Pt-Coated Au Nanoparticle Toxicity Is Preferentially Triggered Via Mitochondrial Nitric Oxide/Reactive Oxygen Species in Human Liver Cancer (HepG2) Cells

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ABSTRACT: Reactive nitrogen species (RNS) that are formed from the reaction of versatile nitric oxide (NO) with reactive oxygen species (ROS) have been less explored in potential cancer therapy. This may be partly due to the fewer available agents that could induce NO in cells. Here, we report platinum-coated gold nanoparticles (Pt-coated Au NPs; 27 ± 20 nm) as a strong inducer of NO (assessed by live-cell imaging under NO-specific DAR-1 probe labeling and indirectly using a Griess reagent) in human liver carcinoma (HepG2) cells. In addition to NO, this study found a critical role of ROS from mitochondrial sources in the mechanism of toxicity caused by Pt-coated Au NPs. Cotreatment with a thiol-replenishing general antioxidant NAC (N-acetyl cysteine) led to significant amelioration of oxidative stress against NP-induced toxicity. However, NAC did not exhibit as much ameliorative potential against NP-induced oxidative stress as the superoxide radical (O$_2^{\cdot-}$)-scavenging mitochondrial-specific antioxidant mito-TEMPO did. The higher protective potential of mito-TEMPO in comparison to NAC reveals mitochondrial ROS as an active mediator of NO toxicity in HepG2 cells. Moreover, the relatively unaltered NP-induced NO concentration under cotreatment of GSH modulators NAC and buthionine sulfoximine (BSO) suggested that NO production due to NP treatment is rather independent of the cellular thiols at least in HepG2 cells. Moreover, toxicity potentiation by exogenous H$_2$O$_2$ again suggested a more direct involvement of ROS/RNS in comparison to the less potentiation of toxicity due to GSH-exhausting BSO. A steeper amelioration in NP-induced NO and ROS and, consequently, cytotoxicity by mito-TEMPO in comparison to NAC reveal a pronounced role of NO and ROS via the mitochondrial pathway in the toxicity of Pt-coated Au NPs in HepG2 cells.

1. INTRODUCTION

Novel approaches and designs are needed to mitigate the intermediate and advanced stages of malignant cancer, including those of hepatocellular carcinoma, the second leading cause of cancer-related death globally. Hepatocellular carcinoma accounts for approximately 90% of among all primary liver cancer cases worldwide; the recurrence rate of hepatocellular carcinoma is almost 70% after 5 years. Most anticancer drugs, such as FDA-approved cisplatin, which lie at the core of their mechanism of action. High levels of ROS have modulatory effects on programmed cell death, which generally refers to the death associated with apoptosis, necroptosis, and autophagy. It is known that ROS also generates other reactive nitrogen species (RNS) upon reacting with the versatile molecule nitric oxide (NO) free radical. Although the potential of RNS in cancer therapy is less discussed about, RNS have been reported to exert strong neoplastic as well as antineoplastic effects in a concentration-dependent manner. Intriguingly, NO released by most chemotherapeutic compounds has been reported to sensitize liver cancer cells. Moreover, NO has been receiving attention for its potential to sensitize many types of cancers that are resistant to both chemotherapy and immunotherapy. Needless to mention that there are fewer NO-inducing agents explored so far compared to ROS-generating agents in oxidative stress-based approaches to cancer therapy. ROS modulators, therefore, have received greater recognition than RNS modulators in cancer therapy.

With the advent of nanotechnology, the landscape of biomedical sciences is changing faster than ever before. Here we report on Pt-coated Au nanoparticles (NPs) defined as particles with at least one dimension that is less than 100
nm) as a strong inducer of NO and ROS, which have emerged as strong antineoplastic agents in human liver carcinoma (HepG2) cells. HepG2 cells are a representative cellular model of hepatocellular carcinoma to carry out studies ranging from toxicity to anticancer therapy. This study was extended further by administring modulators of oxidative stress to understand the toxicity mechanism in greater detail. The combination of a general antioxidant, N-acetyl cysteine (NAC), that acts via restoring cellular antioxidants such as glutathione (GSH) and a mitochondria-specific antioxidant, mito-TEMPO (triphenylphosphonium chloride), that acts by scavenging mitochondrial O$_2$•− (superoxide anion) has been utilized to investigate whether the induced toxicity is primarily due to mitochondrial impairment or is due to some general cellular oxidative stress. It was hypothesized that any difference in the protective potentials of cellular-acting NAC and mitochondrial specific mito-TEMPO would reveal the relative contribution of mitochondrial ROS/NO to the cellular oxidative stress. It was hypothesized that any difference in the protective potentials of cellular-acting NAC and mitochondrial specific mito-TEMPO would reveal the relative contribution of mitochondrial ROS/NO to the cellular oxidative stress caused by Pt-coated Au NP. A pro-oxidant, buthionine sulfoximine (BSO), that can cause oxidative stress by inhibiting GSH biosynthesis and a direct oxidant, H$_2$O$_2$, were used to have further in-depth understanding on the active role of ROS/RNS in the NP-mediated killing potential of HepG2 cancer cells.

