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Enhancement of 5-Aminolevulinic acid-induced oxidative stress on two cancer cell lines by gold nanoparticles

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Abstract

5-Aminolevulinic acid (5-ALA) and its methyl ester (5-ALA-Me) at mM concentration levels induce oxidative stress via the production of reactive oxygen species (ROS). Human cancer cell lines (MCF-7 and HepG2) incubated in the dark in the simultaneous presence of 5.0 mM or more 5-ALA or 5-ALA-Me (for MCF-7) and 7 μg/mL of 15 nm citrate capped gold nanoparticles (AuNPs) were damaged more seriously compared to those in the presence of the levulinic acid alone. Damage is visible in electron micrographs which reveal similar morphology both in the presence or absence of AuNPs. Cytotoxicity was observed irrespective of the presence of serum and medium. Production of ROS in cell free samples containing 5-ALA-Me was monitored by EPR as the DMPO-OH spin adduct and also showed a catalytic effect of AuNPs. Both SOD and CAT inhibited the production of ROS and also reduced cytotoxicity in the cell samples. These observations can be explained by initial attack on the cell membrane by ROS produced in the medium outside the cell and provide insight into possible uses of 5-ALA in cancer chemotherapy.

Keywords: *Gold nanoparticles, 5-Aminolevulinic acid, cytotoxicity, reactive oxygen species*

Abbreviations: *AuNPs, gold nanoparticles; 5-ALA, 5-Aminolevulinic acid; EPR, electron paramagnetic resonance spectroscopy; SOD, superoxide dismutase; CAT, catalase; ROS, reactive oxygen species; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; FBS, foetal bovine serum*

Introduction

5-Aminolevulinic acid (5-ALA) is a precursor in the biosynthesis of heme. Photodynamic therapy in the presence of 5-ALA has been employed widely in cancer treatment since Protoporphyrin IX, an intermediate in heme synthesis, is accumulated in cancer cells and mediates both phototoxicity (via singlet oxygen production) [1,2] and sonotoxicity [3]. At mM levels, 5-ALA produces reactive oxygen species (ROS), causes DNA and cell membrane damage via lipid peroxidation and alterations in membrane fluidity and gives obvious damage to cellular organelles such as mitochondria and microsomes [4]. Monteiro et al. [5,6] concluded that hydrogen peroxide, super-oxide anion radical, hydroxyl radical and ALA enoyl radical were produced by a coupled auto-oxidation of oxyhaemoglobin and 5-ALA (6 mM) by demonstrating inhibition of 5-ALA auto-oxidation and by EPR spin trapping. Also, Hermes-Lima et al. [7,8] demonstrated damage induced by 5-ALA (2-15 mM) for isolated mitochondria from rat liver by studying Ca^{2+} release, which was inhibited by SOD, CAT or the

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Fe(II) chelator o-phenanthroline. Hydroxyl radicals were detected *in vivo* for mice cells treated with 5-ALA and Fe^{3+} by an EPR study using DEPMPO as spin trap [9]. As proposed by Hermes-Lima [8], hydroxyl radicals appear to play an important role in cell damage due to 5-ALA induced oxidative stress. Yet, the chemical mechanisms of formation of ROS mediated by 5-ALA and the damaging process of 5-ALA induced oxidative stress are not completely clear.

5-ALA and its derivatives have potential for new medical applications to kill cancer cells. However, administration of high levels of 5-ALA can cause adverse effects such as skin sensitivity, nausea, vomiting and transient liver dysfunction [10].

Drug delivery using gold nanoparticles (AuNPs) as non-toxic carriers has been demonstrated [11–15]. However, AuNPs can be toxic [16–20]. While particle size can be important, e.g. triphenylphosphine derivative conjugated 15 nm AuNPs were non-toxic for HeLa cells but 1.4 nm particles were toxic [17], toxicity for other, similar-size AuNPs, depends on the type of cell [14,19]. Thus, potential uses of AuNPs require careful study of the target and particle size.

In preliminary experiments, we found that treatment with media containing only 15 nm citrate capped AuNPs (7 μg/mL) inflicted no significant cytotoxity for MCF-7 and HepG2 cells. Based on these observations, we expected that AuNPs might provide a useful tool for delivery of 5-ALA molecules to induce oxidative stress in targeted cells. Hence, we examined the cytotoxicity of 5-ALA in the presence of AuNPs. Here we report the cytotoxic effects on MCF-7 and HepG2 cells by the incubation in the simultaneous presence of 5-ALA (or its methyl ester, 5-ALA-Me, with MCF-7) and 15 nm AuNPs. Moreover, we demonstrate the effect of AuNPs on ROS production for cell-free samples containing 5-ALA-Me by EPR using DMPO as spin trap. The mechanism of increase of the cytotoxicity by the combination of 5-ALA and AuNPs is discussed.

Materials and methods

Cells

Both MCF-7 mammary adenocarcinoma and HepG2 hepatocellular liver carcinoma cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum (FBS).

Chemicals

Non-Phenol-Red RPMI 1640 medium (GIBCO 118 35) was obtained from Invitrogen. FBS was obtained from Atlanta Biologicals (Lawrenceville, GA). 5-Aminolevulinic acid hydrochloride (5-ALA-HCl), 5-Aminolevulinic acid methyl ester hydrochloride (5-ALA-Me.HCl), SOD and CAT were obtained from Sigma-Aldrich (St. Louis, MO). Distilled and deionized water (sterile) and Dulbecco's Phosphate-Buffered Saline (DPBS) were obtained from Mediatech, Inc (Manassas, VA). DMPO was obtained from Alexis Biochemicals (Lausanne, Switzerland).

AuNPs

Spherical AuNPs (15 nm) were stabilized by citrate buffer at pH 6.5 and existed as hydrophilic colloids (we refer to this as gold citrate solution). Citrate buffer used in controls was the supernatant obtained by centrifuging the gold citrate solution. The gold citrate solution (70 μg/mL) and the citrate buffer were from Tanaka Kikinzoku (Tokyo, Japan). Except as noted, all gold-containing cell experiments had 7 μg/mL AuNPs.

Treatment of cells with medium containing 5-ALA (or 5-ALA-Me) and AuNPs

Treatment media containing 5-ALA or 5-ALA-Me were prepared from 1 M stock solutions of the hydrochloride. These were mixed as appropriate with stock solutions of AuNPs, RPMI 1640 growth medium and 10% FBS. Control/comparison solutions lacking one or more of these components also contained distilled water, citrate buffer, DPBS or HCl.

Cell suspension (100 μl) was added to each well of a 96-well cell culture cluster plate (Corning Incorporated, Costar 3596, New York) and the number of intact cells for the cell suspension was determined using a Beckman Coulter multisizer model Z2 (Hialeah, FL). Cells were allowed to attach and grow in darkness for 24 h at 37°C in air containing 5% CO_2, resulting in an approximate doubling of the cell number. Cells were washed and the appropriate treatment medium (100μl) was added under dim white light (1×10^{-2} μW/cm^2). The cell samples were again incubated in complete darkness under the above conditions. Since the volume of reaction solution added to each well was the same as the volume of suspension originally taken, we report cell concentrations for each as twice those of the initial measurement.

