Gold(III) Porphyrin 1a Induced Apoptosis by Mitochondrial Death Pathways Related to Reactive Oxygen Species

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Abstract

Apoptosis is a tightly controlled multistep mechanism of cell death, and mitochondria are considered to play a central role in this process. Mitochondria initiate two distinct apoptosis pathways, one caspase-dependent and the other caspase-independent. In addition, mitochondrial production of reactive oxygen species (ROS) seems to play a role in cell death. Most chemotherapeutic agents induce apoptosis through at least one of these pathways. The post-initiation mechanisms of gold(III) porphyrin 1a were investigated in this study. HONE1 cells exposed to gold(III) porphyrin 1a underwent apoptosis after 24 hours. Functional proteomic studies revealed the alteration of several cytoplasmic protein expressions in HONE1 cells after treatment with the drug. These proteins include enzymes participating in energy production and proteins involved in cellular redox balance. There was a quick attenuation of mitochondrial membrane potential (ΔΨm) with the alterations of Bcl-2 family proteins, the release of cytochrome c, and apoptosis-inducing factor (AIF) following gold(III) porphyrin 1a treatment. Cytochrome c in turn activated caspase-9 and caspase-3. Cotreatment with caspase inhibitor (zVAD-fmk) showed that the activated caspases worked in conjunction with AIF-initiated apoptosis pathways. Further study showed that ROS played a part in gold(III) porphyrin 1a–induced apoptosis by regulating ΔΨm. In summary, gold(III) porphyrin 1a induced apoptosis through both caspase-dependent and caspase-independent mitochondrial pathways, and intracellular oxidation affected gold(III) porphyrin 1a–induced apoptosis. These results support a role for gold(III) porphyrin 1a as a promising anticancer drug lead and as a possible novel therapeutic agent directed toward the mitochondria. (Cancer Res 2005; 65(24): 11553-64)

Introduction

Apoptosis is a tightly controlled multistep mechanism of cell death. Mitochondria are believed to play a central role in the process of apoptosis, as it has been observed that the antiapoptotic Bcl-2 protein localizes to the outer membrane of these organelles (1). In recent years, it has become clear that one of the major events during apoptosis is the permeabilization of the mitochondrial outer membrane. This is facilitated by the Bcl-2 family proteins to release proteins from the intermembrane space and initiate both caspase-dependent and caspase-independent apoptosis pathways (2).

Release of cytochrome c from the mitochondrial intermembrane space initiates the caspase-dependent pathway by activating caspase-9 and caspase-3 (3). The hierarchical activation of these caspases ultimately results in the biological hallmarks of nuclear apoptosis, including the cleavage of poly (ADP-ribose) polymerase-1 (PARP-1) and DNA fragmentation and/or DNA laddering (4). The caspase-independent pathway is mainly mediated by nuclear translocation of apoptosis-inducing factor (AIF) from mitochondria (5). Upon induction of apoptosis, AIF translocates from the mitochondria to the nucleus and causes chromatin condensation and large-scale (~50 kb) DNA fragmentation (6).

Mitochondrial production of reactive oxygen species (ROS) also seems to play a role in cell death (7). ROS, the byproducts of normal cellular oxidative processes, have been suggested to regulate the process involved in the initiation of apoptotic signaling. The cytotoxicity of anticancer chemotherapeutic drugs may largely depend on the intracellular level of reduced glutathione (GSH). Altering the GSH level with antioxidant agents that could reduce ROS after drug treatment may change the apoptotic situations inside cells if ROS is the major cause of apoptosis.

We have shown in previous studies that gold(III) porphyrin 1a exhibited higher cytotoxicity than cisplatin and induced apoptosis in a human nasopharyngeal carcinoma cell line (article accepted by Proteomics). In the present study, we applied a two-dimensional electrophoresis-based proteomic approach together with other biochemical methods to identify the major cellular pathways activated upon gold(III) porphyrin 1a treatment. We also studied how these pathways might contribute to gold(III) porphyrin 1a–induced apoptosis and showed that gold(III) porphyrin 1a induced apoptosis by mitochondrial death pathways related to ROS. These findings suggest that gold(III) porphyrin 1a is a promising anticancer drug lead and a possible novel therapeutic agent directed toward the mitochondria.

Materials and Methods

Materials and reagents. Gold(III) porphyrin 1a was synthesized and purified as described previously (8). Cisplatin and chlorophyllin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 4′,6-Diamidino-2-phenylindole (DAPI) was obtained from Roche (Mannheim, Germany). All other chemicals, except otherwise noted, were obtained from either Sigma-Aldrich Chemical or Amersham Biosciences (Piscataway, NJ). Gold(III) porphyrin 1a, cisplatin, and chlorophyllin were dissolved in DMSO and kept as stock solutions.

