24–27). It is a hydroxylated fullerene forming a cage encapsulating gadolinium inside (Fig. S1). The multihydroxylated amphiphilic metallofullerene [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsuperscript{2}\textsuperscript{+} self-assembles in aqueous solution to form nanoparticles. These nanoparticles do


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not exist as individual molecules or molecular ions but as aggregates. The average size of [Gd@C_{82}(OH)_{22}]_n aggregates is 50 ± 12 nm, measured by dynamic light scattering (DSL). The particles form nonuniform quasi-spherical shapes. The [Gd@C_{82}(OH)_{22}]_n nanoparticles self-assemble into a hexagonal microstructures that are visible by scanning electron microscopy (Fig. 1A and B). This multihydroxylated metallofullerene does not show significant in vivo toxicity as evidenced by morphological analysis of HE staining various tissues of tumor-bearing animals (Fig. 1C). Protonation or deprotonation of [Gd@C_{82}(OH)_{22}], varies the negative charges of the hydroxyl groups, which further modulates the repulsive static-electric forces among the [Gd@C_{82}(OH)_{22}], species (28). These repulsive static-electric forces could affect the size of hydrophobic-force-induced self-assembly of [Gd@C_{82}(OH)_{22}]_n nanoparticles. The size determines the nanoparticle-mediated cellular response, and particle size at 40–60 nm appears to have the greatest effects (29).

Development of PC-3 luc Cells Resistant to Cisplatin. In this study, we proposed that [Gd@C_{82}(OH)_{22}]_n nanoparticles reverse tumor resistance by enhancing the endocytosis of cisplatin via nanoparticle-mediated penetration through the plasma membrane of the CP-r cells. To test this hypothesis in vivo and in vitro, human prostate cancer PC-3-luc cells were chosen to establish cisplatin-resistant (CP-r) cells. Luciferase expressed in PC-3 cells was used as a marker to facilitate optical imaging of the CP-r cells. We found that the CP-r PC-3-luc colonies appeared at a frequency of ≈1/10^6 in the presence of cisplatin. The CP-r variants were identified, isolated, and cloned after the parental cisplatin-sensitive (CP-s) cells were exposed to 1 μg/mL cisplatin for 2 months (Fig. S2). To measure cytotoxicity, we cultured the resistant cells in cisplatin-free medium for 10 days before conducting the cisplatin cytotoxicity assay. The resistant index (RI) of the CP-r cells was found to be 6-fold higher than that of the parental CP-s cells (Fig. 2A).

[Gd@C_{82}(OH)_{22}]_n Sensitized CP-r Cells to Cisplatin Treatment in Vitro and in Vivo. The CP-r cells were treated with various concentrations of [Gd@C_{82}(OH)_{22}]_n nanoparticles (1–50 μM) with or without 1 μg/mL cisplatin (Fig. 2B). The IC_{50} value of cisplatin against parental CP-s cells was 1 μg/mL, the concentration that was used for inducing the CP-r variants and was virtually nontoxic to the CP-r cells. Treatment with [Gd@C_{82}(OH)_{22}]_n nanoparticles alone had no significant effect on either CP-s or CP-r cells at concentrations lower than 10 μM (Fig. S2). Treatment with nanoparticles alone did not change proliferation of the CP-r PC-3-luc cells, nor did cisplatin at 1 μg/mL dosage. However, the number of surviving CP-r cells decreased by ~30% after a combined treatment of 1 μg/mL cisplatin and [Gd@C_{82}(OH)_{22}]_n nanoparticles compared with the nanoparticle or cisplatin treatment alone (Fig. 2B). To further confirm the ability of nanoparticles to overcome cisplatin resistance in vivo, we developed a solid tumor mouse model by s.c. injection of 1 × 10^7 cells in 100 μL sterile saline into the left flank (CP-r PC-3-luc cells) and right flank (CP-s PC-3-luc cells) of athymic nude mice (male, 8–10 weeks old), respectively. Fourteen days after injection, CP-s and CP-r PC-3-luc cells grew into solid tumors having similar sizes. The fluorescence intensities of these tumors were measured by optical imaging. The mice were randomly divided into four groups (n = 5 per group) with different treatments for 4 weeks: (i) i.p. injection of 1.0 μmol/kg [Gd@C_{82}(OH)_{22}]_n nanoparticles daily (NP group); (ii) i.p. injection of 10 mg/kg cisplatin two times per

