Mechanism of Alterations in Isolated Rat Liver Mitochondrial Function Induced by Gold Complexes of Bidentate Phosphines*

(Received for publication, October 28, 1987)

Glenn D. Hoke, Glenn F. Rush, Gerald E. Bossard, James V. McArdle, Bruce D. Jensen, and Christopher K. Mirabelli

From the SmithKline and French Laboratories, Philadelphia, Pennsylvania 19101

Au(DPPE)\(_2\) \((\text{bis}[1,2\text{-bis(diphenylphosphino)ethane}]\text{gold(I)})\) is an organo-gold antineoplastic agent that has anti-tumor activity in a variety of \textit{in vitro} cell lines and \textit{in vivo} rodent tumor models. Preliminary studies suggested that this compound represented a novel class of inhibitors of mitochondrial function. Therefore, the purpose of this study was, therefore, to determine the mechanism of mitochondrial dysfunction induced by Au(DPPE)\(_2\). Au(DPPE)\(_2\) induced a rapid, dose-related collapse of the inner mitochondrial membrane potential (EC\(_{50} = 28.0 \, \mu \text{M}\)) that was not potentiated by Ca\(^{2+}\) preloading. Au(DPPE)\(_2\)-induced dissipation of mitochondrial membrane potential was accompanied by an efflux of Ca\(^{2+}\) from mitochondria upon exposure to Au(DPPE)\(_2\). Ca\(^{2+}\) efflux in these experiments was via a reversal of the Ca\(^{2+}\) unipporter as efflux could be inhibited with ruthenium red. Au(DPPE)\(_2\) did not increase the permeability of mitochondria to oxalacetic acid, indicating that the collapse of membrane potential may not be a result of gross increased inner membrane permeability. However, Au(DPPE)\(_2\) may mediate an increased permeability of the inner membrane to cations and protons. Au(DPPE)\(_2\) caused passive swelling in potassium acetate buffer in the absence of valinomycin, suggesting Au(DPPE)\(_2\) facilitated the exchange of H\(^+\) and K\(^+\). Ca\(^{2+}\) cycling was not extensive and did not contribute to the decrease in membrane potential. These data suggest that one possible mechanism of Au(DPPE)\(_2\) toxicities (12).

The demonstration of elevated mitochondrial membrane potential in a variety of cancerous cells, relative to normal cells (1), has been suggested as a rationale for usage of lipophilic cationic compounds as chemotherapeutic agents (2). Therefore, the selective targeting of mitochondria in cancerous cells might be exploited usefully in an antineoplastic regimen. For example Chen et al. (3) have reported relative differences in the abilities of mitochondria from normal and cancerous cells to accumulate lipophilic cationic dyes such as rhodamine 123. Localization of rhodamine 123 (Rho 123) in the mitochondria of cancerous cells appears to result from a relatively higher electropotential across their inner mitochondrial membrane (4). Rho 123 may exert a selective cytotoxic effect in cancerous cells due to impairment of the bioenergetic mechanisms of these cells via mitochondrial disruption (5). Retention of Rho 123 in mitochondria of colorectal carcinoma cell lines for more than 16 h has been demonstrated (3). The retention in normal, nontransformed epithelial cells, however, was for approximately 2 h. The mechanisms for Rho 123 cytotoxicity have been related to the disruption of mitochondrial functions including protein synthesis (5) and oxidative phosphorylation (6).

Certain lipophilic phosphine-coordinated gold complexes have shown evidence of significant anti-neoplastic activity in \textit{in vitro} and \textit{in vivo} experimental models (7-10). A representative of this class of agents is the positively charged complex \((\text{bis}[1,2\text{-bis(diphenylphosphino)ethane}]\text{gold(I)})\) \((\text{Au(DPPE)}\text{)}\). The antitumor properties of Au(DPPE)\(_2\) may be the result of formation of DNA-protein cross-links and DNA strand breaks (11). In addition, low concentrations of Au(DPPE)\(_2\) have been observed to inhibit protein synthesis relative to DNA and RNA synthesis. High concentrations of Au(DPPE)\(_2\) have been shown to be lethal to murine P388 cells, a result which could not be ascribed to its interactions with DNA.