Cell lines and cell culture. Human nasopharyngeal carcinoma cell lines (HONE1, HNE1, SUNE1, and CNE1) and human nasopharyngeal cell NP 69 were generously provided by Prof. S.W. Tsao (Department of Anatomy, The University of Hong Kong). These nasopharyngeal carcinoma cell lines were cultured in RPMI 1640 with 2.0 g/L sodium bicarbonate plus 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 2 mmol/L 1-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies...
Gold(III) porphyrin 1a induces apoptosis in cancer cells. 

A, NBB assay showed that gold(III) porphyrin 1a exhibits about 4.5-fold greater cytotoxicity on a number of human NPC cells than their normal variants, whereas cisplatin exhibits >750-fold greater cytotoxicity on the same cell lines. Columns, mean of triplicates from three separate experiments; bars, SD. 

B, fluorescence microscopy images of DAPI-stained cells showing the appearance of apoptotic morphology in gold(III) porphyrin 1a– and cisplatin-treated cancer cell lines in a dose-dependent manner. Magnification, ×400. 

C, quantitative analysis of % condensed DNA as in (B).
Bethesda Research Laboratories, Grand Island, NY). The NP 69 cell line was cultured in Keratinocyte-SFM (Life Technologies Bethesda Research Laboratories) medium plus Supplements for Keratinocyte-SFM (Life Technologies Bethesda Research Laboratories). All cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO₂ at 37°C.

**Cytotoxicity assay.** The cytotoxicity of gold(III) porphyrin 1a, cisplatin, and chlorophyllin was determined by naphthol blue black (NBB) staining assay, in accordance with a previously reported procedure (9). The NP 69 cells were changed to RPMI 1640 the day before the assay to minimize the effects of different contents in the media on the cytotoxicity of the analyzed drugs.

**Drug treatment.** The cells were grown to about 80% confluence and were then either subcultured or treated with 2 μmol/L gold(III) porphyrin 1a, 60 μmol/L cisplatin, or 2 mmol/L chlorophyllin. Cisplatin and chlorophyllin were used as positive control drugs for different experiments. These doses

![Figure 1](https://www.aacrjournals.org/external/0009780/large.png)

*Figure 1. Continued. D, fluorescence-activated cell sorting analysis showing the presence of a sub-G₁ peak in the adherent cell population 12 and 24 hours after gold(III) porphyrin 1a treatment. The sub-G₁ peak was confirmed to represent apoptotic cells by examination of ethanol-permeabilized cells containing propidium iodide. WinMDI 2.8 software analysis also revealed a decrease in the proportion of cells in G₀-G₁ and an increase in the DNA content at the S and G₂-M phases of cell cycle. E, DNA ladder was formed after gold(III) porphyrin 1a or cisplatin treatment in both a time-dependent and dose-dependent manner in HONE1 cells rather than in NP 69 cells. F, immunoblot of the cleavage form of PARP-1 (~89 kDa) was detectable after 24 hours of treatment with gold(III) porphyrin 1a (2 μmol/L) or cisplatin (60 μmol/L) in HONE1 cells but not in NP 69 cells. Representative from three separate experiments (B, D, E, and F).
were about twice the IC₅₀ value. To determine whether gold(III) porphyrin 1a induced cell death via the activation of caspase, pan-caspase inhibitor zVAD-fmk (0.1 mmol/L) was pretreated 24 hours before the addition of apoptosis-inducing agents. In some experiments, cells were pretreated with 15 mmol/L N-acetyl-l-cysteine (NAC), 10 mmol/L GSH, 2 mmol/L DTT, 500 units/mL superoxide dismutase (SOD), 500 units/mL catalase, 0.5 mmol/L desferrioxamine, 1.5 mmol/L ATP, 50 μmol/L/astolic acid, 5 μmol/L cyclosporine A, 50 μmol/L/astolic acid, plus 5 μmol/L cyclosporine A, 1 μmol/L trifluoperazine, and 1 μmol/L trifluoperazine plus 5 μmol/L cyclosporine A for 1 hour, before the addition of gold(III) porphyrin 1a.

Morphologic changes. To detect morphologic changes in the apoptosis process, cell nuclei were stained with 1 μg/mL DAPI, and the cells were analyzed with a fluorescence microscope (Olympus IX71 CTS Chinettek Scientific Microscope, Hong Kong, China).

Flow cytometric analysis of apoptosis. Gold(III) porphyrin 1a-induced apoptosis was determined by propidium iodide staining with FACStar Plus flow cytometry, in accordance with a previously reported method (9).

DNA ladder. DNA laddering was assessed by a protocol developed by Fukuda et al. (10). Equal quantities (5-30 μg) of DNA extracted from gold(III) porphyrin 1a or cisplatin treatments were run on 1.4% agarose gels in Tris-borate EDTA buffer. Bands were detected by ethidium bromide staining.