![Fig. 1. Characterization of [Gd@C_{82}(OH)_{22}]_n nanoparticles. (A) [Gd@C_{82}(OH)_{22}]_n nanoparticles characterized by scanning electron microscopy (SEM). (Scale bar, 100 nm.) (B) Size of [Gd@C_{82}(OH)_{22}]_n nanoparticles, as measured by dynamic light scattering (DSL). (C) Representative histological HE staining of various organ tissues from mice treated with [Gd@C_{82}(OH)_{22}]_n nanoparticles or saline. (Scale bar, 50 μm.)](image-url)
week (cisplatin group), the same administration schedule used for treatment of cancer patients (to allow comparison across groups, on the day when cisplatin was not injected, 0.15 mL sterile saline was injected into each mouse); (iii) i.p. injection of both cisplatin (10 mg/kg, 2 times per week) and [Gd@C₇@C(OH)₂]ₙ nanoparticles (1.0 μmol/kg, daily) (cisplatin+NP); and (iv) i.p. injection of saline solution alone (0.15 mL, daily). All mice were weighed daily. Tumor weight was calculated by caliper measurement and tumor volume was determined by MRI analysis (Fig. 2C). The results indicated that [Gd@C₇@C(OH)₂]ₙ nanoparticles improved the inhibition of CP-r tumors growth by cisplatin.

As luciferase is expressed in both the CP-s and CP-r PC-3-luc cells, these prostate cancer cells growing as s.c. tumors on the flanks of the mice can be readily optically imaged in vivo. Representative images of the mice following the 4-week treatment protocol confirmed similarities in CP-s and CP-r tumor proliferation between groups treated with saline or NP. Cisplatin alone was highly efficient at inhibiting CP-s PC-3-luc tumors in vivo but had virtually no effect on the growth of CP-r PC-3-luc tumors in vivo. In contrast, cisplatin combined with the nanoparticles (cisplatin+NP group) inhibited growth of both the cisplatin-sensitive PC-3-luc tumors (as expected) and the cisplatin-resistant PC-3-luc tumors. The sizes of the CP-s PC-3-luc and CP-r PC-3-luc tumors after the combined treatment were very similar (Fig. 2C and Fig. 3A). MRI images also showed reduced sizes of

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**Fig. 2.** [Gd@C₇@C(OH)₂]ₙ nanoparticles induced sensitivity enhancement of CP-r cells and tumors to cisplatin. (A) Cellular viability curves of CP-s and CP-r PC-3-luc cells treated with cisplatin. Cells (3 x 10⁵) were plated in 96-well plates with 100 μL medium per well. After 6 h, various concentrations of cisplatin in 50 μL medium were added, and the cells were incubated at 37 °C for 3 days. Before the cell viability measurement, 10 μL of MTT solution (Kumamoto) was added to each well and incubated for 2 h. Cell viability was measured using spectrophotometry. (B) Measurement of [Gd@C₇@C(OH)₂]ₙ nanoparticle cytotoxicity in CP-r cells treated or untreated with cisplatin (1 μg/mL). The protocol was the same as described in Fig. 2A. Results shown are the average of three different experiments. (C) Tumor weight was measured by caliper quantification (tumor weight (mg) = tumor density (1 mg/mm³) x length (mm) x [width (mm)²/2]) [i], CP-r PC-3-luc; ii, CP-r PC-3-luc). Nanoparticles (20 μM) were used. Tumor volumes were derived from consecutive multiple MRI images using Image J software (iii, CP-s PC-3-luc; iv, CP-r PC-3-luc). Statistical analysis of Inset C using ANOVA shows a significant difference between two sets of data when P < 0.05.