2. RESULTS

2.1. Pt-Coated Au NP-Induced Concentration-Dependent Cytotoxicity in HepG2 Cancer Cells. According to the supplier (Sigma-Aldrich) specification, the concentration of gold was 45.0–55.0 ppm in a product (Pt-coated Au NP) concentration of 90.0–110.0 ppm. Field emission transmission electron microscopy (FETEM, JEM-2100F, JEOL, Inc., Tokyo, Japan) measurements of the Pt-coated Au NPs confirmed the data provided by the supplier that the NPs’ average size was 27 ± 20 nm. The TEM image taken at a resolution of 50 nm (Figure 1A) appears mostly cuboidal in shape and the 5 nm image (Figure 1B) depicts matte planes found in the crystal structures and coating of NPs, respectively. The scanning electron microscopy (SEM) image is provided in Figure 1C. A ζ potential value of −28 to −43 mV and a hydrodynamic size of 64–110 nm for the Pt-coated Au NPs in complete culture media suggest a fair distribution of NPs. Figure 1D depicts the cytotoxicity profile of NPs in HepG2 cells at the indicated concentrations of NPs treated for 24 h. The concentration of Pt-coated Au NPs that caused cytotoxicity by 50% (CC50) in HepG2 cells was estimated to be 148 ± 22 ng/mL (or 0.740 ± 0.11 μM according to the molecular weight of 196.97 of an NP). Table 1 summarizes the findings from TEM, SEM, DLS, and cytotoxicity analysis.

2.2. Induction of NO Was Significantly Higher in All Treatment Groups Except the Mito-TEMPO Cotreatment Group. In the treatment groups, NO level was induced to 8-fold by Pt-coated Au NPs. NAC and mito-TEMPO cotreatments decreased NP-mediated NO induction to 1.12- and 2.63-fold, respectively, in comparison to NP induced alone (DAR-1 images in Figure 2 A and its fluorescence quantification in Figure 2B). From this data, it is clear that the mitochondrial specific O$_2$•−-scavenging antioxidant mito-TEMPO has a significantly higher NO-inhibitory potential than the thiol-replenishing antioxidant NAC (see the bar
diagrams in Figure 2B). Surprisingly, NPs and BSO cotreatment, which induced an 8-fold higher intracellular NO concentration, was not different from the NPs-alone treatment. BSO-alone treatment led to 2-fold higher production of NO, whereas H$_2$O$_2$ treatment led to 4-fold higher NO production in comparison to the nontreated control HepG2 cells. Contrary to BSO, exogenous H$_2$O$_2$ administration appears to potentiate NP-mediated NO production. As stated, treatment with H$_2$O$_2$ alone led to 4-fold higher NO generation, while it was 10-fold higher in the presence of NPs. NO production was compared with cell viability by co-labeling 10-fold higher-concentration cells with calcein-AM dye (see the middle panel of cells with green fluorescence in Figure 2A and its quantification in Figure 2C). Indirect quantification of NO production by measuring with a Griess reagent has been provided in Figure 2D, which largely confirms the NO quantification conducted by DAR-1 imaging.

2.3. NPs Exhibited a Saturation Tendency in Generation of ROS with Respect To Cellular GSH Level. NP-mediated ROS induction was observed to be 1.7-fold and 1.6-fold that of control when measured by DCF probe and DHE probe, respectively. It was reduced to 1.4-fold in the presence of NAC and 1.2-fold in the presence of mito-TEMPO (Figure 3A,B), suggesting mito-TEMPO to be a more potent antioxidant than NAC. Thus, thiol-replenishing NAC appears to be less effective in ameliorating NP-induced ROS generation, whereas O$_2$$^-$$^-$-scavenging mito-TEMPO is more effective. Moreover, noncytotoxic BSO-only treatment led to significant elevation of ROS, measured by DCF and DHE, as was the case with H$_2$O$_2$ at its IC50 treatment.