Subcellular fractionation isolation. Nuclear protein was extracted as follows. PBS-washed cells were resuspended in extraction buffer [10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L KCl, 5 mmol/L MgCl₂] with protease inhibitors [1 mmol/L phenylmethylsulfonyl fluoride and 0.1% protease inhibitor cocktail (Oncogene, Uniondale, NY)]. The cells were incubated on ice for 10 minutes and lysed by addition of Triton X-100. Nuclei were removed by centrifugation, and nuclear protein was extracted with radioimmunoprecipitation assay buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mmol/L DTT] supplemented with protease inhibitors. The supernatant from nuclei isolation was further centrifuged at 10,000 × g for 30 minutes. The supernatant was saved as cytoplasmic fraction, and the pellet was saved as heavy membrane fraction containing mitochondria. Cytoplasmic proteins were precipitated by 10% trichloroacetic acid for proteomic analysis or Western blot, and heavy membrane fraction was extracted with radioimmunoprecipitation assay buffer for Western blot.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was carried out with Amersham Biosystem IPGphor IEF and Hoefer Tank (13 cm) units, in accordance with a previously described protocol (11). Protein samples (100 μg) of cytoplasmic fractions extracted from untreated control, 2 μmol/L gold(III) porphyrin 1a, and 60 μmol/L cisplatin treatments, were used for two-dimensional gel electrophoresis analysis. Untreated cells were used as a control. Tricarboxylic acid was done to ensure reproducibility. All gels were visualized by silver staining (11).

Image analysis and mass spectrometry peptide sequencing. Image acquisition and analysis were done with Image Scanner (Amersham, Biosciences) and Image Master 2D Elite software (Amersham; ref. 11). Comparisons were made between gel images of cells treated with gold(III) porphyrin 1a and cisplatin and those of untreated controls. Altered protein spots that changed consistently and significantly (≥2-fold difference) were selected for analysis with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). In database matching using MS-Fit,4 proteins with 25 ppm or better mass accuracy and MOWSE scores of-flight mass spectrometry (MALDI-TOF MS). In database matching using selected for analysis with matrix-assisted laser desorption/ionization-time-...
decrease in both S and G0-G1 phases; however, the effect of cisplatin on cell cycle was not as dramatic as gold(III) porphyrin 1a (Fig. 1D).

Gold(III) porphyrin 1a-induced apoptosis was also confirmed by the oligonucleosomeic degradation of cellular DNA (Fig. 1E), as this type of chromatin degradation is characteristic in apoptotic cell death (10). DNA ladder was detected in gold(III) porphyrin 1a treatment in both time-dependent and dose-dependent manners in HONE1 cells (Fig. 1E, right), whereas no such DNA fragmentation was detected in NP 69 cells under similar conditions (Fig. 1E, left). The proteolytic cleavage of PARP-1 between Asp216 and Gly217 may also serve as an early indicator of apoptosis. This action cleaves the 116-kDa native enzyme into 89- and 36-kDa fragments (4). The appearance of the 89-kDa fragment was detected in HONE1 cell extracts after treatment with both gold(III) porphyrin 1a and cisplatin, in a time-dependent manner in HONE1 cells, but not in NP 69 cells (Fig. 1F). Again, this finding confirmed that gold(III) porphyrin 1a induced apoptosis in cancer cells.

Altered cytoplasmic proteins are related to energy production and oxidative stress. Because cytoplasm is the generative site for protein synthesis, energy production, glycerol use, and oxidative stress, we applied proteomic technology to study the regulation of cytoplasmic proteins in gold(III) porphyrin 1a treatment. More than 1,000 distinct protein spots were resolved on two-dimensional gel within the ranges of pH 3 to 10 and molecular weights of 6 to 200 kDa. After the silver-stained gels from three independent experiments were analyzed, the protein spots, which differed significantly (>2-fold difference) from the untreated control samples after gold(III) porphyrin 1a and cisplatin treatment, were subjected to MALDI-TOF MS analysis for protein ID. Of the several clusters of identified proteins (>20 altered proteins), the two most notable groups of altered proteins were enzymes that participate in energy production, including 3-phosphoglycerate dehydrogenase (PHGDH), glutamate dehydrogenase 1 (GDH), actin-related protein 1 homologue A (ARP1), sorbitol dehydrogenase (SDH), and glucose-6-phosphate dehydrogenase (G6PD; Fig. 2), and proteins involved in cellular redox balance, including thioredoxin peroxidase and peroxiredoxin 3 isoform (Fig. 2). Table 1 shows protein ID through peptide fingerprinting matching, fold differences of expression, and functional groups of the identified proteins. These alterations of protein expression suggest that gold(III) porphyrin 1a executes cytotoxicity through mitochondria-initiated apoptosis pathways. We next investigated this possibility by biochemical methods and further determined the role of mitochondria in the process of gold(III) porphyrin 1a–induced apoptosis.