**Fig. 3.** Sensitizing CP-r tumors to cisplatin treatment by [Gd@C₇@C(OH)₂]ₙ nanoparticles in vivo. (A) Optical imaging for comparison of the sizes of CP-s and CP-r PC-3-luc tumors treated with either [Gd@C₇@C(OH)₂]ₙ nanoparticles (NP, 20 μM), cisplatin, cisplatin plus nanoparticles (cisplatin + NP), or saline solution alone as a control. (B) MRI images of CP-s and CP-r PC-3-luc tumors after 4 weeks of various treatments described in A. (Right) CP-s tumor. (Left) CP-r tumor. (C) Weights of tumors were measured at the end of 4 weeks’ treatment. MRI images were analyzed by Image J software for tumor volume. Tumor weight was calculated by conversion of volume to weight. NP (20 μM) treatment significantly enhanced the ability of cisplatin to inhibit growth of CP-r tumors.
CP-r and CP-s tumors following the cisplatin plus nanoparticle treatment. A series of multislice MRI images were used to study tumor morphology (shape and location) (Fig. 3B). The tumor volume was calculated based on these consecutive MRI images, after which the tumor weight was also statistically calculated and deduced (Fig. 3C). The effect of the nanoparticles on cisplatin treatment was clearly demonstrated in Fig. 3A and B and as shown by the images of the individual groups (Fig. S3). These studies confirmed that [Gd@C₈₂(OH)₂₂]n nanoparticles increased the sensitivity of CP-r PC-3-luc tumors to cisplatin in vivo.

Intracellular Cisplatin Accumulation by Reactivating the Defective Endocytosis in CP-r Cells. Nanoparticles have been used to deliver drugs or genes for cancer treatment using the unique characteristics associated with the nanoscale size (30, 31). It is known that tumors become resistant to cisplatin, partially because of reduced uptake of cisplatin resulting from an endocytic defect following defective formation of the endocytic recycling compartment (ERC) (32). Although the specific molecular or regulatory defect responsible for the reduced accumulation of cisplatin in the CP-r cells has not been identified, several proteins have been found to be associated with this phenotype (33, 34). To investigate how metallofullerene [Gd@C₈₂(OH)₂₂]n nanoparticles increase cisplatin toxicity in the CP-r cancer cells, the CP-s and CP-r PC-3-luc cells were labeled with Texas Red–transferrin, a bona fide marker of the endocytic recycling pathway. In the parental CP-s PC-3-luc cells, we observed that transferrin localized close to the nucleus as a bright patch of fluorescence. Unlike the pattern observed in the CP-s cells, transferrin was distributed more peripherally in the CP-r cells as discrete punctate structures. In general, there was less intracellular transferrin in CP-r cells compared with CP-s cells (Fig. 4).

![Confocal microscopy images](image_url)

Fig. 4. [Gd@C₈₂(OH)₂₂]n nanoparticles increased transferrin-mediated endocytosis. Texas Red–transferrin conjugates were used to label CP-s and CP-r PC-3-luc cells for endocytotic measurement. Cisplatin concentration was 1 μg/mL, and [Gd@C₈₂(OH)₂₂]n was 20 μM. (A–E) Confocal microscopy images. (A) CP-s cells. (B) CP-r cells. (C) CP-r cells treated with cisplatin. (D) CP-r cells treated with nanoparticles. (E) CP-r cells treated with cisplatin and nanoparticles. (F) ICP-MS/MS measurements of DNA adducts in CP-s treated cisplatin; CP-r cells treated with cisplatin; CP-r cells treated with cisplatin with/without [Gd@C₈₂(OH)₂₂]n. Data presented are the integrated ICP-MS signals as mean values of yield measurements for various DNA adducts measured by ICP-MS in cells after various treatments. (G) CP-s and CP-r cells were labeled with Texas Red–transferrin for endocytotic measurement. Fluorescence intensity of internalized Texas Red–transferrin was measured by spectrophotometer at an excitation wavelength of 595 nm and emission wavelength of 620 nm. Results shown are mean of three different experiments. Comparisons between groups were evaluated by one-way ANOVA. There was a statistically significant difference between the CP-R cells and CP-R cells treated with nanoparticles.