In preclinical toxicity studies with Beagle dogs, Au(DPPE)\(_2\) was shown to produce cardiac, hepatic, and vascular toxicities (12). In vitro Au(DPPE)\(_2\) was also a potent cytotoxicant to dog and rat hepatocytes. Rat hepatocytes exposed to Au(DPPE)\(_2\) underwent pronounced changes in their ultrastructural morphology, the earliest change being mitochondrial swelling (12). Biochemical evaluation of rat hepatocytes revealed that Au(DPPE)\(_2\) caused a rapid initial increase in cellular respiration and a decrease in total cellular ATP content (13). These data are consistent with the hypothesis that mitochondria are target organelles in the cytotoxicity and possibly the hepatotoxicity of Au(DPPE)\(_2\). In subsequent experiments Au(DPPE)\(_2\) was found to stimulate state 4 respiration in isolated rat liver mitochondria, suggesting that Au(DPPE)\(_2\) acts as an uncoupler of oxidative phosphorylation (13).

An understanding of the interrelationships between Au(DPPE)\(_2\)-induced uncoupling of oxidative phosphorylation and its cytotoxic effects is important in attempting to determine the mechanisms responsible for the antitumor and toxic effects of this drug and other lipophilic cations. Additionally, Au(DPPE)\(_2\) represents a novel structural and mechanistic class of mitochondrial inhibitors and, as such, could provide a useful new probe to study the role of mitochondria in certain pathophysiologic states (e.g. malignancy). The purpose of this investigation was, therefore, to more clearly define the biochemical mechanisms involved in the induction of mitochondrial dysfunction by Au(DPPE)\(_2\).
Mitochondrial Dysfunction induced by Gold Complexes of Bidentate Phosphines

EXPERIMENTAL PROCEDURES

Preparation of Compounds—Au(DPPE)\(_2^+\) and the other gold-phosphine complexes were prepared essentially as described previously in the synthesis of Au(DPPE)\(_2^+\)Cl\(_2\) (11). In some cases the gold(III) chloride was reduced with bromide ion in an acetone/ethanol solution (14). These compounds were recrystallized from a variety of solvents. The chloro-complexes used were highly crystalline materials which gave correct elemental analyses and showed no impurities by IR, UV spectroscopy, or cyclic voltammetry. Percollate and hexafluorophosphate salts of the gold complexes were made by metathesis from the corresponding chloride complexes. The Au(III) complex [Au(DPBPb)]Cl\(_2\) was synthesized by refluxing a concentrated solution of the Au(I) complex in ethanol with concentrated HCl under air. The Au(III) complex [Au(DPBPb)]Cl\(_2\) (ClO\(_4\)) was synthesized by suspending Au(DPPE)Cl\(_2\) in 70% perchloric acid and adding a few drops of nitric acid. The solid product was filtered off and washed with isopropanol and ether. The identity of several of the complexes was confirmed by x-ray crystallography. Stock solutions were prepared fresh daily in dimethyl sulfoxide (Me\(_2\)SO). Fig. 1 shows the schematic structures of the various compounds and the abbreviations used in this report.

Isolation of Mitochondria—Liver mitochondria were prepared from male rats (150–200 g) by differential centrifugation essentially as described previously (15). Briefly, livers were homogenized in ice cold 0.25 m sucrose, 20 mM Tris HCl (pH 7.4), with 0.1% bovine serum albumin (STB buffer) containing 2 mM EGTA. The final pellet was suspended in the same buffer without EGTA. Final mitochondrial protein concentration was determined as described by Bradford (16) using bovine serum albumin as the standard.

Determination of Mitochondrial Membrane Potential—Semi-quantitative measurements of mitochondrial membrae potential were determined spectrophotometrically using the indicating dye safranin O (17,18). Mitochondria (1 mg of protein/ml) in STB buffer containing 2 mM rotenone and 9.6 mM safranin O were mixed at 25°C. Absorbance changes were monitored on an SLM Amico DW2C spectrophotometer at the wavelength pair 533-511 nm. Succinate was added to 1.2 mM to energize the mitochondria.