The cytotoxic concentration of NP treatment that caused exhaustion of GSH in cells was not as high as the noncytotoxic concentration of the GSH synthesis inhibitor BSO (Figure 3C). It is known that low concentrations of BSO, 200 μM in this study for example, can induce significant GSH depletion without altering the cell viability. 30 Cytotoxic as well as noncytotoxic BSO treatment led to significant elevation of ROS, measured by DCF and DHE, as was the case with H$_2$O$_2$ at its IC50 treatment.

![Figure 2](https://doi.org/10.1021/acsomega.1c01882) Modulatory potentials of NAC (2 mM), mito-TEMPO (100 μM), BSO (200 μM), and H$_2$O$_2$ (CC50 in HepG2 cells; 136 ± 12 μM) on the NO generation due to Pt-coated Au NP treatment in HepG2 cells were evaluated by imaging an NO-specific DAR-1 fluorescent probe that emits fluorescence in the far-red region of the spectrum (see the top and bottom images in (A)). For each DAR-1 image, a corresponding superimposable image under live-cell calcein-AM dye fluorescence is provided (green images in the middle panel). Quantitative data of DAR-1 and calcein-AM fluorescence is presented in (B) and (C), respectively. Indirect measurement of NO by using the Griess reagent is given in (D). The scale bar, marked only in the initial images as a general convention, represents 40 μm captured by a 20X objective. Data represented are mean ± SD of three identical experiments (n = 3) done in triplicates. *Statistically significant difference as compared to the controls (p < 0.05). α, β, γ, and δ, if present, denote significant difference in response to cotreatments with NAC, mito-TEMPO, and H$_2$O$_2$, respectively, against the response induced by NP treatment alone (p < 0.05). For example, the missing γ (referring to the BSO cotreatment) indicates a lack of significance due to NP treatment against that due to NP plus BSO cotreatment in DAR-1 fluorescence (C) and Griess reagent absorption (D).

Table 1. Physicochemical Characteristics of the Pt-coated Au NPs, Their Hydrodynamic Properties in Culture Media, and CC50 Value in HepG2 Cells Exposed for 24 h

| Characteristic                        | Value               |
|--------------------------------------|---------------------|
| TEM size                             | 27 ± 20 nm          |
| SEM NP size                          | NPs having an average length-to-diameter of 57–25 nm |
| Agglomeration and ζ Potential        | -28 to -43 mV       |
| Hydrodynamic size                    | 64–110 nm           |
| ζ potential                          | -28 to -43 mV       |
| CC50* of Pt-coated Au NPs in HepG2   | 148 ± 22 ng/mL      |

*Statistically significant difference as compared to the controls (p < 0.05). α, β, γ, and δ, if present, denote significant difference in response to cotreatments with NAC, mito-TEMPO, and H$_2$O$_2$, respectively, against the response induced by NP treatment alone (p < 0.05). For example, the missing γ (referring to the BSO cotreatment) indicates a lack of significance due to NP treatment against that due to NP plus BSO cotreatment in DAR-1 fluorescence (C) and Griess reagent absorption (D).
noncytotoxic concentrations of BSO, therefore, are used as a standard tool to decipher the role of GSH in ROS induction in the toxicity mechanism of diverse toxicants.31,32 BSO-treated cells adapt to a progressively oxidizing environment that can be recovered if the stimulus is removed.32 BSO-treated cells, however, can be sensitized to undergo apoptosis following toxicant exposures or abrupt GSH depletion.32 In this study, BSO could not induce as much cytotoxicity as induced by the NP treatment alone though BSO treatment-induced GSH depletion (73 ± 4.3% of the control) was steeper than that caused by NPs alone or under cotreatment conditions, suggesting a limiting capacity of NPs in causing GSH depletion. Similarly, NAC cotreatment increased the GSH to a higher level than that in control cells. *Statistically significant difference as compared to the controls (p < 0.05). α, β, γ, and δ, if present, denote the significant difference in response to cotreatments with NAC, mito-TEMPO, BSO, and H2O2, respectively, against the response induced by NP treatment alone (p<0.05). Note the missing α in (B), and γ and δ in (C).