![ICP-MS/MS measurements of DNA adducts](image_url)

Fig. 5. [Gd@C₈₂(OH)₂₂]n nanoparticles enhance CP-r cell sensitivity to cisplatin. (Top) Normal endocytosis that includes binding of ligands (e.g., transferrin: Tf) to their Tf-receptors on plasma membrane followed by binding, ingestion into cytoplasm, intracellular vesicle transportation, payload release, and vesicle recycle. (Middle) Receptor-mediated endocytosis of cisplatin in the CP-r cells. Because of defective endocytosis, there is less intracellular accumulation of cisplatin and therefore less formation of cisplatin-DNA adducts in the CP-r cells. (Bottom) Nanoparticle-activated endocytosis in the CP-r cells, resulting in more efficient transportation of cisplatin-containing vesicles and more cisplatin binding to nucleic acid to sensitize the CP-r cells.
4). The difference in transferrin distribution between the CP-r and CP-s cells may result from defective endocytosis of the CP-r cells as demonstrated by our previous studies (32, 35). Cisplatin passes through the plasma membrane and enters cells in part by endocytosis (36–38). We believe that multihydroxylated metallofullerene [Gd@C_{82}(OH)_{22}]_{n} nanoparticles reactivate endocytosis through its unique nanoscale properties. The reasoning was confirmed by increased nanoparticle-mediated endocytosis in CP-r cells compared with CP-s cells treated with cisplatin alone (Fig. 4 A–E and G). This was also confirmed by increased cisplatin adducts in NP+CP-treated CP-r cells measured using inductively coupled plasma mass spectroscopy (ICP-MS) in the negative ion mode (Fig. 4F). Inhibition of endocytosis by cytochalasin D of Bafllomyisin A1 decreased the nanoparticle-activated cisplatin intracellular accumulation (Figs. S4 and S5). This suggests that [Gd@C_{82}(OH)_{22}]_{n} nanoparticles restore endocytotoxic function and increase intracellular cisplatin accumulation in CP-r PC-3-luc variants.

**Summary.** Cisplatin is commonly used to treat prostate cancer at early clinical stages. However, the ability of prostate cancer cells to become resistant to cisplatin remains a significant impediment to successful chemotherapy of prostate cancer patients. It is known that cisplatin enters cells by different pathways (39, 40), and endocytosis (14–16), which may be cell-line dependent. Until now, the generally accepted mechanisms by which cells become resistant to cisplatin have been (i) enhanced repair ability and tolerance of nuclear lesions leading to apoptosis; (ii) increased detoxification of cisplatin by metallothionein and glutathione; and (iii) diminished accumulation of cisplatin (2, 6, 8). Of these mechanisms, accumulation defect seems to be dominant in various cell lines (8, 43). As mentioned above, CP-r cells have a defect in endocytosis (32), which may lead to diminished accumulation of cisplatin and confer the cells on resistance against extracellular cisplatin. The restored endocytosis of transferrin by [Gd@C_{82}(OH)_{22}]_{n} nanoparticles indicates that the nanoparticles can circumvent the acquired resistance of the CP-r PC-3-luc variants by enhancing uptake of cisplatin (Fig. 5 and Movie S1). Cisplatin sensitivity was also increased by nanoparticles in CP-r KB-3-1 and BEL 7404 cells (Fig. S6). Because intracellular accumulation of cisplatin is reduced in CP-r cells due to a pleiotropic defect, other mechanisms such as reduced fluidity of plasma membrane or altered cytoskeleton may also contribute to the [Gd@C_{82}(OH)_{22}]_{n} nanoparticle reversal of the cisplatin resistance in vitro and in vivo (9, 10). Bioeffect of nanoparticles was measured with LIVE/DEAD Viability/Cytotoxicity by using confocal microscopy (Fig. S7). These nanoparticles are surprisingly nontoxic to cancer cells in vitro, yet can successfully enhance the growth inhibition by cisplatin on CP-r tumor in vivo. The in vivo enhancing effect of the nanoparticles may also partially contribute to their penetration and accumulation in the leaky vasculature of tumors (44). In conclusion, using nanomaterials to overcome the drug resistance of malignant tumors could lead to new therapies for cancer patients. This provides a promising chemotherapeutic method to treat tumors at lower, nontoxic dose levels.

