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Modulation of gold(III) porphyrin 1a-induced apoptosis by mitogen-activated protein kinase signaling pathways

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ABSTRACT

Gold(III) porphyrin 1a is a novel gold(III) complex with selective anticancer effect in a number of human carcinoma cell lines. We have previously shown that gold(III) porphyrin 1a mediated mitochondrial transmembrane potential ($\Delta\Psi_m$) depletion, leading to cytochrome c release, nucleus translocation of apoptosis-inducing factor (AIF), and generation of reactive oxygen species (ROS). The current study addressed gold(III) porphyrin 1a-induced phosphoproteome alterations and modulation of cell death by the mitogen-activated protein kinase (MAPK) family proteins. ERK and $p38^{MAPK}$ were transiently activated upon gold(III) porphyrin 1a treatment. Inhibition of $p38^{MAPK}$ phosphorylation rescued gold(III) porphyrin 1a-induced cell death upstream of caspase activation. Attenuation of $\Delta\Psi_m$ was the primary effect of gold(III) porphyrin 1a leading to $p38^{MAPK}$ phosphorylation. Further functional proteomic study suggested that differential regulation of phosphotyrosine proteins were related to $p38^{MAPK}$ activation in gold(III) porphyrin 1a-induced signal transduction cascade. In summary, $p38^{MAPK}$ modulated gold(III) porphyrin 1a-induced cell death downstream of mitochondria, and phosphorylation of multiple proteins also involved in this process. These results suggested that gold(III) porphyrin 1a is a promising anticancer agent directed toward the mitochondria.

1. Introduction

Apoptosis is a prominent mechanism of cell death following the exposure of many cell types to toxic agents. This process occurs under the tight control of many pro- and anti-apoptotic molecules and are typically executed by the caspase family of cysteine proteases [1]. A series of recent studies has established that the phosphorylation and dephosphorylation of key regulatory proteins are two of the most essential cellular events with regard to both cell growth and apoptosis.

In order to elucidate the signaling pathways responsible for the regulation of apoptosis, the roles of the members of the mitogen-activated protein kinases (MAPKs) family have been extensively studied. These kinases are important intermediates that convert extracellular signals into intracellular responses. The MAPKs contain three parallel serine–threonine kinases, including the extracellular signal-regulated kinase (ERK), $p38^{MAPK}$, and c-Jun NH_2-terminal protein kinase/stress-activated protein kinases (JNK/SAPKs). ERK is generally considered to be a survival mediator involved in the protective actions of growth factors in apoptosis [2]; whereas $p38^{MAPK}$ and JNK are usually referred to as stress-stimulated MAPKs required for the induction of apoptosis by diverse stimuli such as UV irradiation, osmotic shock, and oxidants [3,4]. The

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detailed functions of these MAPK member proteins vary in different kinds of stimuli and are cell type dependent.

Besides these MAPK member proteins, signal transduction events involve transmission and amplification of signals by phosphorylation and dephosphorylation of key enzyme proteins. Among them, tyrosine phosphorylation is a fundamental mechanism for communication between and within cells compared with serine or threonine phosphorylation [5]. Phosphotyrosine resides play a crucial role in signal transduction from transmembrane receptors to the nucleus, affecting biological processes including cell cycle control, cell proliferation and differentiation, cell movement, gene transcription, and cell death [6]. Combined with mass spectrometry that can identify proteins with phosphorylation, functional proteomics using proper antibodies to enrich phosphotyrosine proteins enables us to study the altered phosphotyrosine proteins in a large scale.

Gold(III) tetraarylporphyrin 1a (gold(III) porphyrin 1a) is a novel gold(III) complex that induces apoptosis by caspase-dependent and -independent mitochondrial death pathways related to reactive oxygen species (ROS) [7,8]. The cytotoxic effect of gold(III) porphyrin 1a is not due to its photosensitizing activity [9], and is closely related to both the porphyrin ligand and the central gold atom [10]. The functional relationship between activation of MAPKs, mitochondrial dysfunction, and apoptosis induction by gold(III) porphyrin 1a were investigated. Additionally, we examined the regulation of phosphortyrosine proteins upon gold(III) porphyrin 1a treatment by functional proteomic approach in the present study. Results from the current study demonstrated that activation of $p38^{MAPK}$ plays important roles in gold(III) porphyrin 1a-induced apoptosis downstream of mitochondrial permeabilization. Moreover, $p38^{MAPK}$ was also involved in regulating phosphorylation state of proteins involved in the process mediating cell morphology and cell cycle reentry. Analysis of signaling mechanisms that may be involved in gold(III) porphyrin 1a-mediated apoptosis could provide valuable insights into how this drug lead exhibits effects on cell death.

2. Materials and methods

2.1. Materials and reagents

Gold(III) porphyrin 1a, gold(III) porphyrin 1e, and zinc(II) tetraarylporphyrin [ZnII(TPP)] were synthesized and purified as described previously [10]. 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 Co. (St. Louis, MO) or Amersham Biosciences (Piscataway, NJ). Gold(III) porphyrin 1a, 1e, and ZnII(TPP) was dissolved in DMSO, and kept as stock solution.