2.4. Pt-Coated Au NP-Induced Mitochondrial Membrane Potential (MMP) Was Significantly Attenuated by mito-TEMPO. Apart from tracing the mitochondrial involvement in NP-induced toxicity by the use of mito-TEMPO, we determined MMP as it is an important marker of the mitochondrial functionality. A significant decline in Rh123 fluorescence intensities suggested Pt-coated Au NPs as a strong disrupter of the mitochondrial function in HepG2 cells (Figure 4A,B). NP caused a loss in MMP of 51%, which was recovered to 63% by NAC and 74% by mito-TEMPO. BSO and H2O2 treatments caused an MMP loss of 79 and 67%, respectively, in comparison to the 100% MMP present in control cells. NP treatment caused further loss in MMP of 44 and 37%, respectively, in the presence of BSO and H2O2. In comparison to NAC, a better recovery in the MMP fluorescence in the cell group co-treated with mito-TEMPO again demonstrated a prominent role of mitochondria in the mechanism of toxicity elicited by Pt-coated Au NPs.

2.5. Pt-coated Au NP-dependent Autophagy Was Significantly Diminished by Mito-TEMPO. MDC and LTR co-imaging (Figure 5A) and their respective fluorescence (Figure 5B for MDC and Figure 5C for LTR) were significantly elevated due to treatment with NP alone and in combination with BSO and H2O2. Autophagy activation, however, appears to be a universal pathway activated under ROS/RNS induction by toxic levels of NPs and H2O2, as well as under antioxidant depletion caused by noncytotoxic concentrations of BSO.

2.6. Preventive Potential of NAC Was Weaker than Mito-TEMPO against Pt-Coated Au NP-Induced Cytotoxicity in HepG2 Cells. Finally, the modulating potential of each cotreatment on the NP-mediated cytotoxicity was
assessed to get an idea of the influence of these markers at the gross level of cell viability. Cell viability due to CC50 concentration of Pt-coated Au NP was increased to 57 and 83%, respectively, due to cotreatments with NAC and mito-TEMPO in HepG2 cells (Figure 6A). The ability of a mitochondria-targeted antioxidant to significantly prevent the NP-mediated cytotoxicity clearly demonstrates the central role of mitochondria in the toxicity mechanism of Pt-coated Au NPs. The zoomed-in images carved out from the treatment groups (uppermost panel in Figure 6B) for a magnified visualization of the cell vacuolations show that in the presence of the most-effective antioxidant, mito-TEMPO, NP-induced vacuolations disappear, but still appear in the presence of the least-effective NAC. In Figure 6C, NO production has been compared with the cell viability data obtained from MTT assay (see Figure 1C) and calcein-AM (see Figure 2C), which reveals NO production highly aligned with the induction of toxicity due to NPs.

3. DISCUSSION

Ultra-thin platinum coating is known to increase the catalytic activity of Au NPs. The Pt-coated Au NPs chosen in this study elicited a concentration-dependent cytotoxicity (CC50 = 148 ± 22 ng/mL for a 24 h exposure) in HepG2 cells. With a reluctant role of the induced NO and ROS in aneoplastic activity, the ability of NPs to induce NO and ROS was estimated and these NPs proved to be a strong inducer of ROS and NO. It is known that NO is a hydrophobic radical molecule that is generated in mammalian cells by a family of enzymes known as nitric oxide synthases (NOSs). The three isoforms of NOSs are designated as endothelial (e) NOS, neuronal (n) NOS, and inducible (i) NOS. Overexpression of iNOS and other NOS isoforms in response to exogenous agents can lead to a high concentration of NO in immune and tumor cells with often contradictory outcomes depending on the NO concentration and other NO-derived RNS in the microenvironment. NO has low reactivity but can generate deleterious RNS such as peroxynitrite (ONOO−) in a diffusion-controlled reaction between NO itself and O2•− (superoxide anion). To explain the opposing effects of NO in the biologic system, it has been proposed that lower concentrations of NO (1−300 nM) are responsible for cell survival and proliferation, while higher concentrations (0.5−1.0 μM) favor phosphorylation of p53, cell cycle arrest, and cell death. It should, however, be noted that the cellular sources of NO are not as diverse as those of ROS. A high-concentration pre-requisite of NO in cells is generally considered as a limiting factor in NO-mediated toxicity, while chances are more to observe NO-mediated cell signaling that requires a low level of intracellular NO. A live-cell calcein-AM fluorescence indicator was found to have a strong correlation with the cytotoxicity-dependent induction of NO due to NPs in HepG2 cells, i.e., higher the cytotoxicity, greater the induction of NO. In essence, these NPs appear to be an inducer of NO up to the level enough to be implicated in the killing of HepG2 cells. Interestingly, NAC has been reported to enhance NO production by the drug Imatinib in Bcr-Abl+ chronic myeloid leukemia (CML) cells by activating...
endothelial NO synthase, and in endotoxin-treated rats. In other reports, NAC was implicated to reduce NO production in many rat macrophage cells and HepG2 cells. It follows that NAC is not uniform in NO modulation in different models and this discrepancy may be dependent on specific intracellular sites of NO production. Of the two antioxidants explored, only mitochondria-targeted mito-TEMPO was able to significantly diminish NP-mediated NO induction in this study, whereas NAC did not appear to have as much appreciable effect on NO induction. This observation suggests a definite role of mitochondria in NO production by Pt-coated Au NPs in HepG2 cells.