Assay of cell viability

After replacing the treatment medium with DPBS, cell viability was determined using a Calcein-AM based cytotoxity detection kit (Cell Counting Kit F, Dojindo, Kumamoto, Japan) and fluorometric micro-plate reader (HTS 7000, Perkin Elmer, MA) with 485 nm excitation and 535 nm emission filters. Manipulations were carried out under dim white light (1×10^{-2} μW/cm^2) and cells were allowed to stand for a few hours before measurement. In this assay, the fluorescence of Protoporphyrin IX is negligible compared to the Calcein-AM induced fluorescence from living cells. The relative cell viability

for each sample was calculated by comparison with that of the control in medium containing neither 5-ALA (or 5-ALA-Me) nor AuNPs (diluted with deionized water).

Transmission electron microscopy (TEM) studies of AuNPs uptake and cell morphology

Treated sample and controls of MCF-7 cells were prepared in the same way as described above using a cell culture cluster plate with six wells (Corning Incorporated, Costar 3506). After incubation under complete darkness at 37°C in air containing 5% CO_2, cells were processed, embedded *in situ* and thin-sectioned for TEM analysis, as previously described [21].

EPR spin trapping studies

All EPR experiments were done in cell free media in the presence of 0.3 M DMPO as the spin trap. Samples were prepared and put into gas-permeable Teflon tubes (Zeus, Orangeburg, SC). EPR signals were recorded at 10 min intervals on a Varian E-9 X-band EPR spectrometer under flowing air (5% CO_2) at 40–45°C. The EPR spectrometer was operated with modulation frequency of 100 kHz, microwave power of 10 mW and microwave frequency of 9.3 GHz.

Results

Cytotoxic effects of 5-ALA and AuNPs on MCF-7cells

MCF-7 cell samples with concentrations between 3×10^4 and 6×10^4 cells/mL were incubated in the dark for 18 h with FBS-medium containing 2.5-10 mM 5-ALA and gold citrate solution (AuNPs; 7 μg/ mL). The population of MCF-7 cells doubles about every 18 h when incubated with FBS-medium. As shown in Figure 1A, cell viabilities were essentially the same for gold-free samples diluted with water or citrate buffer both in the presence and absence of 5-ALA. While 5-ALA itself shows moderate to almost complete loss of viability at higher concentrations (10 mM

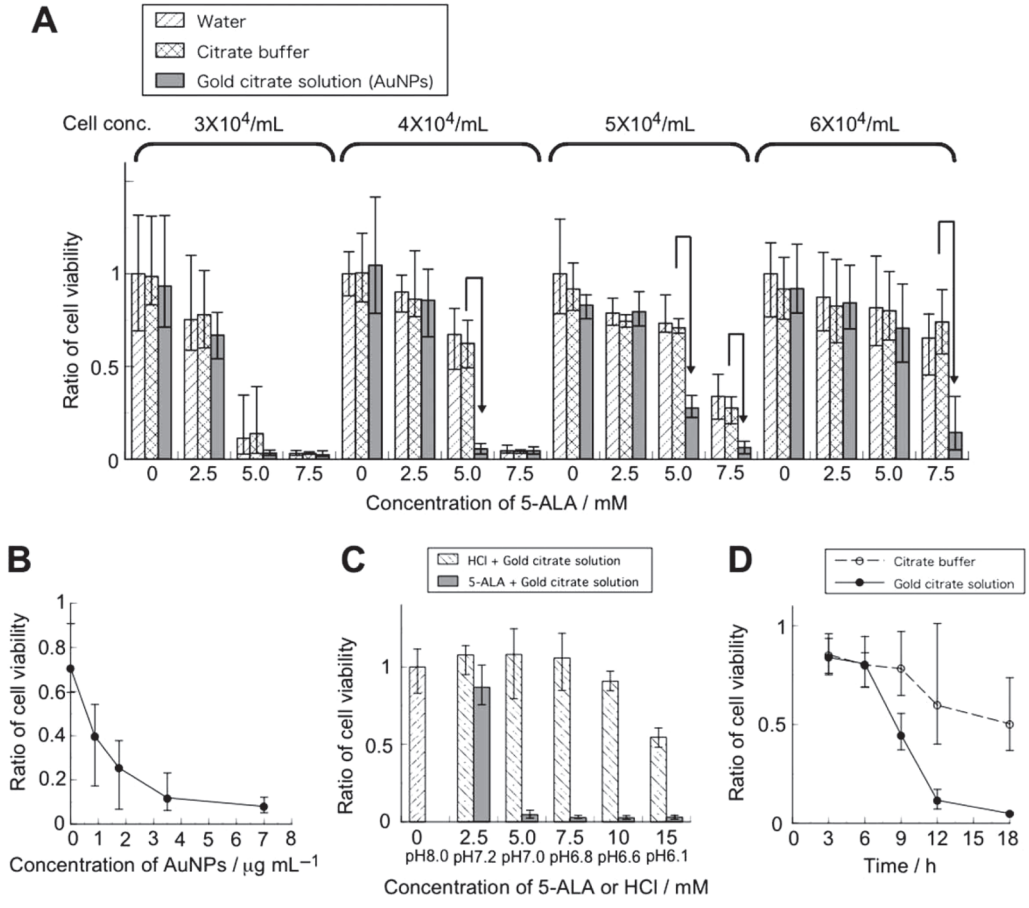

Figure 1. Cytotoxic effects for MCF-7 cells incubated for 18 h in the simultaneous presence of 5-ALA and 15 nm AuNPs in FBS-medium. Viability for each cell sample was referenced to that of the control sample incubated with medium containing neither 5-ALA nor AuNPs. (A) Dependence on concentration of cells and 5-ALA. Cell samples contained 7 μg/mL AuNPs. Significant effects are indicated by arrows. (B) Concentration dependence on AuNPs for samples containing 5.0 mM 5-ALA, and 4×10^4 cells/mL. (C) Comparison of cell viability between cell sample containing 5.0 mM 5-ALA (from 5-ALA HCl) and AuNPs and a control sample adjusted to the same pH with HCl. (D) Variation of viability with incubation time (5×10^4 cells/mL, 7.5 mM 5-ALA, 7 μg/mL AuNPs). Data indicate mean ± SD for four (B), five (A), six (D) and eight (C) replicates.

data is not shown), there is a definite synergistic effect of the gold nanoparticles at intermediate concentrations. Viability remained high and similar for 2.5 mM 5-ALA both in the presence and absence of AuNPs. As might be expected, higher cell concentrations were more resistant to loss of viability. Figure 1B shows the effect of AuNPs concentration on samples containing 4×10^4 cells/mL, 5 mM 5-ALA and FBS-medium. In this range, as the concentration of AuNPs increased, the cell damage became more and was almost total at 7 μg/mL.