Determination of Calcium Uptake and Release—Ca\(^{2+}\) movements across the inner mitochondrial membrane were followed by dual wavelength spectrophotometry using purified Arsenazo-III as the indicating dye (19). Absorbance changes were monitored at the wavelength pair 685-654 nm. Mitochondria (1 mg of protein/ml) were incubated at 25°C in STB buffer containing 1.2 mM succinate, 2 mM rotenone, and 48 mM Arsenazo-III. Au(DPPE)\(_2^+\) and/or ruthenium red were added at various time intervals.

Oxidation Status of Pyridine Nucleotides—The oxidation-reduction status of pyridine nucleotides was followed by dual wavelength spectrophotometry utilizing the wavelength pair 370-340 nm. Mitochondria (1 mg of protein/ml) were incubated in STB buffer at 25°C under constant stirring with 1.2 mM succinate and 2 mM rotenone. As indicated, Ca\(^{2+}\) (40 nmol) was added followed by Au(DPPE)\(_2^+\) (35 μM). tert-Butylhydroperoxide (t-BuHP) produced a maximum oxidation of pyridine nucleotides and was used as a positive control. After addition of Au(DPPE)\(_2^+\), t-BuHP (50 μM) was added to induce the oxidation of remaining endogenous pyridine nucleotides.

Membrane Permeability—State 4 respiration was monitored in the presence of 3 mM oxalacetate (OAA) and/or 50 μM Au(DPPE)\(_2^+\) to determine changes in inner mitochondrial membrane permeability (20). Oxygen consumption was measured with a Clark-type electrode in a temperature-regulated water bath using a YSI O\(_2\) monitor (Yellow Springs Instrument Co., Yellow Springs, OH). Mitochondria (2 mg of protein/ml) were incubated in 70 mM succrose, 220 mM d-mannitol, 5 mM triethanolamine, pH 7.4, 0.5 mM EDTA, 0.97 mM KH\(_2\)PO\(_4\), 1.53 mM KHPO\(_4\), 2 mM rotenone, and 1 mM MgCl\(_2\). State 4 respiration was monitored in the presence or absence of 3 mM OAA and 50 μM Au(DPPE)\(_2^+\) Oxidized cytochrome c was used to permeablize the mitochondrial membranes to OAA demonstrating full inhibition of succinate-supported state 4 respiration.

Mitochondrial Swelling—Mitochondrial swelling was determined by absorbance changes at 700 nm. Swelling was monitored under the conditions stated in the figure legends. The wavelength was chosen due to interfering absorbance of Au(DPPE)\(_2^+\) at 540 nm.

RESULTS

Mitochondria respiring on succinate were capable of establishing and maintaining a membrane potential (Fig. 2a). Addition of Au(DPPE)\(_2^+\) (12.5–100 μM) caused a concentration-dependent increase in the rate of dissipation of the membrane potential (EC\(_{50}\) = 26.0 ± 5.3 μM). In isolated hepatocytes 20 μM Au(DPPE)\(_2^+\) produced 100% cell death, as determined by lactate dehydrogenase leakage, within 2 h (data not shown). All concentrations tested eventually caused a complete dissipation of the membrane potential. Preloading of mitochondria with Ca\(^{2+}\) (40 nmol) caused a transient depolarization of the membrane due to Ca\(^{2+}\) uptake (Fig. 2b). In the Ca\(^{2+}\)-preloaded mitochondria, Au(DPPE)\(_2^+\) caused a concentration-dependent increase in the rate of collapse of membrane potential with an EC\(_{50}\) of 25.0 ± 2.9 μM which was not statistically different from non-Ca\(^{2+}\)-loaded mitochondria. Thus Ca\(^{2+}\) preloading did not affect the Au(DPPE)\(_2^+\) concentration-response curve. The classical uncoupler 2,4-dinitrophenol (DNP) also caused dissipation of the membrane potential (Fig. 2c). The initial rate of decrease at various concentrations of DNP were the same. However, the total extent of membrane potential dissipation increased with increasing concentrations of DNP (12.5–50 μM). The difference between the gold compound and DNP may be due to the differences in the solubility of the two compounds with Au(DPPE)\(_2^+\) being the less soluble.