ROS generation has been regarded as a core mechanism in the toxicity of NPs, and alleviating this ROS generation by using NAC generally translates to the abrogation of NP-induced toxicity. The combined use of NAC with another antioxidant, TEMPOL (not mito-TEMPO), has delineated a prominent role of GSH disruption and O$_2$$^•$$−$ production in the mechanism of phenyl isothiocyanate-mediated toxicity. Further, Pt-coated Au NPs significantly induced MMP, as evidenced by the low Rh123 fluorescence when compared with control cells. Interestingly, treatment with either mito-TEMPO or NAC led to the mitochondria being healthier than they were in control HepG2 cells. As expected, mito-TEMPO completely prevented the anticancer activity of resveratrol-006 by suppressing ROS generation and MMP loss in HepG2 cells, whereas the nonmitochondrial antioxidant TEMPOL did not exhibit as pronounced inhibitory effects as those of mito-TEMPO. In our study, ROS induction due to NP treatment in HepG2 cells is not different from the ROS induction due to NP cotreatment in HepG2 cells that were exhausted of GSH by GSH inhibitor BSO. This data, therefore, suggests a saturation in NP-mediated ROS generation with respect to GSH level. NP-induced ROS, however, appeared to be potentiated in the presence of exogenous H$_2$O$_2$. A mitochondrial specific antioxidant, mito-TEMPO, is known to accumulate in the mitochondria, where it can scavenge O$_2$$^•$$−$ mimicking superoxide dismutase. This study, however, found superoxide dismutase mimicking mito-TEMPO to be more effective than GSH-restoring NAC, reflecting a critical role of mitochondria in Pt-coated Au NP-mediated toxicity in HepG2 cells. Although GSH depletion has been implicated in the toxicity mechanism of a number of NPs, it is not a sole contributor to nanotoxicity.

Figure 5. Autophagy modulating potential of Pt-coated Au NPs alone and under cotreatment with NAC, mito-TEMPO, BSO, and H$_2$O$_2$ as determined by co-imaging (A) under MDC and LTR probes. Respective fluorescence data of MDC and LTR have been depicted in (B) and (C). Note that all treatment and cotreatment conditions exhibit significant difference in comparison to control cells, suggesting autophagy as a sensitive and one of the earliest responses to subtle and rough cues. The scale bar, marked only in the initial images as a general convention, represents 20 μm captured by a 40× objective. Data represented are the mean ± SD of three identical experiments (n = 3) done in triplicates. Statistically significant difference as compared to the controls (p < 0.05). α, β, γ, and δ, if present, denote significant difference in response to cotreatments with NAC, mito-TEMPO, and H$_2$O$_2$ respectively, against the response induced by NP treatment alone (p < 0.05). Note the lack of significance of MDC fluorescence in NP-treated cells against NP treatment in combination with BSO (γ) and H$_2$O$_2$ (δ). Similarly, note the lack of significance in LTR fluorescence in NP-treated cells with NAC (α) and BSO (γ) cotreatments.
conclusion, the rather neutral effects of NAC and BSO (the two modulators exerting opposing activities on the intracellular GSH concentration) on the NP-induced NO level demonstrate that NP-mediated toxicity is relatively independent of the intracellular redox system but dependent on the mitochondrial redox system. From the GSH depletion data, it is clear as in several other studies that GSH depletion itself is not sufficient to attribute the measured toxicity; rather, it may further sensitize cells towards the ROS-inducing toxicant.51,52

Pt-coated Au NPs significantly diminished the MMP in HepG2 cells at the CC50 of NPs; the highest loss in MMP was observed in cell groups that received H2O2 in conjunction with NPs. NAC appears to restore the MMP due to the mediation of the NPs in HepG2 cells, but it was not as pronounced as was mito-TEMPO. BSO-only treatment, however, suggested that mere GSH depletion without apparent cytotoxicity could also result in significant loss in MMP, confirming GSH exhaustion as an early hallmark in the progression of cell death that can be reversed.51,52 As expected, cotreatments with BSO and H2O2 further potentiated the NP-mediated MMP. Of cellular ROS, mitochondria-generated ROS are considered to sensitize cancer cell in favor of cell death via activating specific or overlapping pathway(s) of programmed death in cancer therapy.53