To examine the combined 5-ALA/pH dependence of the cytotoxic effect, MCF-7 cell samples were treated with FBS-medium containing 2.5-15 mM 5-ALA and 7 μg/mL AuNPs. Control samples were adjusted to the same pH values with HCl. Cell samples with 5-ALA at concentrations ranging from 5.0-15 mM and AuNPs lost cell viability completely, while only the pH 6.1 control showed any significant loss (Figure 1C). This result suggests that the large increase of cytotoxicity induced by the combination of 5-ALA and AuNPs does not depend on changes in pH. All the above samples were incubated for 18 h. For samples with 7.5 mM 5-ALA and 5×10^4 cells/ mL, viability remained very high ($> 80\%$) up to 9 h incubation and there was no effect of AuNPs up to at least 6 h (Figure 1D). Since 5-ALA produces ROS even in the absence of cells, this indicates that there is a definite threshold for cell killing.

Figure 2 shows TEM images of MCF-7 cell samples incubated for 18 h. For the control cell sample with AuNPs alone, almost all cells showed normal appearance and uptake of AuNPs with aggregation in vesicles, such as membrane-bound vesicles, multivesicle bodies and endosomes, in the cytoplasm (Figure 2A). For the sample with 5-ALA and citrate buffer, most cells showed a normal appearance, but some cells were abnormal with many vacuoles in the cytoplasm and swelling of mitochondria (Figure 2B). In contrast, most cells treated with 5-ALA and AuNPs had these abnormalities (Figure 2C). Also, these damaged cells indicated uptake of AuNPs with aggregation in the vesicles. There was no significant difference in the amount or location of AuNPs, compared to the sample with AuNPs alone. None of the cell samples showed any disruption of the nucleus.

Cytotoxic effects of 5-ALA and AuNPs on HepG2 cells

HepG2 cell samples with concentration of ~ 6×10^3 cells/mL were treated with FBS-medium containing 7.5 mM 5-ALA and AuNPs (7 μg/mL) for 36 h. Samples with 7.5 mM 5-ALA and AuNPs lost cell viability almost completely, while the control cell samples with 5-ALA and citrate buffer lost ca. 30%. Samples without 5-ALA containing 7.5 mM HCl indicated normal cell viability, irrespective of the presence of AuNPs.

Cytotoxic effects of 5-ALA-Me and AuNPs on MCF-7 cells; comparison with 5-ALA

MCF-7 cell samples (6×10^4 cells/mL) were treated with FBS-medium containing 5.0 mM 5-ALA-Me and AuNPs (7 μg/mL) for 18 h. As shown in Figure 3A, 5-ALA-Me is significantly more cytotoxic than 5-ALA, especially in the presence of AuNPs. In parallel experiments, 0.1 mM diethylenetriaminepen-taacetic acid (DETAPAC), a metal chelator, had no noticeable effect. As in the case of 5-ALA-Me, cell samples with 7.5 mM 5-ALA-Me lost cell viability completely, irrespective of the presence of AuNPs (Figure 5). Cytotoxicity was also enhanced FBS free

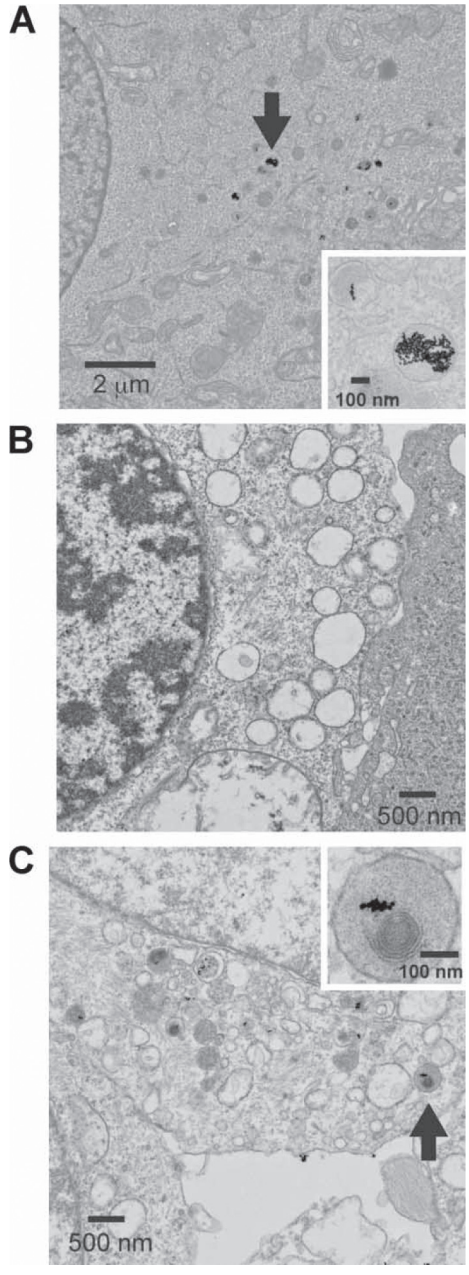

Figure 2. TEM images of MCF-7 cells incubated (18 h) with FBS-medium containing (A) gold citrate solution, (B) 7.5 mM 5-ALA and citrate buffer or (C) 7.5 mM 5-ALA and gold citrate solution. Arrows and insets indicate AuNPs.