Exposure of mitochondria to Au(DPPE)\(_2^+\) prior to Ca\(^{2+}\) addition resulted in a reduced ability for mitochondria to sequester Ca\(^{2+}\) (Fig. 3a). Similarly, Au(DPPE)\(_2^+\) caused efflux...
Mitochondrial Dysfunction induced by Gold Complexes of Bidentate Phosphines

11205

FIG. 2. Depolarization of mitochondrial membrane potential by Au(DPPE)$_2^+$ in the absence (a) and presence (b) of externally added calcium. Mitochondria (1 mg/ml) were incubated and inner membrane potential was monitored as described under "Experimental Procedures." Au(DPPE)$_2^+$ was added from a 10 mM stock in Me$_2$SO. c, effects of 2,4-DNP on membrane potential. DNP was added from a 20 mM stock in Me$_2$SO.

FIG. 3. Effects of Au(DPPE)$_2^+$ on the ability of mitochondria to sequester (a) and retain (b) calcium. Mitochondria (1 mg/ml) were incubated in the presence of 1.2 mM succinate and 2 μM rotenone. The flux of Ca$^{2+}$ was monitored as described under "Experimental Procedures." Au(DPPE)$_2^+$ was added prior to (a) and after (b) the addition of calcium (40 nmol). Ca$^{2+}$ was added as CaCl$_2$.

of Ca$^{2+}$ from Ca$^{2+}$-preloaded mitochondria (Fig. 3b). The interval of time between addition of Au(DPPE)$_2^+$ and the initiation of Ca$^{2+}$ efflux, however, was dependent upon the concentration of Au(DPPE)$_2^+$ added. The total amount of Ca$^{2+}$ released was independent of the concentration of Au(DPPE)$_2^+$ added. Release of Ca$^{2+}$ stimulated by DNP, however, began immediately upon addition at all concentrations used (data not shown). As with the effects on the membrane potential, the total efflux of Ca$^{2+}$ was dependent upon the concentration of DNP.

The efflux of mitochondrial Ca$^{2+}$ induced by exposure to Au(DPPE)$_2^+$ was ruthenium red-sensitive (Fig. 4a). Ruthenium red is a specific inhibitor of the electrogenic flux of Ca$^{2+}$ through the Ca$^{2+}$ uniporter system (21, 22). The data suggest that Ca$^{2+}$ efflux induced by Au(DPPE)$_2^+$ is the result of membrane potential dissipation causing a reversal of the uniporter. Efflux of Ca$^{2+}$ induced by DNP can also be inhibited with ruthenium red (Fig. 4b). DNP causes collapse of the mitochondrial membrane potential by translocation of protons across the inner membrane (23). Both Au(DPPE)$_2^+$ and DNP caused efflux of Ca$^{2+}$ by a ruthenium-sensitive pathway, most likely a reversal of the Ca$^{2+}$ uniporter.

To exclude Ca$^{2+}$ cycling as a mechanism for collapsing the membrane potential, the effects of ruthenium red on the decrease in membrane potential induced by Au(DPPE)$_2^+$ were determined. The term "Ca$^{2+}$ cycling" is applied to those instances where Ca$^{2+}$ is released via the Ca$^{2+}$/2H$^+$ antiport system and then reuptake by the Ca$^{2+}$ uniporter (24). With continued cycling there is a net influx of protons which could lead to a decrease in membrane potential (25). There has been disagreement concerning the actual role of this proposed pathway in mitochondrial physiology (26, 27). Ruthenium red did not alter the decrease in membrane potential induced by 25 μM Au(DPPE)$_2^+$, substantiating that calcium cycling is not involved in dissipation of the mitochondrial membrane potential (Fig. 5). Addition of EGTA to Ca$^{2+}$-loaded mitochondria also did not alter the collapse of the membrane potential caused by Au(DPPE)$_2^+$ (data not shown). The effects of DNP on the mitochondrial membrane potential were not altered by either ruthenium red or EGTA (data not shown).