2.2. Cell culture and generation of mitochondrial DNA (mtDNA)-deficient cells

Human nasopharyngeal carcinoma HONE1 cells from Prof. S.W. Tsao (Department of Anatomy, The University of Hong Kong) were cultured in RPMI 1640 medium with 2.0 g/l sodium bicarbonate plus 10% fetal bovine serum (JRH Bioscience, Lenexa, KS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Grand Isle, NY), and maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37 °C. HONE1 cells lacking mtDNA (Rho^0) was generated by growing HONE1 cells in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM pyruvate, 50 μg/ml uridine, and 100 ng/ml ethidium bromide for 20 generations [11]. The absence of mtDNA was confirmed by PCR analysis using the following specific human mtDNA specific primers [11]: forward primer 5′-aacatacccatggccaacct-3′ and reverse prime 5′-ggcaggagtaatcagaggtg-3′, which gave a 532-bp product. PCR product was normalized to G3PDH primer suitable for human, rat and mouse: forward primer 5′-accacagtccatgccatcac-3′ and reverse primer 5′-tccaccaccctgttgctgta-3′. The size of G3PDH PCR product was 450-bp.

2.3. Drug treatment

The cells were grown to about 80% confluence and were then either subcultured or treated with 2 μM gold(III) porphyrin 1a. This dose was about twice the IC_{50} value (half-maximal inhibitory concentration). The same concentration of gold(III) porphyrin 1e and ZnII(TPP) was used as control. In some experiments, cells were pretreated with 5 μM SB 203580 (CalBioChem, La Jolla, CA), 10 μM PD 98059 (CalBioChem), and 10 μM SP 600125 (CalBioChem), respectively, 1 h prior to the addition of gold(III) porphyrin 1a.

2.4. Cytotoxicity assay

The cytotoxicity of drug treatment was determined by naphthol blue black (NBB) staining assay, in accordance with a previously reported procedure [7].

2.5. Clonogenic assay

Mid-log phase cells were exposed to serial dilutions of drugs for one-generation time (~24 h). After drug exposure, cells were harvested and plated in six-well plate at the density of 200 cells/well and cultured for 8–12 generation times. The resulting colonies were stained with 0.5% methylene blue solution and counted to obtain the surviving fraction (%) [12].

2.6. Mitochondrial transmembrane potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ were assayed by Rhodamine 123 (Rho-123, Molecular Probes, Eugene, OR) staining in accordance with a procedure described previously [7].

2.7. Caspases activities

The activities of caspase 3 and caspase 9 were determined by caspase activity fluorometric assay kits (Oncogene, CN Biosciences), in accordance with the experimental protocol recommended by the supplier and described early [7].

2.8. Western blot analysis

Western blot analysis was performed using primary antibodies against phospho-ERK (Tyr^{204}, Santa Cruz Biotechnol-

ogy), ERK (Santa Cruz Biotechnology), phospho-p38^{MAPK} (Thr^{180}/Tyr^{182}, Cell Signaling Technology, Beverly, MA), p38^{MAPK} (Cell Signaling Technology), phospho-JNK (Thr^{183}/Tyr^{185}, Santa Cruz Biotechnology), JNK (Santa Cruz Biotechnology), Bcl-2 (Santa Cruz Biotechnology), PARP-1 (Oncogene) and β-Actin (Sigma–Aldrich) at optimal dilution.

2.9. *Immunoprecipitation of phosphotyrosine proteins*

About 5×10^7 cells were scraped in ice-cold PBS and lysed in RAPI lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium-deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4 and 50 mM NaF. Lysates were vortexed and incubated for 30 min on ice. Cellular debris was pelleted by centrifugation at 16,000 × *g* for 10 min at 4 °C. Protein concentrations were determined using the Bradford protein assay dye (Bio-Rad Laboratories, Hercules, CA). For each sample, 800 μg-1 mg cell lysate was precleared by incubation with 1 μg of rabbit immunoglobulin G (BD Biosciences, San Jose, CA) and 20 μl of protein G-agarose beads, Fast Flow (Upstate Biotechnology, Lake Placid, NY) at 4 °C for 30 min. The precleared lysate was then separated from the protein G-agarose beads by centrifugation at 1000 × *g* for 5 min at 4 °C. After that, it was incubated with 2–4 μg of anti-phosphotyrosine antibody (Upstate Biotechnology) at 4 °C overnight with rotation. Immunocomplexes were collected using 20 μl of protein G-agarose beads, by rotating at 4 °C for 4 h. Immunoprecipitates were washed three times with 1 ml of wash buffer (0.1% (w/v) Triton X-100, 50 mM Tris–HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.02% (w/v) sodium azide) and bound proteins were eluted by boiling in sample buffer (50 mM Tris–Cl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT). The proteins were finally separated by SDS-PAGE.

The SDS-PAGE gels were silver stained by a method compatible with mass spectrometric analysis [7]. Briefly, the gels were fixed with 40% ethanol and 10% acetic acid overnight, and then incubated in a buffer solution containing 30% ethanol, 4.1% sodium acetate, and 0.2% sodium thiosulfate for 30 min. After washing three times in water for 5 min each, the gels were stained in 0.1% silver nitrate solution containing 0.02% formaldehyde for 40 min. Development was performed for 15 min in a solution consisting of 2.5% sodium carbonate and 0.01% formaldehyde. EDTA solution (1.46%) was used to stop the development and the stained gels were then washed three times in water for 5 min each. Images acquisition and analysis of stained gels were carried out with Image Scanner (Amersham Biosciences) and Image Master 2D Platinum software (Amersham Biosciences) [7].