Gold(III) porphyrin 1a causes attenuation of $\Delta \psi_m$, alteration of Bcl-2 family proteins, and release of cytochrome c and apoptosis-inducing factor. Because energy production and generation of ROS are directly related to mitochondria (7), we conducted experiments to analyze mitochondria of cells treated with gold(III) porphyrin 1a. During apoptosis, the mitochondrial outer membrane becomes permeable, which is necessary for caspase activation in the mitochondrial pathway (3). Using Rho-123 as a marker of mitochondrial membrane integrity, flow cytometric studies revealed a very quick depletion (within 3 hours) of $\Delta \psi_m$ in gold(III) porphyrin 1a treatment (Fig. 3A). That gold(III) porphyrin 1a caused depletion of $\Delta \psi_m$ was confirmed by JC-1 staining after 3 hours of treatment (Fig. 3B). In fluorescent microscopy images, mitochondrial membrane depolarization was detected due to the lack of orange fluorescence of J-aggregate at hyperpolarized membrane potentials (Fig. 3B, i). This was in accordance with the results of Rho-123 staining (Fig. 3B, i). Western blot analysis revealed that Bcl-2 expression was suppressed immediately after gold(III) porphyrin 1a treatment, whereas the protein expression of Bax did not respond at all to this treatment (Fig. 3C). We next examined the release of cytochrome c from mitochondria. An increased amount of cytochrome c was found in the soluble cytoplasmic fraction of cells treated with gold(III) porphyrin 1a for 6, 12, and 24 hours (Fig. 3D). The enhanced level of AIF was determined in the nucleus of cells treated with gold(III) porphyrin 1a in a time-dependent manner (Fig. 3E). AIF translocation to the nucleus was also detected in chlorophyllin-treated cells but was not observed after cisplatin treatment.

Activation of caspase-9 and caspase-3 partially contributes to gold(III) porphyrin 1a–induced apoptosis. The release of the mitochondrial proteins AIF and cytochrome c precedes the downstream events of nuclear condensation and caspase activation (15, 16). Nucleus translocation of AIF activates endonuclease G and subsequently causes DNA large fragmentation (17, 18). Our results showed a large DNA fragmentation in the cells at 12 hours of treatment with gold(III) porphyrin 1a (Fig. 1E). Caspases are major mediators of apoptosis whose activation depends on proteolytic cleavage of the procaspase to a smaller, enzymatically active form. We directly measured enzyme activities of caspases with a spectrofluorometric assay using their corresponding substrates. The initial enzyme activities of caspase-3 and caspase-9 were observed at about 3 hours. Activity peaked between 6 and 12 hours, then tapered off after 24 hours of treatment with gold(III) porphyrin 1a (Fig. 4A).
Table 1. Altered expressed cellular proteins in gold(III) porphyrin 1a (2 μmol/L, 24 hours) or cisplatin (60 μmol/L, 24 hours) treated HONE1 cell

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>NCBI accession no.</th>
<th>Mass (Da/pI)</th>
<th>Coverage (%)</th>
<th>Mean error (ppm)</th>
<th>Fold differences† (mean ± SD)</th>
<th>Major function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHGDH</td>
<td>23308577</td>
<td>56,651/6.3</td>
<td>28.0</td>
<td>1.05</td>
<td>−1.99 ± 0.55</td>
<td>Not determined</td>
</tr>
<tr>
<td>GDH</td>
<td>4885281</td>
<td>61,398/7.7</td>
<td>16.0</td>
<td>1.71</td>
<td>−1.99 ± 0.58</td>
<td>Involved in amino acid metabolism</td>
</tr>
<tr>
<td>ARPI</td>
<td>5031569</td>
<td>42,614/6.2</td>
<td>23.0</td>
<td>5.46</td>
<td>−1.99 ± 0.58</td>
<td>Involved in glucose metabolism</td>
</tr>
<tr>
<td>SDH</td>
<td>4033691</td>
<td>38,297/8.2</td>
<td>21.0</td>
<td>3.19</td>
<td>−2.18 ± 0.37</td>
<td>Glycerol use</td>
</tr>
<tr>
<td>SDH</td>
<td>4033691</td>
<td>38,297/8.2</td>
<td>19.0</td>
<td>3.74</td>
<td>−2.00 ± 0.11</td>
<td>Glycerol use</td>
</tr>
<tr>
<td>G6PD</td>
<td>26224790</td>
<td>54,824/6.9</td>
<td>14.0</td>
<td>1.84</td>
<td>−1.51 ± 0.36</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Thioredoxin peroxidase</td>
<td>5453549</td>
<td>30,540/5.9</td>
<td>22.0</td>
<td>5.90</td>
<td>−4.72 ± 0.25</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Peroxiredoxin 3 isoform</td>
<td>5802974</td>
<td>27,693/7.7</td>
<td>13.0</td>
<td>0.875</td>
<td>−2.97 ± 0.45</td>
<td>Oxidative stress</td>
</tr>
</tbody>
</table>

Abbreviation: ND, nondetectable.
†Average expression level in gold(III) porphyrin 1a– or cisplatin-treated HONE1 cells to untreated control cells from three independent analyses (−, decrease).