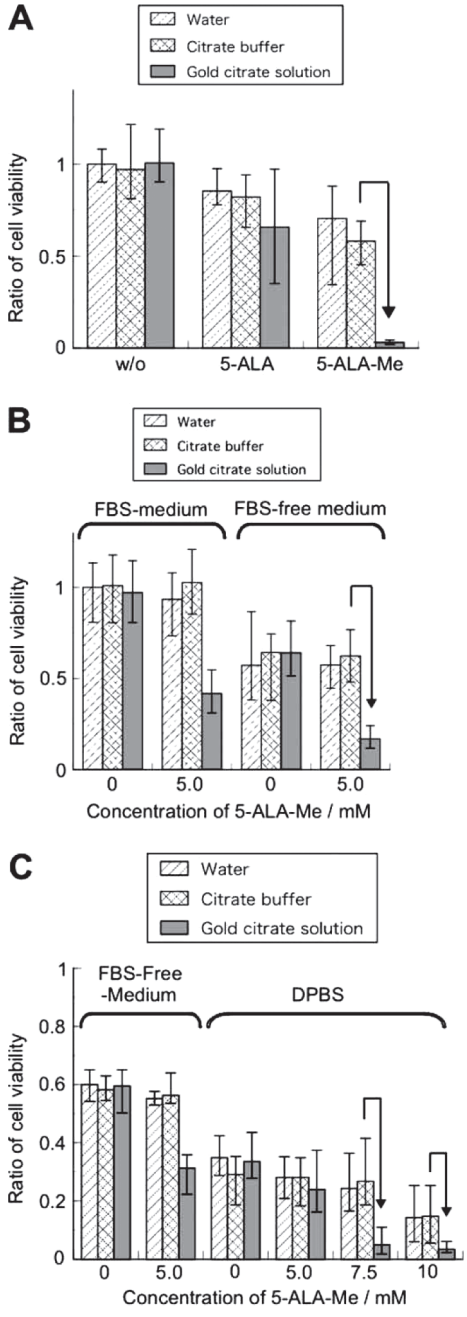

Figure 3. Cytotoxic effects for MCF-7 cells incubated in the simultaneous presence of 5-ALA-Me and AuNPs. Significant effects are indicated by arrows. (A) Comparison of 5-ALA with 5-ALA-Me. Cell samples (6×10^4 cells/mL) were incubated for 18 h with FBS-medium containing 5.0 mM levulinic acid and with or without AuNPs. (B) Serum dependence of cytotoxicity. Cell samples (8×10^4 cells/mL) were incubated with FBS-medium or FBS-free-medium containing 5 mM 5-ALA-Me and AuNPs for 12 h. (C) Medium dependence of cell viability. Cell samples (8×10^4 cells/mL) were incubated with FBS-free-medium or DPBS containing 5-ALA-Me with various concentrations and AuNPs for 12 h. Data indicate the mean ± SD. $n=6$ (A); 9 (B) and (C). Note that, even in the absence of 5-ALA, cells without the nourishment of the medium and/or FBS would (as observed) have much reduced viability compared to the control.

samples (Figure 3B) and in samples without any growth medium (Figure 3C). Similar decreases in viability were observed in 5-ALA samples (data not shown). These results demonstrate that AuNPs enhance the cytotoxicity of both 5-ALA and 5-ALA-Me.

To investigate the possible contributions of superoxide anion radical, hydrogen peroxide and hydroxyl radical to the observed cytotoxicity, some experiments were conducted with added SOD (10 units/mL) or CAT (8.6 units/mL). While the degree of cytotoxicity varies with concentration of 5-ALA and the presence or absence of AuNPs, both SOD (Figure 4A and B) and CAT (Figure 4C and D) were highly protective.

Figure 5 shows TEM images of MCF-7 cells treated with FBS-medium containing 5.0 mM 5-ALA-Me. In the absence of AuNPs, cells appeared normal (Figure 5A). However, for the sample with AuNPs, gold uptake and abnormalities were evident (Figure 5B) which were similar to those caused by 5-ALA. As for the cells treated with FBS-free-medium containing 5-ALA-Me and AuNPs, AuNPs aggregated around the cells (Figure 5C). When the medium was replaced by DPBS, AuNPs entered the cells (not shown).

EPR spin trapping studies

With DMPO (0.3 M) as spin trap, FBS-medium based (cell free) solutions containing 40 mM 5-ALA-Me gave rise to the characteristic four line DMPO-OH spectrum (splittings, $a_N = a_H = 14.9$ Gauss) both in the absence and presence of AuNPs (30 μg/mL, Figure 6A). Control experiments without 5-ALA-Me had negligible intensity. As shown in Figure 6B, the intensity of the DMPO-OH EPR signal for the solutions containing 5-ALA-Me increased with time, saturating within ~ 50 min, presumably due to the limited lifetime of the spin adduct. Addition of 0.74 mM DETAPAC gave no significant change in the DMPO-OH signal. Signal intensity depends on the concentration of AuNPs (Figure 6C). Figure 6D compares intensities after 20 min in the presence of SOD (77 units/mL) and CAT (6.6×10^3 units/mL). Noting the different time scales and concentrations, we cannot make quantitative comparisons between these cell free spin trapping experiments and the experiments with cells. However, we can say that in both situations the presence of AuNPs catalyses the production of ROS from the reaction between 5-ALA-Me and oxygen and that both superoxide ion and hydrogen peroxide are produced. In fact, the signal in the presence of SOD became more pronounced with time compared to the control. Figure 6 also shows the growth of the DMPO-OH signal in the absence of FBS and when the FBS medium is replaced by DPBS. While the removal of FBS has little effect (cf. Figure 6B and E), the DPBS sample without AuNPs showed significantly less production of the spin adduct.