2.10. *Mass spectrometric analysis*

Mass spectrometric identification of proteins was done essentially according to a strategy described previously [7]. The gel slices corresponding to the bands indicated in Fig. 4a were excised and transferred into siliconized 1.5 ml Eppendorf tubes. Gel slices were destained in a 1:1 mixture solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate and then equilibrated in 50 mM ammonium bicarbonate to pH 8.0. After hydrating with acetonitrile and drying in a SpeedVac, the proteins were digested with an excess of modified sequencing grade trypsin (Promega, Madison, WI) overnight at 37 °C. After in-gel digestion, the supernatant was spotted onto an Applied Biosystems MALDI plate. MS/MS spectra were acquired on a 4700 Proteomics Analyzer (''TOF/TOF'', Applied Biosystemes Inc., Foster City, CA). Spectra were collected from the successive spots by selecting the most abundant ions from each replicate and excluding previously selected peaks until reasonably sized MS peaks could no longer be detected. The resulting data were processed by using the 4700 Explorer^{TM} software. Duplicate or triplicate runs were made to ensure the accuracy of the analysis.

2.11. *Statistical analysis*

Statistical analysis was performed using two-tailed Student's *t*-test, and $p < 0.05$ was considered significant. Data were expressed as mean ± S.D. of triplicate samples, and reproducibility was confirmed in three separate experiments.

3. Results

3.1. *Gold(III) porphyrin 1a activated ERK and p38^{MAPK}, but not JNK*

Several studies have reported that the MAPK signaling pathway play an important role in the activities and effects of chemotherapeutic drugs [13]. Therefore, we next determined whether the MAPKs were activated in gold(III) porphyrin 1a-induced apoptosis in HONE1 cells by Western blot analysis using phosphorylated protein-specific antibodies. Phosphorylation of ERK 1/2 was detected as a sustained activation from 2 to 3 h after exposure to 2 μM gold(III) porphyrin 1a (Fig. 1a). Gold(III) porphyrin 1a also induced a sustained activation of p38^{MAPK} from 2 to 4 h treatment (Fig. 1b). However, JNK was not phosphorylated at any time in gold(III) porphyrin 1a treatment (Fig. 1c). Phosphorylated c-

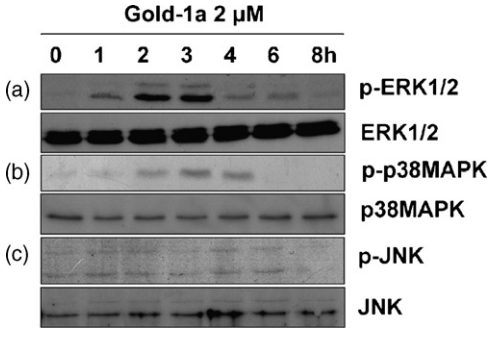

Fig. 1 – Gold(III) porphyrin 1a treatment activated members of MAPK in HONE1 cells. HONE1 cells were untreated or treated with 2 μM gold(III) porphyrin 1a for different period of times as indicated (h). Western blot analysis of the cell lysates was performed using antibodies against (a) phospho-ERK 1/2, (b) phospho-p38^{MAPK}, and (c) phospho-JNK 1/2, respectively. The blots were then stripped and reprobed with antibodies against ERK 1/2, p38^{MAPK}, and JNK 1/2 accordingly. These results were representative from three independent experiments.