At the same time, we only detected a slight increase in caspase-8 activity (Fig. 4A). zVAD-fmk, a pan-caspase inhibitor, was then used to verify the involvement of caspases in the mechanism(s) of gold(III) porphyrin 1a–induced apoptosis. Cells pretreated with 0.1 mmol/L zVAD-fmk were analyzed by NBB assay for cytotoxicity. The viability of cells treated with cisplatin increased from 46% to 90% after pretreatment with zVAD-fmk. However, cell viability in gold(III) porphyrin 1a treatment only increased from 40% to 65% in the same circumstances (Fig. 4B). Furthermore, nuclear morphologic changes detected by DAPI staining (Fig. 4C) revealed that the percentage of the cell population showing typical apoptotic morphology decreased from 63.4% to 30.5% on pretreatment with zVAD-fmk in gold(III) porphyrin 1a treatment but changed from 59.4% to 15.5% in cells cotreated with cisplatin and zVAD-fmk (Fig. 4C). In addition, the nuclear translocation of AIF was detected in the presence of zVAD-fmk at 6 hours of treatment of gold(III) porphyrin 1a (Fig. 4D).

Reactive oxygen species plays a role in the cytotoxicity of gold(III) porphyrin 1a. In these experiments, we showed that mitochondria initiated gold(III) porphyrin 1a–induced apoptosis. Because the mitochondrial respiratory chain on the inner mitochondrial membrane is a major intracellular source of ROS (19), we investigated the relevance of oxidation to apoptosis. Possible generation of intracellular oxidants by gold(III) porphyrin 1a was assessed by measuring the extent of oxidation with sensitive dye DCFH-DA, which reacts with cytosolic H₂O₂ and other peroxides in the presence of peroxidase. Reaction of DCFH-DA with H₂O₂ results in the formation of a fluorescent product that can be quantified by fluorescence-activated cell sorting. In this experiment, we examined the oxidative status in gold(III) porphyrin 1a– or cisplatin-treated HONE1 cells, using H₂O₂ as a positive control. Figure 5A shows that gold(III) porphyrin 1a generated an increase in DCFH-DA fluorescence within 30 minutes. Exposure of HONE1 cells to gold(III) porphyrin 1a or cisplatin resulted in a drop in intracellular GSH level within 5 minutes of treatment, and then steady increases were detected in both agents until 2 hours of treatment (Fig. 5B). However, GSH depletion was found after 4 hours of treatment, corresponding to the phenomenon of apoptosis (20). These results suggest that gold(III) porphyrin 1a generated ROS in HONE1 cells.

To further investigate the role of oxidative stress in gold(III) porphyrin 1a–induced apoptosis, we evaluated the effects of a series of ROS detoxification agents and mitochondrial permeability transition regulating agents on the change of ΔΨᵐ in induced by gold(III) porphyrin 1a after 3 hours of treatment. We found that the mitochondria lost nearly 90% of their ΔΨᵐ (Fig. 3A and Fig. 5C). Pretreatment with NAC, GSH, DTT, catalase, desferrioxamine, trifluoperazine, and trifluoperazine plus cyclosporine A could partially prevent the depletion of ΔΨᵐ caused by gold(III) porphyrin 1a (Fig. 5C). By contrast, pretreatment with SOD, ATP, astrolithic acid, cyclopurine A, or astrolithic acid plus cyclosporine A had no effect on mitochondrial permeabilization under the same conditions. These agents alone did not affect ΔΨᵐ significantly (Fig. 5C). These data suggest that cellular oxidative status could affect gold(III) porphyrin 1a–induced apoptosis by regulating ΔΨᵐ, the central component of the apoptosis pathways.

Discussion

Novel anticancer chemotherapeutic drugs with different modes of action are needed for the treatment of cancer patients, particularly for those refractory to standard treatment (e.g., cisplatin-resistant patients). The potential application of gold(III) complexes as a new class of anticancer drugs with higher cytotoxicity and fewer side effects than existing metal anticancer drugs has been eagerly explored recently. Our laboratories have been attempting to develop gold(III) meso-tetraarylporphyrins as potential chemotherapeutic leads by elucidating their action mechanisms (8). Determining the detailed molecular pathways through which gold(III) porphyrin 1a induces apoptosis will provide valuable information for the further development of novel anticancer drug leads. Cisplatin was chosen as a control drug in most of the mechanism studies because it is one of the most widely used metal anticancer drugs, with well-understood action mechanisms (21). In addition, cisplatin induces drug resistance, and its side effects still pose major problems for its
clinical use (22). There is an urgent need for the development of new anticancer drug candidates, which exhibit cytotoxicity in a different way from cisplatin and can be applied to patients whose responses to cisplatin treatment are poor.