Au(DPPE)$_2^+$ did cause partial oxidation and/or hydrolysis of mitochondrial pyridine nucleotides (Fig. 6). The oxidation and/or hydrolysis of pyridine nucleotides was not complete, even at concentrations above 35 μM (data not shown). Subsequent addition of t-BuHP (50 μM) caused complete oxidation of the remaining endogenous pyridine nucleotides (28).

Exposure of mitochondria, respiring in the presence of succinate, to Au(DPPE)$_2^+$ resulted in a concentration-dependent increase in swelling (Fig. 7). This induced swelling may result from increased permeability of the inner membrane or stimulation of respiration. “To determine if increased inner membrane permeability occurs in response to Au(DPPE)$_2^+$, the ability of OAA to traverse the normally impermeable inner mitochondrial membrane and inhibit succinate-supported respiration was monitored (20). An increased rate of state 4 respiration caused by Au(DPPE)$_2^+$ (Fig. 8) indicated uncoupling of oxidative phosphorylation, as observed previ-
Mitochondrial Dysfunction induced by Gold Complexes of Bidentate Phosphines

Fig. 4. The ability of ruthenium red to inhibit the efflux of Ca\(^{2+}\) from mitochondria induced with Au(DPPE)\(^{2+}\) (a) and DNP (b). Calcium efflux was monitored as described in Fig. 3 and under "Experimental Procedures." 10 \(\mu\)M ruthenium red (RR) was added prior to the addition of Au(DPPE)\(^{2+}\) (100 \(\mu\)M) or DNP (40 \(\mu\)M).

Fig. 5. The effect of ruthenium red on Au(DPPE)\(^{2+}\)-induced decrease in the mitochondrial membrane potential. Membrane potential was monitored as described in the legend to Fig. 2 and under "Experimental Procedures." Ruthenium red (RR) was added 0.5 min prior to addition of 25 \(\mu\)M Au(DPPE)\(^{2+}\). Au(DPPE)\(^{2+}\) only (dashed line) or Au(DPPE)\(^{2+}\) in the presence of ruthenium red (solid line) are shown.

Fig. 6. Oxidation of endogenous mitochondrial pyridine nucleotides induced by Au(DPPE)\(^{2+}\) and t-BuHP. Oxidation of pyridine nucleotides was monitored at the wavelength pair 340–370 nm as described under "Experimental Procedures." Mitochondria were monitored in the presence of 1.2 mM succinate, 2 \(\mu\)M rotenone, and 40 nmol of Ca\(^{2+}\). t-BuHP (50 \(\mu\)M) was added following exposure to Au(DPPE)\(^{2+}\) to elicit complete oxidation of pyridine nucleotides (dashed lines).

Fig. 7. Au(DPPE)\(^{2+}\)-induced swelling of energized mitochondria. Mitochondrial swelling (downward deflection) was monitored at 700 nm in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin, 1.2 mM succinate, and 2 \(\mu\)M rotenone. The protein concentration was 1 mg/ml. Au(DPPE)\(^{2+}\) was added at the arrow, and the numbers represent the micromolar concentration of Au(DPPE)\(^{2+}\).

Oxidation of pyridine nucleotides was substantially altered in a nonselective way by Au(DPPE)\(^{2+}\); OAA should traverse the membrane and inhibit succinate dehydrogenase (20, 29, 30). As shown in Fig. 8, Au(DPPE)\(^{2+}\) does not cause increased permeability to OAA. Addition of deoxycholate to permeabilize the inner membrane to OAA led to inhibition of succinate-supported respiration. Results with DNP were similar to those obtained with Au(DPPE)\(^{2+}\) (data not shown).