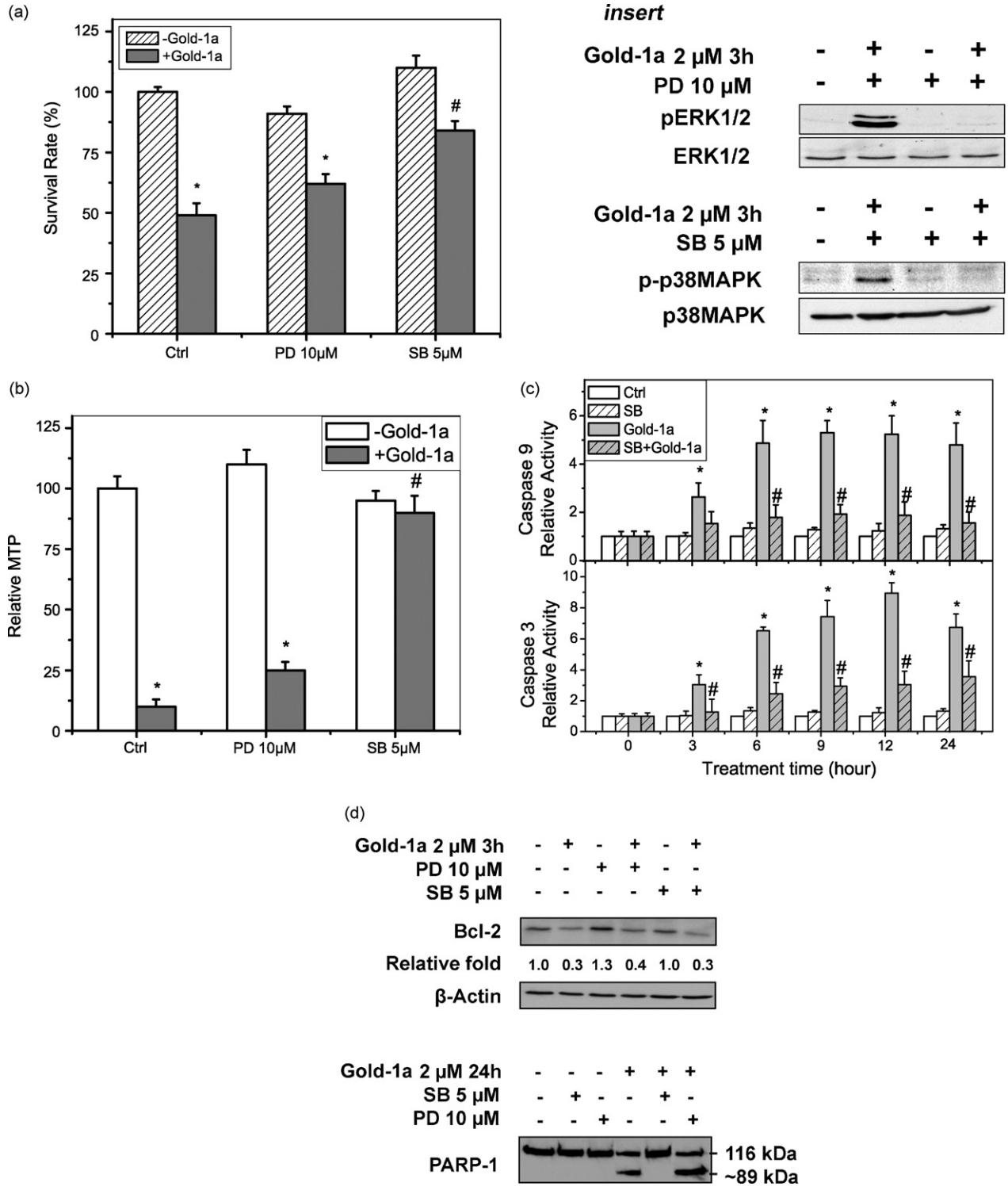

Fig. 2 – Inhibition of $p38^{MAPK}$ activation abrogated gold(III) porphyrin 1a-induced cell death in HONE1 cells. (a) The effect of ERK and $p38^{MAPK}$ inhibitors PD98059 and SB203580 on the colony survival of HONE1 cells after gold(III) porphyrin 1a treatment (*insert*, effects of SB 203580 and PD 98059 on ERK and $p38^{MAPK}$ activation by gold(III) porphyrin 1a showing that the concentrations used in the present study are sufficient to block ERK and $p38^{MAPK}$ phosphorylation). (b) Measurement of $\Delta\Psi_m$ under gold(III) porphyrin 1a treatment in the presence of PD98059 and SB203580. (c) Gold(III) porphyrin 1a activated caspase 9 and caspase 3 was blocked by $p38^{MAPK}$ inhibitor, SB 203580 in a time dependent manner. (d) Effects of PD 98059 and SB 203580 on Bcl-2 expression and PARP-1 cleavage after gold(III) porphyrin 1a treatment for 3 h and 24 h, respectively. Results in (d) were representative from three independent experiments, and results in (a), (b), (c) and (d) are presented as mean ± S.D. of triplicates and are representative data from three separate experiments (*$p < 0.05$ statistically significant difference compared with control; #$p < 0.05$ statistically significant difference compared with gold(III) porphyrin 1a treatment).