**Materials and Methods.** 

[Gd@C_{82}(OH)_{22}]_{n} Nanoparticles. Gd@C_{82} was synthesized by the method of Krâtschmer-Huffman (45) and extracted under high temperature and high pressure (46). A high performance liquid chromatography (LC908-C60, Japan Analytical Industry Co.) coupled with SPPB and Buckysup columns (Nacalai) was used to separate Gd@C_{82} from other metallofullerenes (47). The final purity of the Gd@C_{82} was greater than 99.5% as measured by MALDI-TOF-MS (Auto-Flex, Bruker). Hydroxylation was performed by alkaline reaction (47, 48). The Gd@C_{82}(OH)_{n} was isolated using Sephadex G-25 column chromatography (5 x 50 cm) with an eluent of neutralized water (47). A fraction of the eluate was collected over a short time interval to ensure that the hydroxyl number distribution was in a narrow range.

MRL. Athymic nude mice were anesthetized using 2% isoflurane, and the tumors were positioned at the center of the RF coil. The physiologic conditions of the animals were monitored using a respiratory monitoring device during the scanning. The animals were scanned using a Bruker 400 MHz, 89-mm NMR spectrometer. After a pilot scan for determining the region of interest, a multislice spin–echo sequence was used with repetition time (TR) of 2 s, echo time (TE) of 25 ms, and slice thickness of 1 mm. The tumor sizes were calculated using Image J software (National Institutes of Health).

**Optical imaging.** Luciferase was used as a marker in optical images to evaluate the growth of tumors by using the Xenogen IVIS Imaging System (Caliper Life Sciences). Anesthetized mice were i.p. injected with 75 mg/kg D-Luciferin (Caliper Life Sciences) in PBS. Eight minutes after injection, bioluminescence images were acquired using optical imaging. The acquisition time was 0.1 s. Images were set at the indicated pseudocolor scale to show relative bioluminescence changes over time.

**Endocytosis of Texas Red-Transferrin.** The CP-s and CP-r PC-3-luc cells were labeled with 10 μg/mL Texas Red-transferrin for 20 min at 37 °C. Texas Red-transferrin was removed by a PBS rinse. Cells labeled with Texas Red-transferrin were cultured in RPMI medium with 1 μg/mL cisplatin for 40 min, fixed by 70% ethanol in PBS, then imaged by spinning disk confocal microscope (Yokogawa). Gd@C_{82}(OH)_{22} nanoparticles (20 μM) and cisplatin (1 μg/mL) were used in each individual experiment to measure the endocytosis of Texas Red-transferrin.

**DNA Adducts Measured by Inductively Coupled Plasma Mass Spectroscopy.** The CP-s and CP-r PC-3-luc cells were grown at a density of 10^{5} cells/mL. Cells were exposed to 1 μg/mL cisplatin for 40 min with or without 20 μM Gd@C_{82}(OH)_{22} nanoparticles. Cells were trypsinized, washed with PBS, and centrifuged for 6 min at 1,000 x g. The DNA was then extracted and digested as reported in the previous studies (49, 50). The mass spectrometer used was a Thermo Bioxytoms X7 inductively coupled plasma mass spectroscopy (ICP-MS) instrument. Measurements were made in the negative ion mode with the source temperature.

**Experimental Details.** Experimental details on preparation of [Gd@C_{82}(OH)_{22}]_{n} nanoparticles, establishment of the cisplatin-resistant cells, transferrin-mediated endocytosis, and cell proliferation assay are included in SI Text.