The capacity of Au(DPPE)\(^{2+}\) to translocate protons or ions across the inner membrane would result in dissipation of the membrane potential. Under passive conditions (i.e. respiration inhibited) the ability of various salts of permeant anions to induce swelling has been characterized (31–33). In the presence of KNO\(_3\), passive swelling of mitochondria requires the presence of nigericin, to mediate K\(^+\) uptake, and an uncoupler, to translocate protons (32). The translocation of protons is necessary to drive nigericin-mediated exchange of protons for K\(^+\). Fig. 9 shows the effects of DNP and Au(DPPE)\(^{2+}\) on passive swelling of mitochondria in KNO\(_3\) buffer in the presence and absence of nigericin. DNP in the presence of nigericin caused an increased rate of swelling, while Au(DPPE)\(^{2+}\) in the presence of nigericin had no effect on swelling. To substantiate the inability of Au(DPPE)\(^{2+}\) to induce proton translocation, DNP and Au(DPPE)\(^{2+}\) were analyzed for their ability to support passive swelling in potassium acetate in the presence of valinomycin. Swelling under these conditions requires a pathway for K\(^+\) influx (via valinomycin) and efflux of accumulated H\(^+\) (via uncoupler) (33). Fig. 10 shows that DNP in the presence of valinomycin results in

---

2 P. F. Smith, G. D. Hoke, D. W. Alberts, P. J. Bugelski, S. Lupo, C. K. Mirabelli, and G. F. Rush, manuscript in preparation.
FIG. 8. The effects of Au(DPPE)$_2$ on the permeability of the inner mitochondrial membrane to OAA. Succinate-supported respiration was monitored as described under "Experimental Procedures." Respiration was monitored in the presence and absence of 3 mM OAA. Deoxycholate (DOC) was added to permeabilize the inner membrane and inhibit respiration. Traces represent respiration rates ± 50 μM Au(DPPE)$_2$.

FIG. 9. Passive swelling of mitochondria in KNO$_3$ upon exposure to DNP (a) or Au(DPPE)$_2$ (b). Mitochondria (2 mg/ml) were suspended in 100 mM KNO$_3$, 2 mM Tris (pH 7.3), and 40 μM rotenone. Swelling (downward deflection) was monitored at 700 nm. Nigericin (NIG) at [4 μM] was added 20 s prior to addition of either DNP [80 μM] or Au(DPPE)$_2$ [80 μM]. a, nigericin + DNP (---), ±DNP (solid lines); b, without Au(DPPE)$_2$ (solid line), with Au(DPPE)$_2$ (---), with nigericin and Au(DPPE)$_2$ (---).

FIG. 10. Passive swelling of mitochondria in potassium acetate upon exposure to DNP (a) or Au(DPPE)$_2$ (b). Mitochondria (2 mg/ml) were suspended in 100 mM potassium acetate, 2 mM Tris (pH 7.3), and 40 μM rotenone in the presence or absence of 40 μM valinomycin (VAL). Swelling was monitored at 700 nm. DNP [80 μM] or Au(DPPE)$_2$ [80 μM] was added as indicated in the figure. a, without valinomycin (---), valinomycin without DNP (solid line), valinomycin with DNP (---): b, without valinomycin and Au(DPPE)$_2$ (---); valinomycin without Au(DPPE)$_2$ (top solid line); with Au(DPPE)$_2$ without valinomycin (---); with Au(DPPE)$_2$ and valinomycin (bottom solid line).

FIG. 11. Effect of DNP upon Au(DPPE)$_2$-induced passive swelling in potassium acetate in the absence of valinomycin. Swelling was monitored under the conditions in Fig. 10 except in the absence of valinomycin. DNP [80 μM] was added 15 s following addition of Au(DPPE)$_2$ [80 μM].