Gold(III) porphyrin 1a induces apoptosis in cancer cells. Apoptosis is a process of gene-mediated programmed cell death essential for the elimination of unwanted cells in various biological systems and is the key mechanism of chemotherapeutic agents. There are several characteristic cellular and biochemical hallmarks of apoptotic cell death, including oligonucleosomal DNA fragmentation (23), nucleus condensation, DNA laddering, and PARP-1 cleavage (24, 25). In the present study, we discovered that gold(III) porphyrin 1a–treated HONE1 cells showed marked changes in several of the molecular markers examined (Fig. 1).

Interestingly, gold(III) porphyrin 1a stimulated cell cycle arrest at different phases and induced significantly lower cleavage of PARP-1 than cisplatin, suggesting that these two agents cause cell death through different pathways. Because these markers were downstream phenomena of apoptosis, we then did functional proteomic analysis in conjunction with other biochemical techniques to elucidate the detailed action mechanisms of gold(III) porphyrin 1a.

Cellular protein alterations are related to gold(III) porphyrin 1a–induced apoptosis. Using functional proteomic approaches, we examined the proteomic changes associated with gold(III) porphyrin 1a–induced apoptosis in cellular fraction, the site of most biological activities. From the protein targets identified, we revealed several cellular pathways that are potentially involved in this apoptotic process. Two major groups of protein targets altered

Figure 3. Gold(III) porphyrin 1a exhibits cytotoxicity through mitochondria-initiated apoptosis pathways. A, measurement of ΔΨm depolarization in gold(III) porphyrin 1a–treated HONE1 cells by Rho-123 within 3 hours at 30-minute intervals. B, confirmation that gold(III) porphyrin 1a caused ΔΨm depolarization after 3 hours of treatment by (i) Rho-123 using flow cytometric analysis (ii) and JC-1 detected by fluorescent microscopy. C, Western blot analysis of expression of Bcl-2 and Bax under gold(III) porphyrin 1a treatment, using different positive controls from our laboratory that have been confirmed to induce expression of these proteins.
by treatment with this agent are enzymes involved in cellular metabolism and redox balance.

As shown in Table 1 and Fig. 2, the majority of proteins that are negatively regulated are related to energy production. Under apoptotic conditions, cellular energy production is reduced due to mitochondrial dysfunction (26, 27). These proteins are known to influence ATP hydrolysis (e.g., ARP1) and glucose metabolism (e.g., G6PD, SDH, and GDH). G6PD catalyzes the first and the rate-limiting step in several pathways of glucose metabolism, including glycolysis and glycogen synthesis. Altered expression of G6PD has been reported in p53-induced apoptosis in human colorectal cancer cells (28) and in a 14-3-3 knockout mouse model (29). In these cases, apoptosis is mediated through the mitochondria-initiated signaling pathways. Another protein whose expression is significantly down-regulated under gold(III) porphyrin 1a treatment is GDH. GDH, which is known to be involved in glucose metabolism (30), has also been found to be down-regulated in several types of cell death mediated by mitochondria (31). Down-regulation of this group of energy producing proteins in gold(III) porphyrin 1a–induced apoptosis suggests that the mitochondria, the site for the production of ATP through glycolysis and Krebs cycle, may serve as a target for gold(III) porphyrin 1a.

The mode of drug-induced apoptosis has also been described at the level of modifying intracellular oxidative state. Table 1 and Fig. 2 show that thioredoxin peroxidase and peroxiredoxin 3 were down-regulated in gold(III) porphyrin 1a–induced apoptosis. These two proteins are essential for maintaining mitochondrial mass and ∆Ψm and were reported to be suppressed under oxidative state (32, 33). Zhang et al.’s study found that thioredoxin peroxidase could prevent hydrogen peroxide accumulation in cells and serve as an inhibitor of apoptosis acting upstream of Bcl-2 (32). Peroxiredoxin 3, whose overexpression was reported to protect cells against H2O2, t-butylhydroperoxide, and the anticancer drug imexon induced apoptosis (34), regulating apoptotic signaling through the mitochondria (35). Down-regulation of this group of proteins indicates that gold(III) porphyrin 1a induces apoptosis, in part, through the generation of ROS and alteration of cellular oxidative status.

Both thioredoxin peroxidase and peroxiredoxin 3 isoform are associated with the mitochondria, which play a pivotal role in the apoptosis signaling pathways. We further investigate the possibility that gold(III) porphyrin 1a exerts cytotoxicity through mitochondria-mediated apoptotic pathways.