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Supporting Information

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SI Materials and Methods

Preparation of Gd@C_{82}(OH)_{22}. Gadolinium metallofullerenes (Gd@C\textsubscript{82}) were synthesized by arc-burning composite rods consisting of Gd\textsubscript{2}O\textsubscript{3} and graphite in a He atmosphere. The Gd@C\textsubscript{82} was separated by a two-step high-performance liquid chromatography (HPLC; LC908-C60, Japan Analytical Industry Co.) technique with COSMOSIL 5PBB guard columns and Buckyprep guard columns (Nacalai Tesque). The purity of the final Gd@C\textsubscript{82} product was greater than 99.5%. Gd@C\textsubscript{82}(OH)\textsubscript{x} was synthesized using an alkaline reaction. Briefly, Gd@C\textsubscript{82} toluene solution was first mixed with a 50% NaOH aqueous solution. A few drops of 40% tetrabutylammonium hydroxide (TBAH) were added to the mixture as catalyst. After vigorous stirring at room temperature, a brown sludge precipitate appeared and the toluene solution became colorless. Remaining TBAH and NaOH were removed with water and methanol. The brown precipitate was then dissolved in deionized water with continuous stirring for 24 h until the solution became reddish brown. Finally, the solution was purified by a Sephadex G-25 column chromatography (5 × 50 cm\textsuperscript{2}) with distilled water as an eluent. To obtain a final Gd@C_{82}(OH)\textsubscript{x} product with a narrow range of attached hydroxyl groups, the fraction (eluative) was collected in several fractions. The molecular weight was determined by elemental analysis, MALDI-TOF-MS, and x-ray photoemission spectroscopy. The number of attached hydroxy groups for Gd@C\textsubscript{82}(OH)\textsubscript{x} was determined to be 22 ± 2. The dry Gd@C\textsubscript{82}(OH)\textsubscript{22} powder was weighed and dissolved in 0.9% sterile saline solution.

Cell Lines and Establishment of Cisplatin-Resistant Cells. PC-3-luc cells, a human prostate cancer cell line transfected with luciferase gene, were the parent cell line for the selection of drug-resistant variants. The PC-3-luc cells were maintained in RPMI medium 1640 supplemented with 10% FBS (HyClone) and 100 U/mL of penicillin and streptomycin each (Invitrogen). To establish cisplatin-resistant cells, PC-3 luc cells were first cultured in RPMI medium with 0.5 μg/mL cisplatin. The surviving cells were then maintained in medium containing 1 μg/mL cisplatin for 4 weeks until the growth of the cells was stable enough to form solid colonies. Individual colonies were harvested as cisplatin-resistant PC-3-luc cells. The cisplatin-resistant variants were cultured with 1 μg/mL cisplatin to maintain cellular resistance to cisplatin. All cell lines were grown as monolayer cultures at 37 °C.

Animals. The animals used were 8–to 10-week-old male athymic nude mice (Harlan). The mice were kept in a pathogen-free (SPF) animal facility under a controlled environment (22 ± 1 °C, 60 ± 10% humidity, and a 12-h light/dark cycle) with free access to food and water. All animal experiments were performed in compliance with the local ethics committee guidelines.

Cytotoxicity Assay. Cytotoxicity was determined by the viability of cells with MTT assay. Cells were seeded in 96-well plates at 3 × 10\textsuperscript{3} cells with 100 μL medium per well. After 6 h incubation, the test compounds dissolved in 50 μL medium were added to each well, and the cells were incubated for 3 days. Next, 10 μL MTT solutions (5 mg/mL in PBS) were added to each well and incubated at 37 °C in 5% CO\textsubscript{2} for 2 h. The medium in the wells was then aspirated gently, and 150 μL DMSO was added to each well to dissolve the formazan crystals. After shaking for 10 min, the absorbance of each well at 490 nm was measured using a multimode plate reader (Infinite M200, Tecan Group). Results were expressed as the mean percentage of cell viability relative to untreated cells. To measure the adjuvant effect of metallofullerene nanoparticles, 0.2 μM cytochalasin D (MW:507.62) and 0.01μM bafilomycin A1 (MW:622.83, Sigma-Aldrich) as intracellular endocytotic vesicle trafficking inhibitors were used to pretreat cells for 30 min before nanoparticles and/or cisplatin treatment for cytotoxic measurement. The intracellular platinum amount was measured with inductively coupled plasma mass spectroscopy (ICP-MS) technique (Elemental x7 ICP-MS, Thermo Fisher Scientific).