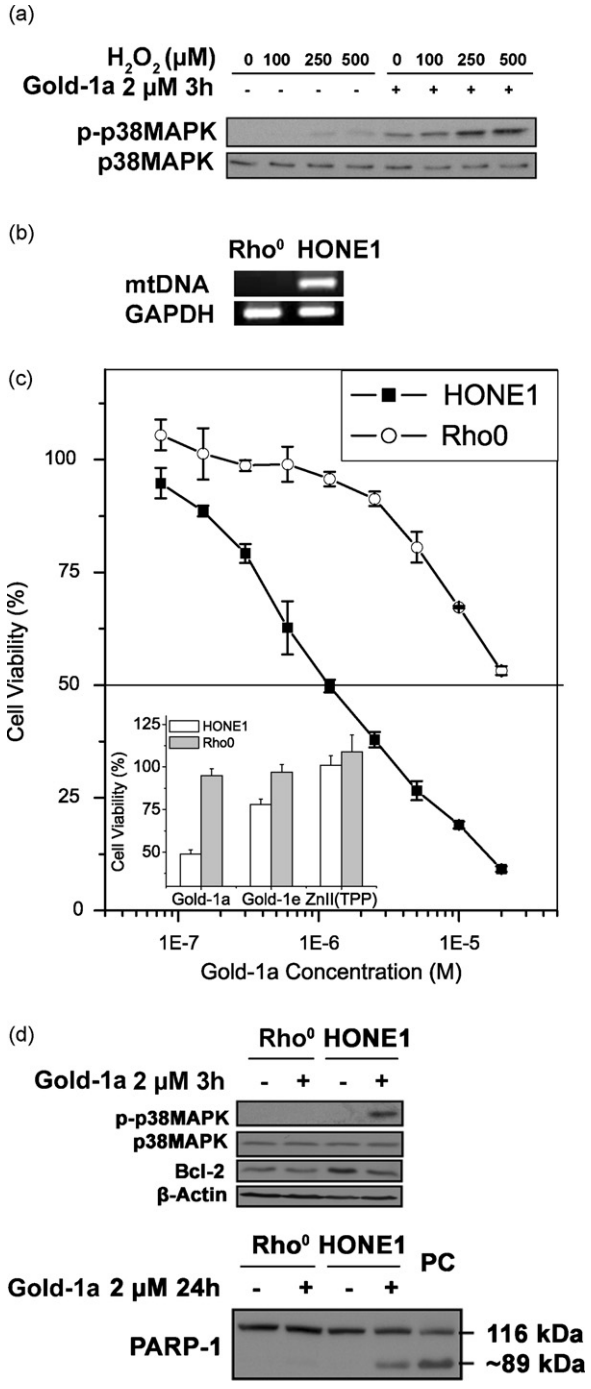

Fig. 3 – Mitochondrial permeabilization was a major effect of gold(III) porphyrin 1a that activated p38^{MAPK}. (a) HONE1 cells exposed to increasing doses of H_2O_2 (0, 100, 250 and 500 μM) under gold(III) porphyrin 1a treatment leading to enhanced p38^{MAPK} activation. (b) Determination of the level of mtDNA in Rho^0 HONE1 cell by RT-PCR analysis. (c) Cytotoxicity of gold(III) porphyrin 1a on HONE1 and Rho^0 cells by NBB staining assay (*insert*, cytotoxicity of 2 μM gold(III) porphyrin 1a, gold(III) porphyrin 1e, and ZnII(TPP) on HONE1 and Rho^0 cells). (d) Effects of gold(III) porphyrin 1a treatment on p38^{MAPK} activation, Bcl-2 suppression, and PARP-1 cleavage in Rho^0 cells compared to HONE1 cells by Western blot analysis. Results in (a), (b) and (d) were representative from three independent experiments, and error bars in (c) represent S.D. of an average from three independent experiments.

Jun, a target of JNK, could not be detected all the time in gold(III) porphyrin 1a treatment, also confirming that JNK was not activated by this agent (data not shown). The total cellular ERK, p38^{MAPK} and JNK level remained constant over the time course (Fig. 1).

3.2. *Inactivation of p38^{MAPK}, but not ERK played a functional role in gold(III) porphyrin 1a-induced apoptosis*

We then directly examined the detailed functions of ERK and p38^{MAPK} activation in gold(III) porphyrin 1a-induced cell death. As shown in Fig. 2a insert, gold(III) porphyrin 1a-mediated phosphorylation of ERK 1/2 and p38^{MAPK} was reduced to untreated level in the presence of 10 μM PD 98059 and 5 μM SB 203580 respectively, indicating the efficiency of these inhibitors. Clonogenic assay showed that inhibition of p38^{MAPK} by SB 203580 increased cell survival in gold(III) porphyrin 1a-treated group from about 49% to 84%; whereas, inhibition of ERK by PD 98059 only elevated cell survival from about 49% to 62% (Fig. 2a). Prior exposure of cells to p38^{MAPK} inhibitor SB 203580, which by itself exerted minimal effects on mitochondrial function or viability, effectively counteracted the attenuation of mitochondrial transmembrane potential ($\Delta\Psi_m$) mediated by gold(III) porphyrin 1a (Fig. 2b). In contrast, ERK inhibitor PD 98059 only slightly increased $\Delta\Psi_m$ from 10% to about 26% compared to untreated control cells (Fig. 2b). Further studies on the caspase 9 and caspase 3 activities showed that prior treatment with SB 203580 significantly reduced activation of caspase 9 and caspase 3 in gold(III) porphyrin 1a treatment (Fig. 2c). Experimental data also revealed that gold(III) porphyrin 1a-induced Bcl-2 down-regulation could not be blocked by the inhibitors of ERK and p38^{MAPK} (Fig. 2d). Furthermore, inhibition of p38^{MAPK} activation abrogated gold(III) porphyrin 1a-caused PARP-1 cleavage, while co-treatment with PD 98059 did not have similar effect (Fig. 2d). These observations suggest that activation of p38^{MAPK}, but not ERK, contributed to gold(III) porphyrin 1a-induced apoptosis upstream of the mitochondrial death pathway.

3.3. *Mitochondrial permeabilization and ROS regulated gold(III) porphyrin 1a-elicited p38^{MAPK} activation*

The results obtained thus far in this study led us to propose that p38^{MAPK} activation may be the major cellular response to gold(III) porphyrin 1a upstream of mitochondria. To validate this speculation, we examined the effect of cellular ROS and mitochondrial permeabiliation on p38^{MAPK} activity in gold(III) porphyrin 1a-treated HONE1 cells. Fig. 3a shows that altering cellular ROS status by co-treatment with increasing amount of H_2O_2 resulted in enhanced expression of phospho-p38^{MAPK}. Since there is no any reagent available to abrogate gold(III) porphyrin 1a-induced mitochondrial permeabilization completely [7], we generated mitochondrial respiratory chain deficient Rho^0 HONE1 cells that are not capable of carrying out oxidative phosphorylation to study the relation between attenuation of $\Delta\Psi_m$ and p38^{MAPK} activation [14]. Reverse