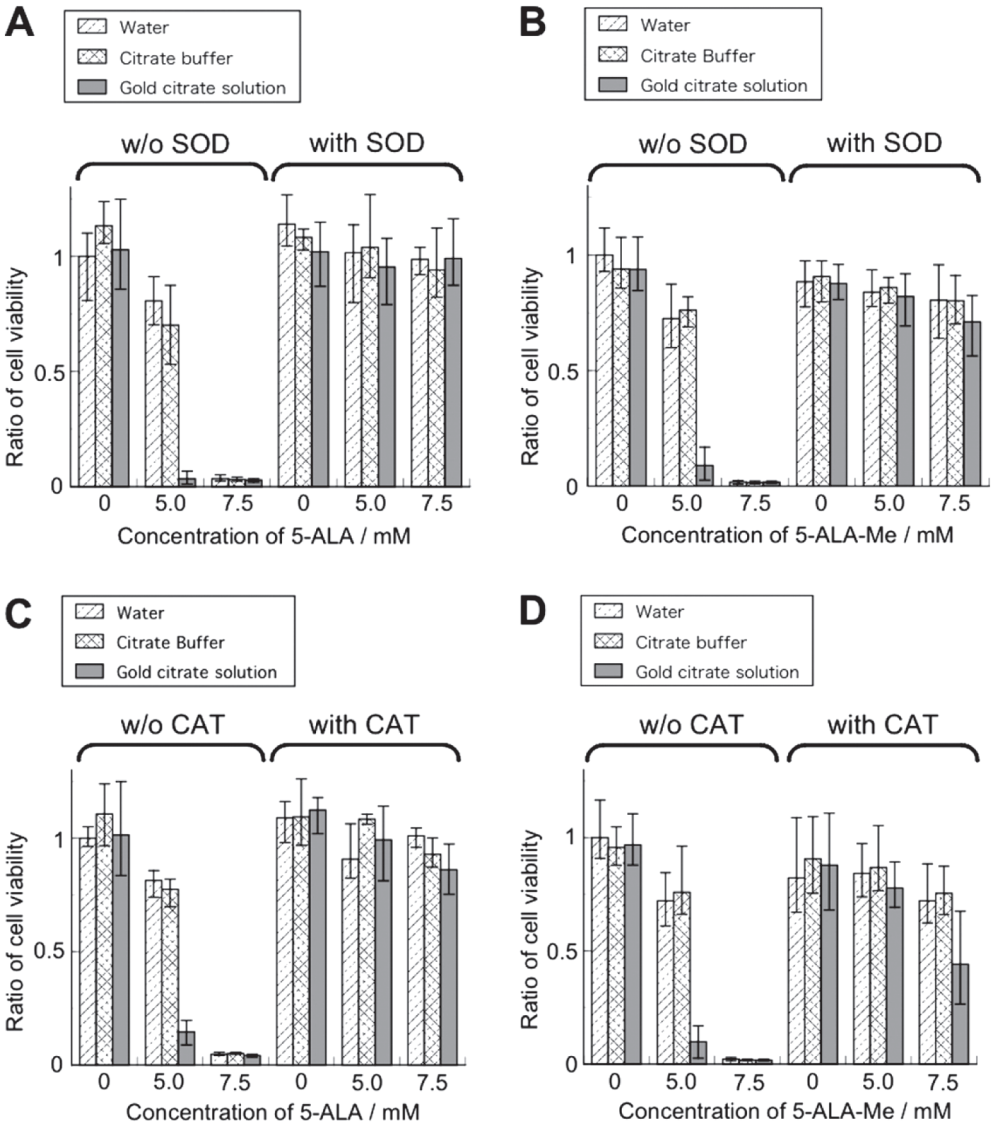

Figure 4. Protective effects of SOD (10 units/mL) (A and B) and CAT (8.6 units/mL) (C and D) for MCF-7 cells. Samples contained 6×10 cells/mL cells in FBS-medium containing 5.0 mM or 7.5 mM 5-ALA or 5-ALA-Me with or without AuNPs. Data indicate the mean ± SD. $n = 3$ for (A) and (C) and 7 for (B) and (D).

Discussion

In this study, we have demonstrated that two human cancer cell lines (MCF-7 and HepG2) incubated, in the dark, with 5-ALA (5.0 and 7.5 mM) and 15 nm citrate capped AuNPs show increased cytotoxicity compared to the incubation in the presence of 5-ALA alone. With MCF-7 cells, this effect was a factor of 1.5 for 5.0 mM 5-ALA and even greater for 5-ALA-Me.

Addition of SOD or CAT provided substantial protection to the cells for samples containing 5-ALA or 5-ALA-Me both with and without AuNPs (Figure 5). Furthermore, the ESR spin trapping experiments show that AuNPs increase the yield of ROS and that SOD and CAT are each effective in reducing the total amount of ROS. The morphological appearances of the damaged MCF-7 cells were the same whether or not AuNPs were present. These results suggest that the mechanism of cell damage by the combination of the levulinic acid and AuNPs is essentially the same as that by the levulinic acid alone and due to enhanced production of both superoxide and hydroxyl radicals (perhaps from hydrogen peroxide) in the presence of AuNPs. Similar conclusions have been reported for metal catalysed 5-ALA auto-oxidation [5] and the 5-ALA induced release of Ca^{2+} from mitochondria [7,8]. In this connection, super-oxide radicals have long been shown to propagate x anthine/xanthine oxidase-initiated dihydroxyacetone phosphate oxidation via steps similar to those of 5-ALA [22]. With regard to the spin trapping results, we note that the superoxide adduct of DMPO is unstable and converts to DMPO-OH in a process that involves free hydroxyl radicals as an intermediate [23]. The fact that CAT partially inhibits the DMPO-OH yield is an indication that some of the trapped radical comes from hydrogen

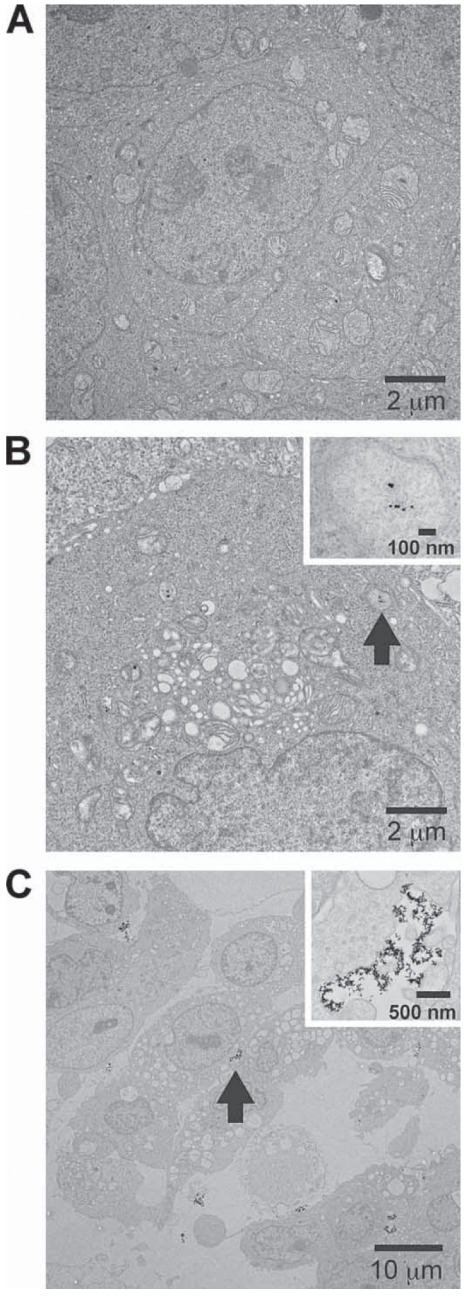

Figure 5. TEM images of MCF-7 cells incubated in the simultaneous presence of 5-ALA-Me and AuNPs. Cells were incubated with FBS-medium containing (A) 5.0 mM 5-ALA-Me and citrate buffer or (B) 5.0 mM 5-ALA-Me and AuNPs, for 18 h. (C) Cell sample incubated with FBS-free-medium containing 5.0 mM 5-ALA-Me and AuNPs for 12 h. Arrows and insets indicate AuNPs.

peroxide which is the product of the two-electron redox reaction between 5-ALA and oxygen [6].

While 15 nm AuNPs alone gave negative results for both cell damage and for ROS production in the EPR experiments, AuNPs are clearly catalysing ROS production in the presence of the levulinic acids. On the other hand, Zhang et al. [24] have observed a reduction in EPR signal for nitroxides in the presence of 15 nm AuNPs which they attribute to the association of the polar nitroxide molecules with the gold surface. This is followed by enhanced superoxide formation in the presence of O_2. Also, 13 nm AuNPs catalyse nitric oxide production by reacting with S-nitrosothiols in a blood serum [25]. This is not unexpected since gold has a great affinity for sulphur. A report of oxidative stress being produced by (only) 13 nm AuNPs involved a complete organism, *Mytilus edulis* [26], which provides ample opportunity for ROS production with no other chemical additive. Catalysis of oxygen reductions by AuNPs appear to involve Au-O_2 binding only for very small (3-5 nm or less) particles [27,28]. Thus, it seems reasonable to postulate that the enhanced formation of ROS in our system is the result of a mechanism in which 5-ALA initially interacts with AuNPs followed by interaction with oxygen to form ROS. In our experiments, 5-ALA is a zwitterion (pK_{a1} = 4.1, pK_{a2} = 8.7; pK_a = 8.4 for 5-ALA-Me) [29] and it is likely that the higher reactivity of the ester is related to decreased charge interaction with the negatively charged, citrate capped AuNPs.