Gold(III) porphyrin 1a exhibits cytotoxicity through mitochondria-initiated apoptosis pathways. Attenuation of ∆Ψm was observed within 3 hours under gold(III) porphyrin 1a treatment (Fig. 3A and B), consistent with the hypothesis that mitochondria are affected particularly early during the apoptotic process. The mitochondria play a crucial role in both caspase-dependent and caspase-independent apoptosis (3). The depletion of mitochondrial membrane potential (∆Ψm) is an early hallmark of apoptosis induced by a variety of stimuli. The mitochondrial membrane potential (∆Ψm) serves as a gatekeeper for the release of proapoptotic factors, such as cytochrome c, from the mitochondrial intermembrane space to the cytosol, which triggers the activation of caspases and the execution of apoptosis.

Figure 3 Continued. D, Western blot analysis of cytochrome c (Cyt c) expressed in heavy membrane containing mitochondria and cytosolic fractions obtained from HONE1 cells treated by gold(III) porphyrin 1a for 6, 12, and 24 hours, using cisplatin treatment as a positive control. Representative of three independent experiments (A, B, C, and D). E, AIF immunostaining (green) of HONE1 cells treated with 2 μmol/L gold(III) porphyrin 1a for 1.5, 3, 6, 9, 12, and 24 hours, using 60 μmol/L cisplatin and 1 mmol/L chlorophyllin (CHL) treatment as negative and positive controls, with DAPI staining of the nucleus (blue). Magnification, ×1,000. Repeated twice with similar results.
Figure 4. Activation of caspases contributed partly to gold(III) porphyrin 1a–induced cell death. A, whole cell lysates from HONE1 cells treated with 2 μmol/L gold(III) porphyrin 1a were assayed for caspase-3, caspase-8, and caspase-9 activities. **, * P < 0.001; *, * P < 0.005 compared with control. B, pan-caspase inhibitor zVAD-fmk could partially inhibit gold(III) porphyrin 1a–induced apoptosis (NBB assay). C, nucleus morphologic changes detected by DAPI stain showing effects of zVAD-fmk on gold(III) porphyrin 1a– and cisplatin-induced cell apoptosis. Magnification, ×400. Result from three separate experiments were quantitated and presented as a chart. D, AIF mitochondria-nuclear translocation could be detected in the presence or absence of zVAD-fmk after 6 hours of treatment with gold(III) porphyrin 1a. Columns, means of triplicates from three separate experiments; bars, SD.
of $\Delta \Psi_{m}$ was facilitated by the suppression of Bcl-2 expression at an early stage (Fig. 3C), which was reported as an early and essential apoptotic response to photodynamic therapy (36). The loss of $\Delta \Psi_{m}$ preceded the release of cytochrome c and AIF upon gold(III) porphyrin 1a treatment (Fig. 3D and E) and further activated downstream effectors caspase-9 and caspase-3 (Fig. 4A). Furthermore, AIF mitochondria-nucleus translocation was not blocked in the presence of pan-caspase inhibitor, zVAD-fmk (Fig. 4D). This result indicates that nucleus translocation of AIF may also partially contribute to gold(III) porphyrin 1a–induced apoptosis and that it does not depend wholly on caspase activation. Our results, together with the results of Susin et al. (6) and Arnoult et al. (37), suggest that the mitochondria-nucleus translocation of AIF does not require caspases activation.

**Figure 5.** Role of ROS in gold(III) porphyrin 1a–induced cell death. A, HONE1 cells were labeled with DCFH-DA and then treated with 2 μmol/L gold(III) porphyrin 1a, 60 μmol/L cisplatin, or 100 mmol/L H$_2$O$_2$ for 30 minutes. Fluorescence intensity in individual cells was measured by fluorescence-activated cell sorting analysis, as described in Materials and Methods. Representative from three separate experiments. B, measurement of GSH level in HONE1 cells treated with gold(III) porphyrin 1a (2 μmol/L) or cisplatin (60 μmol/L). Points, mean of triplicate experiments; bars, SD. C, profile of gold(III) porphyrin 1a–induced $\Delta \Psi_{m}$ alteration regulated by oxidative stress. Before gold(III) porphyrin 1a treatment, HONE1 cells were incubated for 1 hour with NAC, GSH, DTT, SOD, catalase, desferrioxamine (DFO), ATP, aristolochic acid (ArA), cyclosporine A (CyA), aristolochic acid plus cyclosporine A (ArA + CyA), trifluoperazine (TFZ), and trifluoperazine plus cyclosporine A (TFZ + CyA). The relative Rho-123 fluorescent intensity was determined as $\Delta \Psi_{m}$. Columns, means from two separate experiments; bars, SD.
Cisplatin-induced apoptosis is mediated by both the death receptor- and mitochondria-initiated activation of caspase-3 and caspase-8 (38, 39). However, the 2-fold up-regulation of caspase-8 activity (Fig. 4A), together with the failure to detect Bid and lamin A/C cleavage (data not shown), ruled out the possible participation of death receptors and their downstream cascades in gold(III) porphyrin 1a–induced apoptosis (21, 40). These results support the notion that the mitochondria play a central role in the death pathways induced by gold(III) porphyrin 1a other than as mediators of the apoptosis processes induced by cisplatin.