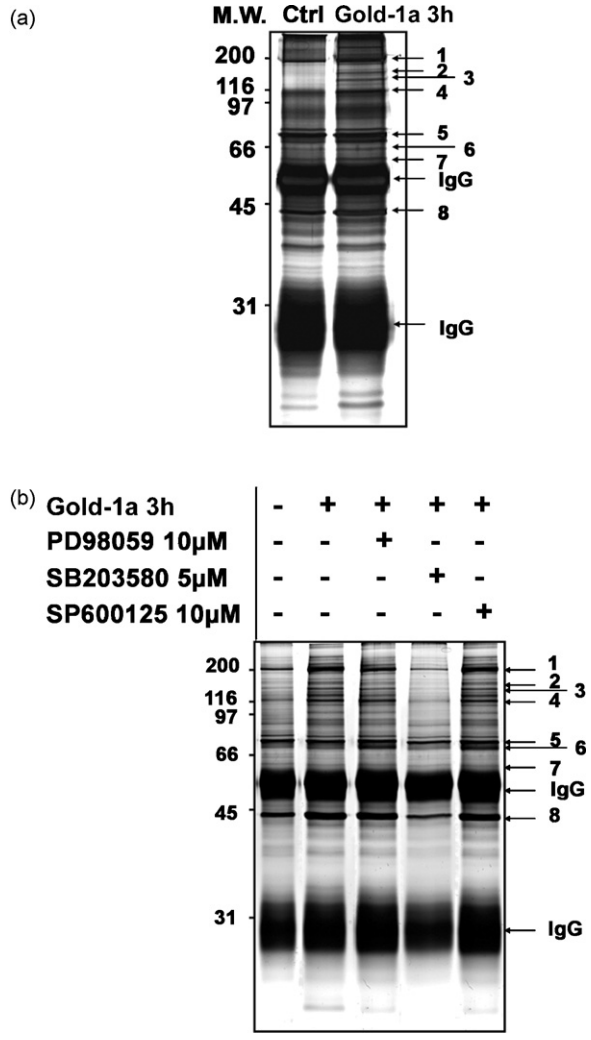

Fig. 4 – Gold(III) porphyrin 1a-induced alterations in tyrosine phosphorylated proteins were related to p38^{MAPK} activation. (a) Alteration of tyrosine phosphorylated proteins under gold(III) porphyrin 1a-treatment. (b) Effect of mitogen-activated protein kinases (MAPKs) specific inhibitors on gold(III) porphyrin 1a-induced alteration of phosphoproteome. Protein bands in SDS-PAGE image of immunoprecipitated (IP) tyrosine phosphorylated proteins were visualized using silver staining. Numbers indicate the position of protein bands from gold(III) porphyrin 1a treatment, which were excised, digested by trypsin for mass spectrometry analysis. Results in (a) and (b) were representative of two independent experiments.

transcription-PCR (RT-PCR) results showed that mtDNA content was decreased by >95% in the Rho^0 HONE1 cells (Fig. 3b). NBB staining assay indicated that Rho^0 cells were more than 12-fold tolerance to the cytotoxicity of gold(III) porphyrin 1a over HONE1 cells (Fig. 3c). Fig. 3c insert shows that gold(III) porphyrin 1a exhibited higher cytotoxicity towards HONE1 cells than the other two compounds with either modified porphyrin ligand (gold-1e) or different central atom [ZnII(TPP)], respectively. Chemical structures of these compounds are given in supplemental data. This result together with our previous reports [9,10,15] suggests that the significant effects triggered by gold(III) porphyrin 1a are due to the influence of both the gold central atom and the porphyrin ligand. Western blot analysis showed that gold(III) porphyrin 1a treatment led to phosphorylation of p38^{MAPK}, suppression of Bcl-2, and cleavage of PARP-1 in HONE1 cells but not in Rho^0 cells (Fig. 3d). Taken together, these results support that mitochondria are the major cellular target of gold(III) porphyrin 1a, and that the activation of p38^{MAPK} and further cascades depend on gold(III) porphyrin 1a-induced mitochondrial permeabilization.

3.4. *Gold(III) porphyrin 1a altered tyrosine-phosphorylated proteins*

It is conceivable that phosphorylation and dephosphorylation of cellular proteins by protein kinases play an important role in regulation of cellular functions including cell growth, differentiation, and the response to extracellular stimuli. Among these alterations of phosphor status, tyrosine phosphorylation was revealed as a major mechanism of transmembrane signaling [6]. Western blot analysis of different time of exposure to gold(III) porphyrin 1a suggested that 3 h of treatment caused most significant alterations in phosphotyrosine proteins (data not shown). We subsequently applied immunoprecipitation by anti-phosphotyrosine antibody combined with SDS-PAGE and mass spectrometry to identify proteins that were tyrosine-phosphorylated as a result of 2 μM gold(III) porphyrin 1a stimulation of HONE1 cells for 3 h. After immunoprecipitated using anti-phosphotyrosine antibody, the immune complexes were boiled and resolved by one-dimensional SDS-PAGE, and then visualized by silver staining (Fig. 4a). Eight major protein bands that were altered significantly after gold(III) porphyrin 1a treatment were excised, digested by trypsin, and analyzed by MALDI-TOF and tandem mass spectrometry. These altered proteins were identified to be myosin heavy polypeptide 9 (non-muscle), myosin I beta, F-box and WD-40 domain protein 10, heat shock 70 kDa protein 9B (mortalin-2), cyclin T2, phosphate cytidylyltransferase 2, and actin gamma 1. Table 1 lists IDs of these proteins, together with their fold differences after gold(III) porphyrin 1a treatment, phosphorylation data and corresponding references. The band numbers correspond to the bands excised from the silver-stained gel shown in Fig. 4a. These results suggested that phosphorylation of multiple-function proteins participated in gold(III) porphyrin 1a-induced cell death.

Several studies have reported that the MAPK signaling pathways play important roles in the activities and effects of external stimuli-induced cell death [16]. We then investigated the effect of specific MAPK inhibitors on the alteration of phosphotyrosine proteome. Fig. 4b shows that co-treatment with p38^{MAPK} inhibitor SB203580 reduced gold(III) porphyrin 1a-induced alteration in phosphoproteome to untreated control level; while ERK inhibitor PD98059 and JNK inhibitor SP600125 did not have such effects (Fig. 4b). These inhibitors themselves did not induce changes in tyrosine phosphorylated proteins compared to untreated control cells (data not shown).