Confocal Microscopy for Measurement of Viability Using LIVE/DEAD Viability/Cytotoxicity Kit-Labeled Cells. The cytotoxicity measurement of Gd@C\textsubscript{82}(OH)\textsubscript{22} nanoparticles was compared to cisplatin in CP-s and CP-r PC-3 cells exposed to nanoparticles and/or cisplatin for 24 h. Briefly, ≈.2.0 × 10\textsuperscript{4} cells were plated onto 30-mm glass microscope coverslip and cultured at 37 °C overnight until the monolayer was confluent. The cells were washed with PBS (pH 7.4). The cell monolayer was then incubated with serum-free medium containing different concentrations Gd@C\textsubscript{82}(OH)\textsubscript{22} nanoparticles and/or cisplatin for 24 h. The cytotoxicity was measured using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Rockville, MD) for staining with calcein AM (live cells fluoresce green) and ethidium homodimer (dead cells fluoresce red) and viewed by confocal microscopy (Ultraview ERS, Perkin-Elmer).

Measurement of Transferrin-Mediated Endocytosis. Cisplatin-sensitive and cisplatin-resistant PC-3-luc cells were labeled with 10 μg/mL Texas Red-transferrin for 20 min at 37 °C. Free Texas Red-transferrin was removed by washing three times with PBS. Cells labeled with Texas Red-transferrin were incubated in RPMI medium 1640 at 37 °C for 30 min, and labeled cells were suspended by detachment with trypsin/EDTA without calcium. Suspended cells were collected collected by centrifugation. Cell pellets were resuspended in PBS and fixed with 70% ethanol in PBS. In each individual experiment, 20 nmol/mL [Gd@C\textsubscript{82}(OH)\textsubscript{22}]\textsubscript{n} nanoparticles and 1 μg/mL cisplatin were used to measure the endocytosis of Texas Red-transferrin. Cisplatin-sensitive and cisplatin-resistant cells were counted, and the same numbers of cells labeled with Texas Red–transferrin were measured by spectrophotometer.
Fig. S1. Formation and structures of Gd@C$_{82}$, Gd@C$_{82}$(OH)$_x$, Gd@C$_{82}$(OH)$_{22}$, and Gd@C$_{82}$(OH)$_{22}$ in saline solution.

Fig. S2. Effects of [Gd@C$_{82}$(OH)$_{22}$]$_n$ nanoparticles on cisplatin cytotoxicity (1 $\mu$g/mL). CP-s cells (3 $\times$ 10$^3$) were plated in 96-well plates with 100 $\mu$L medium per well. After 6 h, cisplatin (in 50 $\mu$L medium) was added, and the cells were incubated at 37°C for 3 days. Next, 10 $\mu$L of MTT solution (Kumamoto) was added per well and incubated for $\sim$2 h. Cell viability was measured using a spectrophotometer at the wavelength suggested in the kit’s protocol. The results shown are the mean of three different experiments. A significant difference was assumed to exist when $P < 0.05$.