NPs are well-known agents of autophagy induction. In this study, Pt-coated Au NP treatment caused a significant induction of autophagy in HepG2 cells that were significantly protected by mito-TEMPO in comparison to NAC. NAC and mito-TEMPO are known to inhibit the autophagic marker LC3B induced by CO in A549 cells.54 Recently, mito-TEMPO has been reported to decrease the autophagy induced by arsenic oxide (NaAsO2) by inhibiting ROS and MDA and increasing the GSH in mouse insulinoma (MIN6) cells.55 In this study, mito-TEMPO offered a more preventive potential in comparison to NAC against acidic vesicle formation that was induced by NP treatment, thus confirming the ROS/NO signals emanating from mitochondria as the causative agent of autophagy induced by NPs.56,57

4. CONCLUSIONS

Data on cell viability and MMP also resemble significantly the toxicity recovery capacity of mito-TEMPO, concluding a mitochondrial contribution in Pt-coated Au NP-mediated toxicity. Pt-coated Au NPs could be potentially effective agents in cancer therapy in that they not only produce mitochondrial

Figure 6. Modulatory potentials of NAC, mito-TEMPO, BSO, and H2O2 on the cell viability due to NPs was again evaluated by MTT (A) and extended by imaging under phase contrast (B). Zoomed images carved out from the treatment groups (see upper panel of Fig B), to visualize the cell vacuolations better, represent equal areas (highlighted in yellow squares). (C) compares the NO induction (obtained from live-cell imaging) with cell viability data (obtained from calcein-AM and MTT), which highlights the fact that NO induction is highly correlated with increase in toxicity. The scale bar, marked only in the initial images or control as a general convention, represents 40 μm captured by a 20× objective. Data represented are the mean ± SD of three identical experiments (n = 3) done in triplicates. *Statistically significant difference as compared to the controls (p < 0.05). α, β, γ, and δ, if present, denote significant difference in response to cotreatments with NAC, mito-TEMPO, and H2O2, respectively, against the response induced by NP treatment alone (p < 0.05).
ROS but also demonstrate the capacity to generate NO, the cumulative activity of which leads to a kind of cytotoxicity that is not preventable by general antioxidants like NAC.

5. MATERIALS AND METHODS

5.1. Chemicals and Reagents. Fetal bovine serum, penicillin-streptomycin, and LTR (LysoTracker Red DND-99) were purchased from Invitrogen Co. (Carlsbad, CA). DMEM F12, MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], NADH, pyruvic acid, perchloric acid, DCFH-DA, DHE (dihydroethidium), DAR-1 (4,5-diamo-

5N,N′N″-tetraethylrhodamine), MDC (monodansylcadaverine), Rh123, Hoechst (bisBenzimid H 33342 trihydrochlor-

eide), PI (3,8-diamo-5-[3-(diethylmethylammonio)propyl]-6-

phenylphenanthridinium diiodide), GSH, o-pthalaldehyde (OPT), Hank’s balanced salt solution (HBSS), NAC, mito-

TEMO, BSO, and Bradford reagent were purchased from Sigma-Aldrich, MO. Ultrapure water was taken from a Milli-Q system (Millipore, Bedford, MA). All other chemicals used were of reagent grade.

5.2. Pt-Coated Au Nanoparticles and Physicochemical Characterization. Pt-coated Au NPs (27 ± 20 nm) were obtained from a commercial source (Sigma-Aldrich, MO). The NP size and coating were confirmed by field emission transmission electron microscopy (FETEM, JEM-2100F, JEOL, Inc., Japan). The shape of these NPs was evaluated by field emission scanning electron microscopy (FE-SEM; JSM-7600F, JEOL Inc., Akishima, Japan) at an accelerating voltage of 5 kV. For carrying out dynamic light scattering (DLS) measurements, the NP suspension in the relevant fluid was freshly prepared at 200 ng/mL and ultrasonicated for 10 min (Ultrasonic Cleaner-8891, Cole-Parmer, 625 Bunker Court Vernon Hills, IL). A phenol red-free culture medium was used for DLS measurement that was performed in special cuvettes supplied by the DLS system (Nano-Zeta Sizer-HT, Malvern Instruments, Malvern, U.K.).