To further characterize our reaction conditions, UV-visible spectra for all treatment media were collected. AuNPs solutions alone are red with a single peak at 520 nm, consistent with other studies [30]. In the presence of FBS-medium, this peak shifted with time to 525 nm and broadened at higher wavelengths. In addition FBS has a peak at 408 nm which disappears over time in the presence of 5-ALA-Me. Changes in the visible spectrum of AuNPs (decrease in the 525 nm peak and a new peak at 635 nm) when 5-ALA-Me was added in the presence of FBS-medium provide evidence for interaction between 5-ALA-Me and AuNPs both in the presence and absence of cells (Figure 7). There are also reports of spectral changes attributed to proteins interacting with AuNPs [31]. Solutions containing DPBS rather than medium seemed to destabilize the gold colloid (not shown). In these solutions, the red colour dissapeared and AuNPs tended to aggregate and precipitate over time. This may account for the lower efficiency of ROS production in the cell free (EPR) experiments (Figure 6F, cf. 6B and E). Note also the large difference in the gold/no gold data in Figure 6F for solutions containing 5-ALA and no medium. This indicates a rather high level of interaction and AuNPs catalysis in simple systems that may have more general implications.

We now consider the location of ROS production during incubation of cells. The TEM analyses have shown the presence of AuNPs both inside and outside the cell membrane. However, neither SOD nor CAT can penetrate to the cell interior. The observation that both SOD and CAT protect against cytotoxicity, then, leads us to conclude that, although the levulinic acids can enter the cells [32,33], the bulk of damaging ROS production occurs extracel-lularly. The ROS thus produced can react with molecules in the cell membrane, leading to cell

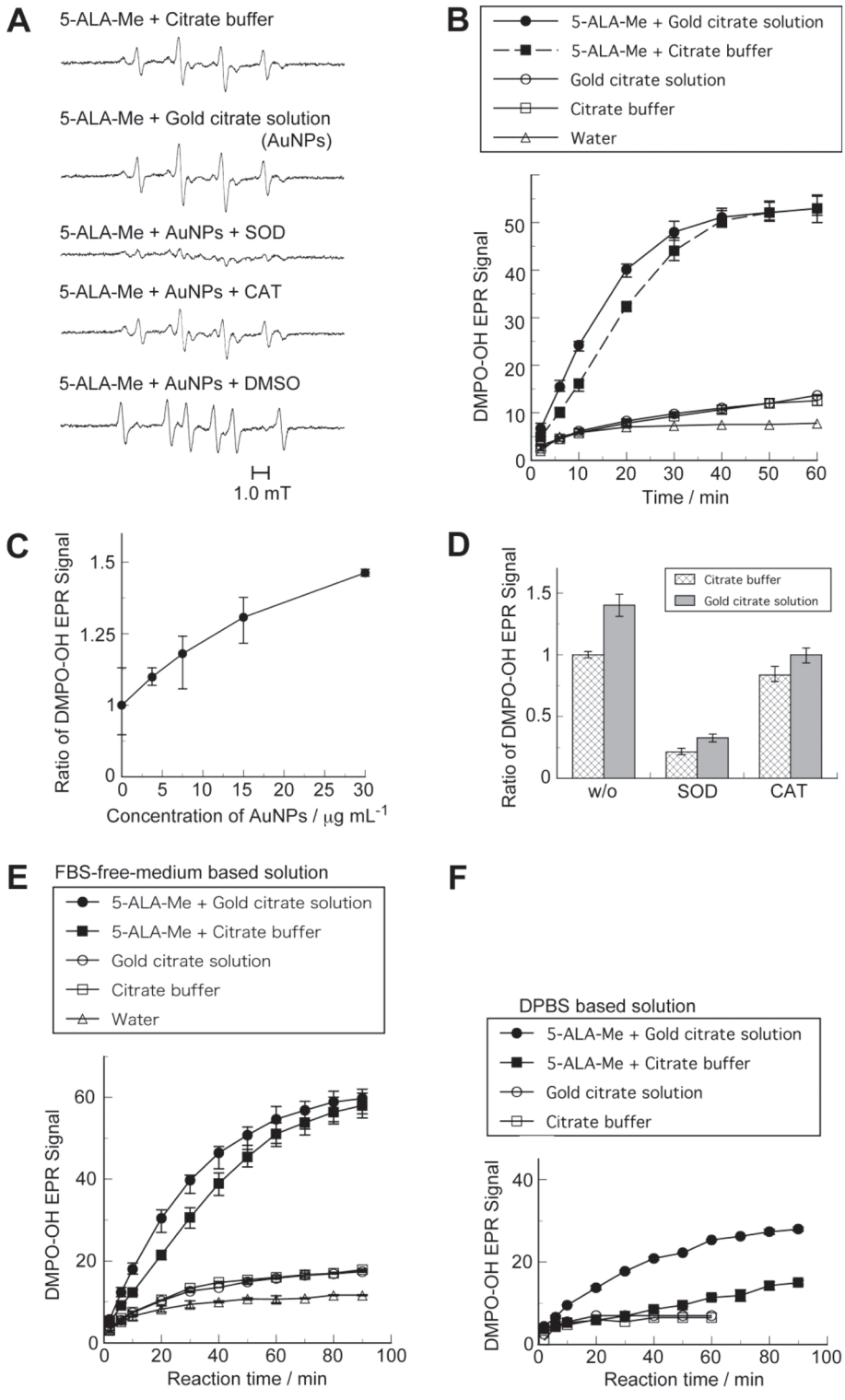

Figure 6. EPR spin trapping studies using DMPO (0.3 M) for FBS-medium based cell-free samples containing 5-ALA-Me and AuNPs. (A) Representative DMPO-OH EPR signals for FBS-medium based cell-free samples for the control containing 5-ALA-Me and citrate buffer, with added gold citrate solution (AuNPs; 30 μg/mL) and with added SOD (77 units/mL) or CAT (6.6×10^3units/mL). The addition of DMSO (8%, v/v) converts hydroxyl radical to methyl radical which gives the six-line DMPO-CH_3 spectrum. (B) Time evolution of the DMPO-OH signal. (C) Dependence of DMPO-OH signal intensity on AuNPs concentration. (D) Inhibiting effect of SOD or CAT. (E) As in (B) but without FBS. (F) As in (B) but with DPBS in place of FBS- medium. In (A, C–F) the EPR signals were measured 20 min after mixing. In (C) and (D), the ratios were calculated by comparing with 5-ALA-Me free controls Concentrations: [AuNPs] =30 μg/mL, [5-ALA-Me] =40 mM for (A–D) and 20 mM for (E) and (F). Data indicate mean ± SD. *n=3* for (C) and (D); 5 for (B); 4 for (E); 2 for (F).