Cellular oxidation is related to cytotoxicity of gold(III) porphyrin 1a. Accumulation and/or action of ROS are secondary to the breakdown of ΔΨm (41), and it has been suggested that the response of mitochondria to ROS could affect drug cytotoxicity (42). Results from Fig. 5A and B indicated that gold(III) porphyrin 1a induced intracellular oxidation, as measured by the formation of oxidized DCFH-DA and the altered level of intracellular GSH. It is known that the toxicity of anticancer agents may largely depend on the intracellular level of reduced GSH, because GSH is the main antioxidant system in the cell. A possible explanation is that GSH depletion facilitates ROS accumulation in cells treated with anticancer agents, which in turn increases their lethality (43).

To clarify the role of ROS in the action mechanisms of gold(III) porphyrin 1a, we evaluated the effects of a series of agents on the depletion of ΔΨm induced by gold(III) porphyrin 1a. These agents can regulate ROS detoxification and mitochondrial permeability transition, such as the scavengers of oxygen-free radicals NAC and GSH, the thiol-reducing agent DTT, and the H2O2-scavenging enzyme catalase, which is known that partially inhibited the membrane permeability induced by gold(III) porphyrin 1a (Fig. 5C). Cotreatment with the iron chelator deferoxamine, which inhibits iron-mediated hydroxyl radical formation from superoxide and hydrogen peroxide (44), also reduced gold(III) porphyrin 1a–induced ΔΨm depletion. In contrast, SOD, a singlet oxygen quencher, failed to inhibit the permeabilization of ΔΨm induced by gold(III) porphyrin 1a (Fig. 5C). These results suggested that gold(III) porphyrin 1a–induced ΔΨm depletion is mediated, at least in part, by cellular H2O2 and/or thiol oxidation. ATP, the physiologic ligand of ANT, could not inhibit the effect of gold(III) porphyrin 1a–induced mitochondrial permeabilization, thus confirming that ANT does not constitute the physical target of this agent (Fig. 5C).

We next investigated whether oxidative stress directly induced mitochondrial permeabilization. The phospholipase A2 inhibitor aristolochic acid and the protein phosphatase 2B inhibitor cyclosporine A have been reported to prevent ΔΨm depletion by inhibiting ROS production alone or in combination (45, 46). HONE1 cells pretreated with these two agents could not block gold(III) porphyrin 1a–induced mitochondrial permeabilization (Fig. 5C). In contrast, trifluoperazine, another well-established mitochondrial permeability transition inhibitor that directly interferes with the formation of membrane protein-thiol cross-linkage (47), partially prevented gold(III) porphyrin 1a–induced ΔΨm depletion. In addition, combined trifluoperazine and cyclosporine A, which block the mitochondrial permeability transition, inhibited about 40% of ΔΨm permeabilization and depolarization in our model (Fig. 5C). Together, these results suggest that gold(III) porphyrin 1a–induced mitochondrial permeabilization is not directly mediated by its production of ROS, but that a reduction of intracellular ROS could at least partially decrease mitochondrial permeabilization, thus affecting the cytotoxicity of gold(III) porphyrin 1a.

In summary, gold(III) porphyrin 1a was shown to induce apoptosis in a HONE1 human nasopharyngeal carcinoma cell line, and intracellular oxidation is related to its cytotoxicity. Based on the results obtained in this study, we constructed a mode of action as shown in Fig. 6. Gold(III) porphyrin 1a caused depletion of ΔΨm soon after cellular uptake (within 3 hours) with suppression of Bcl-2. ΔΨm depletion facilitated the release of AIF and cytochrome c from mitochondria. Subsequently, AIF activated the process of nuclear condensation, and cytochrome c activated the following caspases and caused oligonucleosomal DNA fragmentation and PARP-1 cleavage. Oxidative stress was likely to affect the cytotoxicity of gold(III) porphyrin 1a by regulating mitochondrial permeabilization.

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Figure 6. Proposed model for the cellular mechanisms of gold(III) porphyrin 1a–induced apoptosis in HONE1 cells. Gold(III) porphyrin 1a directly caused depletion of ΔΨm, leading to the alteration of Bcl-2 family proteins, AIF nucleus translocation, and cytochrome c (Cyt c) release, which further activated caspase-9 and caspase-3, and subsequently caused PARP-1 cleavage. ROS were also generated. The altered cellular oxidative state affected cytotoxicity of gold(III) porphyrin 1a by regulating mitochondrial permeabilization.
detailed mechanisms by which gold(III) porphyrin 1a targets the mitochondria remain to be determined.

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References


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