5.3. Cell Culture and Treatments with NPs and Different Oxidative Stress Modulators. Human liver cancer (HepG2) cells (ATCC, US) were maintained in DMEM-F12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO2 incubator. The cells were passaged every 3–4 days. Cells were treated with Pt-coated Au NPs alone or in combination with oxidative stress modulators for a period of 24 h. This study was advanced by analyzing several relevant markers in HepG2 cells at the CC50 (148 ng/mL) of Pt-coated Au NPs alone and in combination with oxidative stress modulators NAC (2 mM) and mito-TEMPO (100 μM) or oxidants BSO (200 μM) and H2O2 (IC50; 275 μM), referred to as “treatment groups.” Similarly, controls of each cotreat-

tment designated as “control groups” in the lower panels of the images have been included for a fair comparison and better understanding. The concentrations of NAC, mito-TEMPO, and BSO were chosen on the basis of their showing a preliminary dosage response curve (data not shown) in HepG2 cells that is in accordance with the available literature.69,46,58

NB. To avoid any ambiguity, a “treatment” word refers to a single-item exposure with either NPs or one of the modulators (NAC, mito-TEMPO, BSO, and H2O2), while a “cotreatment” refers to a co-exposure of NP in conjunction with one of the modulators.

5.4. Determination of Cell Viability by MTT. Cell viability was determined by MTT assay as described by Mosmann.59 Briefly, 2 × 10^4 HepG2 cells were seeded in a 96-

well plate and treated the next day. After a 24 h exposure period, the cells were added to a filtered MTT solution made in HBSS and left for 1.5 h. The formazan crystal thus formed by viable cells was solubilized in 20% SDS prepared in 50% dimethylformamide. Absorbance at 570 nm was measured by a plate reader (Synergy HT, Bio-Tek, Winooski, VT) and cell viability was calculated as % of control. IC50 calculations for the NPs and H2O2 were made from the online IC50 calculator (https://www.aatbio.com/tools/ic50-calculator) provided by AAT Bioquest, Inc. (CA 94085). In addition to MTT assay, cells under various treatment conditions were imaged under the setting of phase-contrast microscopy too.

5.5. Analysis of Intracellular NO. Intracellular NO was determined by imaging a rhodamine-based live-cell-permeable fluorescent probe DAR-1 that reacts specifically with NO and generates intense fluorescence in the infrared region.50–62 Cells were treated with the respective agents for 24 h in a 12-well plate and labeled with DAR-1 at a final concentration of 15 μM for 2 h. Cells were also colabeled with the live-cell fluorescent probe calcein-AM at 1 μM to corroborate the live-cell status. Then, they were carefully washed with cold HBSS three times and imaging was conducted using an appropriate filter in a microscope (Leica DMi8, Wetzlar, Germany). Direct imaging of NO using DAR probes has been successfully reported in RAW264.7 cells,63 PC-12 cells,64 and zebrafish.65 NO was also indirectly quantified by measuring the nitrite liberated into cell culture media using a Griess reagent at 540 nm in a plate reader (Synergy HT, Bio-Tek, Winooski, VT). A standard of sodium nitrite (1–100 μM) prepared in culture media was similarly run for calculation purposes as conducted by various investigators.66,67 Data has been presented as % of NO concentration in untreated control cells.

5.6. Determination of Intracellular ROS. The potential induction of ROS was determined by a 2−/7−-dichlorofluorescin diacetate (DCFH-DA) probe68 that was incubated for 45 min at a final concentration of 50 μM after the treatment period was over. The plate was washed thrice with cold PBS to remove excess dye from each well and DCF fluorescence was measured at 528 nm in the plate reader (Synergy HT, Bio-Tek, Winooski, Vermont). Dihydroethidium (DHE) is a cell-

permeable probe that preferentially reacts with O2−−−producing red fluorescent products ethidium or 2-hydroxyethidium.69 Cells were labeled with DHE at a final concentration of 5 μM and incubated for 30 min. The plates were carefully washed with cold HBSS three times before fluorescence reading in a 590 ± 35 nm emission band pass filter of the plate reader (Synergy HT, Bio-Tek, Winooski, Vermont).