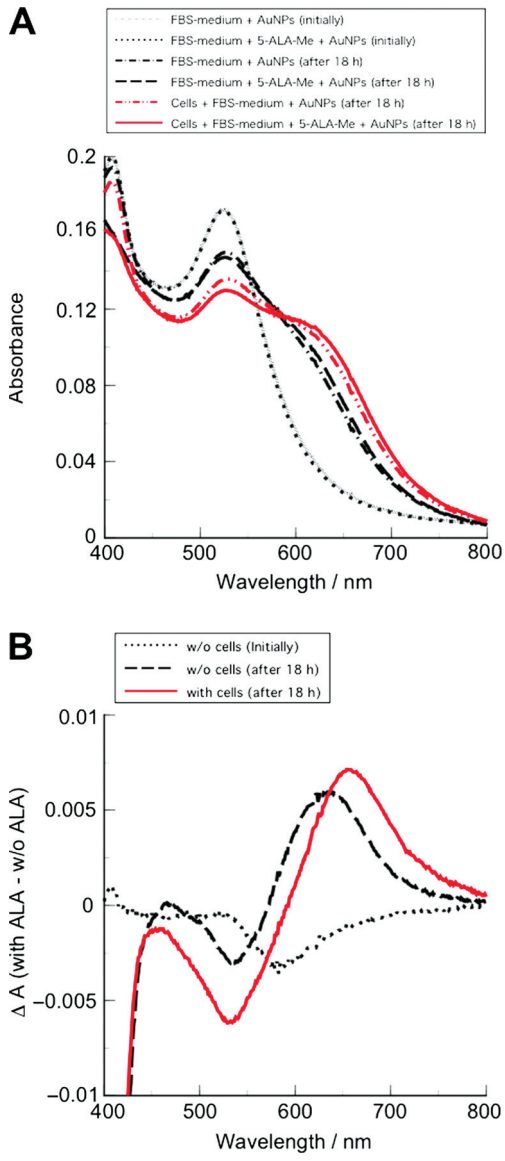

Figure 7. Visible spectra of AuNPs solutions. (A) Effect of standing both in the absence and presence of cells. (B) Effect of 5-ALA-Me. Similar changes were apparent after 6 h.

death. We note, for example, the large aggregation of AuNPs outside, but close to, the cell membrane (Figure 5C) for the highly cytotoxic experiments incubated in the absence of FBS (cf. Figure 3B).

While cell damage is initiated by ROS production, attack at the cell membrane can be direct and/or after formation of organic peroxyl radicals resulting from reactions of the ROS with components of the growth medium. These, then, can attack critical cellular sites due to their ability to diffuse significant distances and to initiate lipid peroxidation in cellular membranes [34–36].

The fact that either SOD or CAT brings cell viability to near normal for 5.0 mM 5-ALA-Me must mean that there is a threshold for cell killing and that either additive brings total ROS below that threshold (Figure 4), an idea that is consistent with the observation of high viability at shorter incubation times (Figure 1D). When the 5-ALA-Me concentration is raised to 7.5 mM, the restoration is not complete, apparently because of the larger steady state yield of radicals.

Although we have concluded that ROS are produced in the medium outside the cell with resulting damage to the membrane, we have also noted uptake of AuNPs without cell damage as well as internally damaged cells from samples containing the levulinic acids. AuNPs enter cells by endocytosis [14,16]. Thus, endocytic uptake of AuNPs may lead to increased cytotoxicity beyond that of the externally produced ROS. In fact, the failure of SOD and CAT to fully protect cells at higher levulinic acid concentrations may be due to some ROS production within the cell, especially after damage at the membrane. The ROS in the interior of cells may attack the cellular organelles such as mitochondria and microsomes, enhancing the oxidative stress. In connection with uptake of 5-ALA with nanoparticles, a recent study using a drug containing 5-ALA and a nanocolloid lotion with sizes ranging from 10-200 nm demonstrated intense fluorescence due to endocytotic uptake of 5-ALA with the polymeric nanoparticles leading to over-production of Protoporphyrin IX and $^1O_2$ photo-toxicity during exposure to white light [37]. Oo et al. [30] demonstrated that uptake of 5-ALA-conjugated 30 nm AuNPs mediated more phototoxicity, but did not increase cytotoxicity in the dark for human tumour cells. We have also observed that larger (40, 80 and 100 nm) AuNPs in the presence of 5-ALA did not increase cytotoxicity for MCF-7 and HepG2 cells (data not shown). Since all of our operations were carried out in the dark, the cytotoxicity we observed cannot be due to analogous over-production of protoporphyrin IX.

In conclusion, incubation of two cell lines with 5-ALA or its methyl ester with 15 nm AuNPs induced greater cytotoxicity that the levulinic acid alone. While experiments with added SOD and CAT point to production of damaging ROS outside the cell, TEM data, UV-visible spectra and spin trapping experiments indicate several possible combinations of interaction between AuNPs, the levulinic acids and medium responsible for loss of cell viability. The combination of the levulinic acids and AuNPs may have potential for new medical applications. It is possible to kill cancer cells by use of the levulinic acids at a low concentration, without photoirradiation, thus decreasing the risk of the adverse effects caused by the levulinic acids. AuNPs bound to a membrane receptor [38,39] can target specific cells. Also, liposomes can enable transport of the levulinic acids and AuNPs to the targeted cells [40]. By using such techniques it may become possible for the combination of the levulinic acids and AuNPs to kill the cancer cells selectively without damaging normal cells. This work is a first step towards

enhancing 5-ALA induced oxidative stress by AuNPs. The chemical and biological mechanisms of putative interaction between the levulinic acids and AuNPs constitutes a challenging subject for future studies.

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References

[1] Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M, Moan J, Peng Q. Review photodynamic therapy. J Natl Cancer Inst 1998;90:889–905.

[2] Fotinos N, Campo MA, Popowycz F, Gurny R, Lange N. 5-Aminolevulinic acid derivatives in photomedicine: characteristics, application and perspectives. Photochem Photobiol 2006;82:994–1015.

[3] Kinoshita M, Hynynen K. Mechanism of porphyrin-induced sonodynamic effect: possible role of hyperthermia. Radiat Res 2006;165:299–306.

[4] Karbownik M, Reiter RJ. Melatonin protects against oxidative stress caused by delta-aminolevulinic acid: implications for cancer reduction. Cancer Invest 2002;20:276–286.

[5] Monteiro HP, Abdalla DSP, Faljoni-Alario A, Bechara EJH. Generation of active oxygen species during coupled autoxidation of oxyhemoglobin and δ-Aminolevulinic acid. Biochim Biophys Acta 1986;881:100–106.

[6] Monteiro HP, Abdalla DSP, Augusto O, Bechara EJH. Free radical generation during δ-Aminolevulinic acid autoxidation: induction by hemoglobin and connections with porphyrinpathies. Arch Biochem Biophys 1989;271:206–216.