Table 1 – Immunoprecipitation of phosphotyrosine proteins in gold(III) porphyrin 1a (2 μM, 3 h) treated HONE1 cells

Band excised	Protein name	NCBI access no.	MW (kDa) (theoretical)	No. of peptides recovered[a]	No. of peptides sequenced[b]	Sequence coverage (%)	Fold difference (gold: control)	Phosphotyrosine data
Band 1	Myosin, heavy polypeptide 9 (non-muscle)	12667788	226.4	45	4	25.4	2.5 ± 0.4	187–199 VIQ**pY**LAYVASSHK, and Ref. [17]
Band 2	Myosin, heavy polypeptide 9 (non-muscle)	12667788	226.4	29	5	22.6	8.1 ± 1.2	187–199 VIQ**pY**LAYVASSHK, and Ref. [17]
Band 3	Myosin I beta	1926311	117.9	25	3	33.4	11.3 ± 1.6	225–239 NPQS**pY**LVKGQCAK
Band 4	F-box WD-40 domain protein 10 (FBXW10)	20306882	120.6	14	8	32.0	3.9 ± 0.5	Phosphorylation not well documented
Band 5	Heat shock 70 kDa protein 9B (Mortalin 2)	24234688	73.6	29	12	54.5	1.7 ± 0.1	Tyrosine phosphorylated in response to oxidative stress [16]
Band 6	Cyclin T2	4502629	73.6	6	2	14.0	1.9 ± 0.3	454–466 DH**pY**IAAQVEQQHK
Band 7	Phosphate cytidylyltransferase 2	14603223	43.8	4	1	14.0	2.1 ± 0.2	Phosphorylated as a lipid second messenger to control cell cycle re-entry [18,19]
Band 8	Actin, gamma 1	54696574	41.7	8	4	66.9	2.4 ± 0.4	Multiple site phosphorylation or dephosphorylation during actin remodeling [20,21]

[a] Unique peptides identified by peptide mass fingerprinting.
[b] Unique peptides identified by MS/MS sequencing.

4. Discussion

4.1. *Activated p38^{MAPK} modulates gold(III) porphyrin 1a-induced apoptosis upstream of caspase activation*

We reported recently that gold(III) porphyrin 1a induces apoptosis in HONE1 cells depending on the mitochondrial death pathway through the activation of caspase 9 and caspase 3 together with AIF nucleus translocation [7]. Additionally, ROS production and the resulting cellular redox change are part of the signal transduction pathway leading to apoptosis. Further examination of the MAPKs family members showed that transient activation of p38^{MAPK}, but not ERK and JNK, was involved in gold(III) porphyrin 1a-mediated cell death (Figs. 1 and 2a).

Recently, activation of p38^{MAPK} has been recognized as a key event upstream of mitochondrial dysfunction in singlet oxygen and bisindolylmaleimide VIII enhanced DR5-meidated apoptosis [22,23]. The role of p38^{MAPK} in the control of apoptosis has been studied using specific inhibitors of this kinase. Frasch et al. reported that stresses, such as UV, hyperosmolarity, or sphingosine, activated p38^{MAPK} and that the resultant apoptosis could be inhibited by the specific inhibitor SK&F86002 [24]. The ability of pharmacologic p38^{MAPK} inhibitor to block potentiation of apoptosis suggests a functional role for p38^{MAPK} in gold(III) porphyrin 1a-triggered apoptosis upstream of caspase activation (Fig. 2c). Protection of cell death by inhibition of p38^{MAPK} activation was also conferred by clonogenic assay (Fig. 2a) and PARP-1 cleavage (Fig. 2d).

Numerous studies indicated a cross-talk of the MAPK activation to mitochondrial death pathways, and to the Bcl-2 family protein-regulated cell death [3,25]. Watabe et al. have reported that Bcl-2 acted on downstream of MKP-1 but upstream of MAPK in bufalin-induced apoptosis in human leukemia U937 cells [26]. Our current results are consistent with the above study indicating gold(III) porphyrin 1a-induced down-regulation of Bcl-2 expression could not be blocked by either ERK or p38^{MAPK} inhibitors (Fig. 2d), which suggested that suppression of Bcl-2 protein was due to other mediators of apoptosis.

4.2. *Mitochondrial permeabilization is the central event governing cell death in gold(III) porphyrin 1a treatment*

Interestingly, inhibition of p38^{MAPK} activation abrogated gold(III) porphyrin 1a-induced $\Delta\Psi_m$ depletion (Fig. 2b). We have demonstrated in the previous study that attenuation of $\Delta\Psi_m$ happened soon after gold(III) porphyrin 1a uptake [7]. If p38^{MAPK} activation was due to $\Delta\Psi_m$ depletion, alteration of mitochondrial status could lead to differential regulation of p38^{MAPK} activation. It has been revealed that altering cellular ROS level could affect $\Delta\Psi_m$ [7], and our current results showed that gold(III) porphyrin 1a-induced p38^{MAPK} phosphorylation was enhanced with increasing concentration of H_2O_2 (Fig. 3a). Results in Fig. 3d showed that gold(III) porphyrin 1a failed to

activate p38^{MAPK} in Rho^0 HONE1 cells, suggesting the essential role of mitochondria in the induction of p38^{MAPK} phosphorylation. However, co-treatment with p38^{MAPK} inhibitor abrogated $\Delta\Psi_m$ depletion (Fig. 2b) and caspases activation (Fig. 2c), suggesting that there may be an off-target effect of p38^{MAPK} inhibition on cells, such as activation of the ERK pathway [27]. This kind of regulation cascade has also been found in doxorubicin-induced endothelial apoptosis, in which inhibition of p38^{MAPK} down-regulated phosphorylation of Akt and Bad [28]. Recent findings suggested that gold compounds are highly specific inhibitors of mitochondrial thioredoxin reductase, with the inhibition influencing mitochondrial membrane permeabilization that ultimately leads to apoptotic cell death [29]. The central role of mitochondria in apoptosis has also been demonstrated in the cell death induced by gold(I) phosphine complex and auranofin [30,31]. These results strongly support the notion that mitochondria are the critical cellular target of gold(III) porphyrin 1a that further activated p38^{MAPK} and caspases.