5.7. Determination of GSH. The cellular content of GSH was quantified according to the method given by Hissin and Hilf.70 After treatment, cells were lysed in an aqueous solution of 0.1% deoxycholic acid plus 0.1% sucrose for 2 h, which included 3 cycles of freeze–thaw and centrifugation at 10 000 g for 10 min at 4 °C. The supernatant was precipitated in the final concentration of 1% perchloric acid and centrifuged at 10 000g for 5 min at 4 °C. Twenty liters of the perchloric acid protein-precipitated cell lysate supernatant was mixed with 160 μL of 0.1M K-phosphate—5 mM EDTA buffer, pH 8.3, and 20 μL of o-pthalaldehyde (OPT, 1 mg/mL in methanol) in a black 96-well plate. After 2.5 h of incubation at room temperature in the dark, the fluorescence was measured at an emission wavelength of 460 nm (Synergy HT, Bio-Tek, Winooski, Vermont). A standard curve was obtained for
calculation from similarly prepared known concentrations of GSH. The protein concentration was estimated from unprecipitated supernatant and the data converted to GSH nmol/mg protein.

5.8. Determination of Mitochondrial Membrane Potential by Rh123. Rhodamine (Rh) 123 is a powerful probe for monitoring the abundance and activity of mitochondria.\(^{71,72}\) To conduct the assay, Rh123 at a final concentration of 20 μM was added to the cells in a 12-well plate for 15 min. The reaction mixture was removed and the cells were washed with HBSS three times carefully so that the cells are not washed off. Imaging was conducted using a blue filter in a microscope (Leica DMi8, Wetzlar, Germany). The resultant green fluorescence intensity is directly proportional to the MMP; higher the green Rh123 fluorescence, greater the MMP and vice-versa.

5.9. Imaging of Intracellular Acidic Vesicles. Mono-dansylcadaverine (MDC) is a popular autophagy marker that preferentially accumulates in autophagic vesicles due to a combination of ion-trapping and specific interactions with lipid molecules in the membrane of the vesicles.\(^{75}\) It has been suggested that MDC fluorescence marks the autophagy event that occurs after the fusion of lysosomes with the autophagosome, forming autolysosomes.\(^{74}\) Another relevant dye, Lysotracker (LTR), is commonly used to detect lysosomes\(^{75}\) that are acidic organelles employed in the turnover of damaged or old macromolecules and organelles. The activity of lysosomes and their perinuclear localization are increased significantly during induced autophagy.\(^{76}\) Because of their distant emission spectra, MDC and LTR (LysoTracker Red DND-99) were simultaneously assayed to follow autophagy in a more dynamic way. The concentrations of MDC and LTR in a 12-well plate containing cells were 50 mM and 1 μM, respectively, when incubated for 60 min. After careful washing, imaging was conducted using a violet filter cube for MDC and a green filter cube for LTR under a fluorescence microscope (Leica DMi8, Wetzlar, Germany). An increase in punctate blue or red fluorescence in treated cells than that in control cells is indicative of induced autophagy in the treated cells.\(^{74,76}\) A higher mito-TEMPO-mediated inhibition of NO, ROS, and toxicity induced by NP reveals a toxicity mechanism that is dependent on the mitochondrial NO/ROS in HepG2 cells. The cumulative data on NO, ROS, autophagy, and MMP suggest Pt-coated Au NPs as a promising killing agent of highly resistant liver carcinoma HepG2 cells via the activating mitochondrial pathway. This study warrants further investigation of the anticancer potential of NO-inducing Pt-Au NPs in in vivo models.

5.10. Protein Estimation. The total protein content was measured by a convenient BCA Protein Assay Kit from Sigma-Aldrich as per instructions.

5.11. Statistics. ANOVA (one-way analysis of variance) followed by Dunnnett’s multiple-comparison tests was employed for statistical analysis of the results. For a particular set of experiments, a burst of images was captured for a constant exposure of time, gain, saturation, and γ. For the calculation of corrected total cellular fluorescence (CTCF), a reasonably constant area was selected and restored via the “restore selection” command to all images once opened in an ImageJ software (NIH, Bethesda, MD). CTCF was calculated by subtracting the fluorescence in the background (without cell) from the mean of individual cellular fluorescence values. The scale bar in the images was set using ImageJ after adjusting the scale of the pixels/micron for a particular objective and then saving all images in a JPEG format. The scale bar, marked only in the initial images as a general convention, represents 40 μm (micrometer or micron) when captured by a 20× objective and 20 μm when captured by a 40× objective. Representative images (captured by a 5-megapixel Leica DFC450C camera, Wetzlar, Germany) from three independent experiments (n = 3) are shown for the particular experimental group. Data represented are the mean ± SD of three identical experiments (n = 3) done in triplicates. Statistical significance was attributed to p < 0.05.

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Notes

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