[7] Hermes-Lima M, Valderez VGR, Vercesi AE, Bechara EJH. Damage to rat liver mitochondria promoted by δ-aminolevulinic acid-generated reactive oxygen species: connections with acute intermittent porphyria and lead-poisoning. Biochim Biophys Acta 1991;1056:57–63.

[8] Hermes-Lima M. How do Ca^{2+} and 5-aminolevulinic acid-derived oxyradicals promote injury to isolated mitochondria? Free Radic Biol Med 1995;19:381–390.

[9] Timmins GS, Liu KJ, Bechara EJ, Kotake Y, Swartz HM. Trapping of free radicals with direct in vivo EPR detection: a comparison of 5,5-dimethyl-1-pyrroline-N-oxide and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide as spin traps for $HO^{\cdot}$ and $SO_4^{\cdot-}$. Free Radic Biol Med 1999;27: 329–333.

[10] Morofuji Y, Matsuo T, Hayashi Y, Suyama K, Nagata I. Usefulness of intraoperative photodynamic diagnosis using 5-aminolevulinic acid for meningiomas with cranial invasion: technical case report. Neurosurgery 2008;62:102–104.

[11] Ghosh P, Han G, De M, Kim CK, Rotello VM. Gold nanoparticles in delivery applications. Adv Drug Deliv Rev 2008;17:1307–1315.

[12] Hainfeld JF, Slatkin DN, Smilowitz HM. The use of gold nanoparticles to enhance radiotherapy in mice. Phys Med Biol 2004;49:N309–N315.

[13] Connor EE, Mwamuka J, Gole A, Murphy CJ, Wyatt MD. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. Small 2005;1:325–327.

[14] Chithrani BD, Ghazani AA, Chan WCW. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett 2006;6:662–668.

[15] Kong T, Zeng J, Wang X, Yang X, Yang J, McQuarrie S, McEwan A, Roa W, Chen J, Xing JZ. Enhancement of radiation cytotoxicity in breast-cancer cells by localized attachment of gold nanoparticles. Small 2008;4:1537–1543.

[16] Lewinski N, Colvin V, Drezek R. Cytotoxicity of nanoparticles. Small 2008;4:26–49.

[17] Pan Y, Neuss S, Leifert A, Fischler M, Wen F, Simon U, Schmid G, Brandau W, Jahnen-Dechent W Size-dependent cytotoxicity of gold nanoparticles. Small 2007;3:1941–1949.

[18] Mukherjee P, Bhattacharya R, Bone N, Lee YK, Patra CR, Wang S, Lu L, Secreto C, Banerjee PC, Yaszemski MJ, Kay EN, Mukhopadhyay D. Potential therapeutic application of gold nanoparticles in B-chronic lymphocytic leukemia (BCLL): enhancing apoptosis. J Nanobiotechnol 2007;5:4.

[19] Pernodet N, Fang X, Sun Y, Bakhtina A, Ramakrishnan A, Sokolov J, Ulman A, Rafailovich M. Adverse effects of citrate/gold nanoparticles on human dermal fibroblasts. Small 2006;2:766–773.

[20] Patra HK, Banerjee S, Chaudhuri U, Lahiri P, Dasgupta AK. Cell selective response to gold nanoparticles. Nanomed 2007;3:111–119.

[21] Gonda MA, Aaronson SA, Ellmore N, Zeve VH, Nagashima K. Ultrastructural studies of surface features of human normal and tumor cells in tissue culture by scanning and transmission electron microscopy. J Natl Cancer Inst 1976;56:245–263.

[22] Mashino T, Fridovich I. Superoxide radical initiates the autoxidation of dihydroxyacetone. Arch Biochem Biophys 1987;254:547–551.

[23] Villamena FA, Merle JK, Hadad CM, Zweier JL. Superoxide radical anion adduct of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). 2. The thermodynamics of decay and EPR spectral properties. J Phys Chem A 2005;109:6089–6098.

[24] Zhang Z, Berg A, Levanon H, Fessenden RW, Meisel D. On the interactions of free radicals with gold nanoparticles. J Am Chem Soc 2003;125:7959–7963.

[25] Jia HY, Liu Y, Zhang XJ, Han L, Du LB, Tian Q, Xu YC. Potential oxidative stress of gold nanoparticles by induced-NO releasing in serum. J Am Chem Soc 2009;131:40–41.

[26] Tedesco S, Doyle H, Redmond G, Sheehan D. Gold nanoparticles and oxidative stress in Mytilus edulis. Mar Environ Res 2008;66:131–133.

[27] Hvolbaek B, Janssens TVW, Clausen BS, Falsig H, Christensen CH, Norskov JK. Catalytic activity of Au nanoparticles. Nano Today 2007;2:14–18.

[28] Cortie MB, van der Lingen E. Catalytic gold nano-particles. Materials Forum 2002;26:1–14.
[29] Uelinger P, Zellweger M, Wagnières G, Juillerat-Jeanneret L, van der Bergh H, Lange N. 5-Aminolevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells. J Photochem Photobiol B 2000;54:72–80.
[30] Oo MK, Yang X, Du H, Wang H. 5-aminolevulinic acid-conjugated gold nanoparticles for photodynamic therapy of cancer. Nanomed 2008;3:777–786.
[31] Horovitz O, Tomoaia G, Mocanu A, Yupsanis T, Tomoaia-Cotisel M. Protein binding to gold colloids. Gold Bull 2007; 40:213–217.
[32] Gaullier JM, Berg K, Peng Q, Anholt H, Selbo PK, Ma LW, Moan J. Use of 5-aminolevulinic acid esters to improve photodynamic therapy on cells in culture. Cancer Res 1997;57:1481–1486.
[33] Rud E, Gederaas O, Høgset A, Berg K. 5-aminolevulinic acid, but not 5-aminolevulinic acid esters, is transported into adenocarcinoma cells by system BETA transporters. Photochem Photobiol 2000;71:640–647.

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[34] Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1–85.
[35] Dix TA, Aikens J. Mechanisms and biological relevance of lipid peroxidation initiation. Chem Res Toxicol 1993; 6:2–18.
[36] Misik V, Riesz P. Free radical intermediates in sonodynamic therapy. Ann N Y Acad Sci 2000;899:335–348.
[37] Hurlimann AF, Hanggi G, Panizzon RG. Photodynamic therapy of superficial basal cell carcinomas using topical 5-Aminolevulinic acid in a nanocolloid lotion. Dermatology 1998;197:248–254.
[38] Lynch I, Dawson KA, Linse S. Detecting cryptic epitopes created by nanoparticles. Sci STKE 2006;pe14.
[39] Jiang W, Kim BY, Rutka JT, Chan WC. Nanoparticle-mediated cellular response is size-dependent. Nat Nanotech- nol 2008;3:145–150.
[40] Kojima C, Hirano Y, Yuba E, Harada A, Kono K. Preparation and characterization of complexes of liposomes with gold nanoparticles. Colloids Surf B Biointerf 2008;15: 246–252.