Fig. S3. Morphological observation of tumor after 4 weeks treatment. Animals treated with the following: (A) Gd@C$_{82}$(OH)$_{22}$$_n$ nanoparticles (NP); (B) cisplatin; (C) cisplatin/nanoparticles (cisplatin + NP); and (D) saline as control. (Right) Drug-sensitive tumor. (Left) Drug-resistant tumor. The experiment is representative of three individual experiments.
Fig. S4. Optimized concentration of Cytochalasin D and Baflomysin A1 without significant toxicity on CP-s and CP-r PC-3 Cells. CP-r PC-3 cells treated with different concentrations of Cytochalasin D (A) and Baflomysin A1 (B), and CP-s PC-3 cells treated with different concentration of Cytochalasin D (C) and Baflomysin A1 (D). Briefly, cells were seeded in the 96-well plastic disk by 5,000 cells/well. After 24 h culture, cells were incubated with cytochalasin D or baflomysin A1 at 37 °C for 30 min. Cytochalasin D (MW: 507.62, 2,000 μM stock solution) was used to treat cells at 0.002, 0.02, 0.2, 2, or 5 μM. Baflomycin A1 (MW: 622.83, 160 μM stock concentration), at 0.0001, 0.001, 0.01, and 0.1, 0.5 μM, was used to inhibit endocytosis. After washing with PBS three times, the cells were incubated with cisplatin, nanoparticles, or cisplatin plus nanoparticles at 37 °C for 48 h. The viability was examined using the MTT method. Briefly, 10 μL of MTT solution was added to each well. Cell viability was measured using a spectrometer at 490 nm. Cytochalasin D and Baflomycin A1 prohibited cell proliferation with increasing concentration. For further experimental treatment, 0.2 μM cytochalasin D and 0.01 μM baflomycin A1 were selected based on their effects on cell viability assay. The result shown is the mean of five individual experiments.
Fig. S5. CPs and CP-r PC-3 cell viability was affected by increased intracellular cisplatin accumulation due to Cytochalasin D or Bafilomycin A1 inhibiting endocytosis. Viability of CP-r PC-3 cells was measured following exposure to cytochalasin D (A) and Bafilomycin A1 (B). Viability of CP-s PC-3 cells was measured after exposure to cytochalasin D (C) and Bafilomycin A1 (D). Intracellular platinum concentration was measured by MALDI-TOF–MS as described in SI Materials and Methods (E). Baf, Bafilomycin A1; CP, cisplatin; Cyt, Cytochalasin D; NP, nanoparticles.
Fig. S6. KB-CP20 (A) and BEL-7404 CP-20 (B) CP-r cells were treated with nanoparticles and cisplatin for measurement of cytotoxicity. Optical absorbance of cells suspended with CCK-8 was measured using a spectrophotometer at 520 nm. Columns indicate mean; bars indicate SD. Comparisons of groups were evaluated by one-way ANOVA. When twice the SD was higher than the mean, a nonvariable test was used to evaluate the difference. A significant difference was assumed to exist when \( P < 0.05 \). Data presented represent the mean of at least three experiments. CP \( x \), \( x \) \( \mu \)g/mL cisplatin; NP20, 20 \( \mu \)mol/mL nanoparticles.

Fig. S7. Cytotoxicity of Gd@C\(_{82}\)(OH)\(_{22}\) nanoparticles was compared with cisplatin in PC-3 and PC-3 CP-r cells that were exposed to nanoparticles and/or cisplatin for 24 h. Cytotoxicity was measured using a LIVE/DEAD Viability/Cytotoxicity Kit for staining with calcein AM (live cells fluoresce green) and ethidium homodimer (dead cells fluoresce red) by confocal microscopy. Results are representative of three individual experiments.
Fig. S8. Biodistribution study of nanoparticles in tumors was measured by inductively coupled plasma mass spectroscopy (ICP-MS) instrument. For intratumor localization of the nanoparticles, \(2 \times 10^6\) CP-s and CP-r PC-3 cells (in 200 \(\mu\)L saline solution) per mouse were injected intraperitoneally (i.p.) into 5-week-old Balb/C mice (five mice per group). Each mouse was administered i.p. with a dose of 20 \(\mu\)mol \([\text{GdBC}82(\text{OH})_{22}]\) in 2 weeks. The weight of the mice was measured every 24 h. Elemental x2 ICP-MS (Thermo Fisher Scientific) was used to quantitatively measure of the amount of cisplatin (Fig. S5E) and gadolium in the isolated tumor tissue. Results represent the mean of three experiments.

Movie S1. A movie is created to make the cartoon in Fig. 5 easily understandable. This dynamic cartoon shows that cisplatin resistant cancer cells can be sensitized by increasing cisplatin intracellular concentration through nanoparticle-mediated endocytosis. This movie is divided into three dynamic cartoons. The first cartoon explains the normal endocytosis mechanism, which includes the ligands binding with receptor on plasma membrane, penetration and ingestion into cytoplasm, intracellular vesicles transportation, releasing the payload, and recycling back to the plasma membrane. The second cartoon shows the cisplatin entry into cells by receptor-mediated endocytosis. Cisplatin is trapped in endocytosis vesicles and is released during endocytotic vesicle trafficking. The defective endocytosis in CP-r cells leads to less effective cisplatin intracellular accumulation. The third cartoon defines nanoparticle-activated endocytosis, by which more cisplatin is transported and released into the cytoplasm of CP-r cells. The active cisplatin can then effectively bind with nucleic acids and sensitize CP-r cells by inhibiting cell proliferation. The movie indicates that cisplatin-resistant cancer cells can be sensitized by increasing cisplatin intracellular concentration through nanoparticle-mediated endocytosis.

Movie S1