4.3. *Multiple phosphotyrosine proteins are involved in gold(III) porphyrin 1a-induced apoptosis related to p38^{MAPK} activation*

Most extracellular signals are transmitted within the cell via a series of phosphorylation and dephosphorylation reactions. Possible involvement of phosphotyrosine proteins in gold(III) porphyrin 1a-induced cell death was studied by functional proteomic approach. The identified phosphotyrosine proteins can be classified into two categories, namely cytoskeleton and cytoskeleton-like proteins, kinase proteins and proteins involved in signal transduction (Fig. 4 and Table 1).

The cytoskeleton and cytoskeleton-like proteins include myosin heavy polypeptide 9 (non-muscle), myosin I beta, and actin gamma 1. Phosphorylation plays a key modulatory role in controlling the functions of cytoskeleton proteins. It has been reported that phosphorylation of myosin heavy chain by endogenous kinase results in a decrease of the actin-activated ATPase, thus leads to prevention of cell cycle progress [17]. Actins, the major structural and functional proteins in all eukaryotic cells, involve in maintaining cell morphology, cell adhension, cell motility, exocytosis and endocytosis, as well as cell division by remodeling [20,21]. Our results suggest that phosphorylation of myosin heavy chain and actin was abrogated by p38^{MAPK} inhibitor SB203980 (Fig. 4b). Potential roles of p38^{MAPK} activation on phosphorylation of actin and myosin during cytoskeleton remodeling have been addressed in osmotic challenges and oxidative stress, respectively [16,32]. These implicate that phosphorylation of cytoskeleton proteins is a primary event in gold(III) porphyrin 1a-induced cell death related to p38^{MAPK} activation.

Changes in phosphorylation state of kinase proteins and proteins involved in signal transduction play regulatory roles in apoptotic cell death [5]. F-box WD-40 domain protein 10 (FBXW10), heat shock 70 kDa protein 9B (mortalin 2), cyclin T2, phosphate cytidylyltransferase 2, thioesterase superfamily member 4, and tyrosine hydroxylase belong to kinase proteins and signal transduction proteins. The functions of most WD-40 repeat-containing proteins remained essentially unknown at present. Van Loo et al. have reported that WD-40 repeats are present in the C-terminal regulatory domain of Apaf-1, and are shown to be involved in self-association of Apaf-1 and the regulation of caspase 9 activation [33]. Cyclin T2, a regulatory unit of cdk9, acts as a cdc 2-related serine/threonine kinase able to phosphorylate p56/pRb [34]. The cdk9/ cyclin T2 phosphorylation of pRb allows the conversion from an inactive complex into a fully active complex that is ready to induce pro-apoptotic signal transcription [35]. Phosphated cytidylyltransferase (CT) is the key enzyme in phosphatidylcholine synthesis, which serves an important role in signal transduction as a source of lipid second messengers. Soluble, inactive forms of CT are more highly phosphorylated than their membrane-bound, active forms [35]. It has been demonstrated that phosphorylation of CT leads to the decrease of phosphatidylcholine synthesis and mass, which is an important factor in cell cycle progression through G_1 and S phase [18,19]. The increased phosphorylated form of these proteins suggest that multiple proapoptotic signals are generated under gold(III) porphyrin 1a stimuli.

Cellular levels of tyrosine phosphorylation normally depend on a balance between cell kinase and phosphatase activities [36]. The activities of these kinase and phosphatase are under control of mitochondrial respiratory by modulation of intracellular ATP and H_2O_2 levels [37]. It has been reported that phosphatase inhibition by oxidative stress can lead to intracellular phosphotyrosine accumulation [38,39]. Experimental results from Livigni and his colleagues also suggested that mitochondrial A-kinase anchor proteins link cAMP and tyrosine kinase signaling to oxidative metabolism [40]. Thus, it is conceivable that p38^{MAPK} inhibitor attenuated mitochondrial permeabilization, leading to diminished tyrosine phosphorylation of these proteins. However, a challenging avenue of future research would be to elucidate the molecular mechanisms underlying how these phosphotyrosine proteins are involved in gold(III) porphyrin 1a-induced apoptosis.

In conclusion, we have described a novel mechanism by which MAPK members regulate the cytotoxicity of gold(III) porphyrin 1a. Prior exposure of HONE1 cells to p38^{MAPK} inhibitor abrogated gold(III) porphyrin 1a-induced cell death, as indicated by the abrogation of $\Delta\Psi_m$ depletion, and the inactivation of caspase 9 and caspase 3. Gold(III) porphryrin 1a also failed to activate p38^{MAPK} in mtDNA deficient Rho^0 HONE1 cells, suggesting that the attenuation of $\Delta\Psi_m$ is an important component of the intrinsic apoptotic pathway in this model. Whereas transient activation of ERK did not affect cytotoxicity of gold(III) porphyrin 1a. Several phosphotyrosine proteins also participate in gold(III) porphyrin 1a-mediated signal transduction cascade that depended on activation of p38^{MAPK}. The identification of the unique property of gold(III) porphyrin 1a-mediated apoptosis by targeting both the mitochondrial pathway and the activation of p38^{MAPK} may be helpful in the design and screening of better chemotherapy drugs directed towards mitochondria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.11.024.

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