TABLE I

| Compound          | EC₅₀ [μM]$^a$ |
|-------------------|---------------|
| Au(DPPE)$_2$      | 28.0 ± 2.9$^b$|
| Au(DPPV)$_2$      | 27.0 ± 6.5    |
| Au(DPPP)$_2$      | 29.3 ± 6.6    |
| Au(DPPBz)$_2$     | 127.7 ± 48.4  |
| Au(DPPBz)$_2$     | 48.7 ± 9.3    |

$^a$ EC₅₀ values represent the effective concentration at which the rate of decreased membrane potential is 50% of the maximally obtainable rate.

$^b$ Mean ± S.E., n = 3.
Mitochondrial Dysfunction induced by Gold Complexes of Bidentate Phosphines

The effects of Au(DPPE)$_2^+$ on isolated mitochondria are in agreement with the observations made in P388 cells and isolated hepatocytes indicating uncoupling of oxidative phosphorylation as a mechanism of injury (13). Au(DPPE)$_2^+$ caused a collapse of the inner mitochondrial membrane potential and efflux of Ca$^{2+}$. The delay in onset of Ca$^{2+}$ efflux at low concentrations of Au(DPPE)$_2^+$ is reflected in the rate of dissipation of membrane potential and suggests that Ca$^{2+}$ efflux is secondary to decreased membrane potential. Ca$^{2+}$ uptake occurs via an electrogenic process through the Ca$^{2+}$ uniporter which is driven by the membrane potential (29). This uptake mechanism would not seem to be solely responsible for maintaining homeostatic concentrations of Ca$^{2+}$ in mitochondria, since the membrane potential could support a Ca$^{2+}$ gradient that is several orders of magnitude greater than the measured Ca$^{2+}$ gradient (34, 35). However, under conditions of decreased membrane potential, Ca$^{2+}$ efflux can occur by reversal of the uniport system (36, 37). The results presented here demonstrate that Au(DPPE)$_2^+$ causes a dissipation of membrane potential which, in intact hepatocytes, could result in impaired ATP synthesis. Mitochondria preferentially use a reduced membrane potential to maintain internal Ca$^{2+}$ concentrations over the maintenance of ATP synthesis (38, 39). Therefore, Au(DPPE)$_2^+$ could inhibit mitochondrial ATP synthesis by decreasing mitochondrial membrane potential in cells. This would explain the decreased cellular ATP found in hepatocytes and P388 leukemia cells exposed to Au(DPPE)$_2^+$ (12, 13). Increased O$_2$ consumption reported in isolated hepatocytes exposed to Au(DPPE)$_2^+$ is most likely a result of increased mitochondrial respiration secondary to dissipation of the membrane potential (40). Au(DPPE)$_2^+$ does not appear to be the result of Ca$^{2+}$ cycling (Figs. 5, 6, and 8). Ca$^{2+}$ cycling has been suggested to be related to permeability changes in the inner membrane of mitochondria (40-42) and implicated in the processes of regulating intramitochondrial Ca$^{2+}$ concentrations. Fig. 8 shows that the inner membrane does not become more permeable to OAA upon exposure to Au(DPPE)$_2^+$. Ca$^{2+}$ flux resulting in a nonselective increased permeability to solutes smaller than 1500 daltons has been reported (43). Ca$^{2+}$ efflux via an increased membrane permeability has been associated with activation of phospholipase A$_2$ (44) and with sulfhydryl modification (20, 45). Dibucaine and other inhibitors of phospholipase A$_2$ have been shown to inhibit increased permeability mechanism as a Ca$^{2+}$ efflux mechanism (44). Addition of dibucaine or thiodiglycol did not alter the Au(DPPE)$_2^+$-induced dissipation of mitochondrial membrane potential (data not shown). These results, taken with those in Fig. 8, lend support to the absence of gross membrane permeability alterations by phospholipase A$_2$ as a result of exposure to Au(DPPE)$_2^+$.

The mechanism by which Au(DPPE)$_2^+$ causes the decrease in membrane potential is not entirely clear. Au(DPPE)$_2^+$ did not appear to induce passive swelling in mitochondria exposed to KNO$_3$ and supplemented with nigericin as would be expected if Au(DPPE)$_2^+$ acted as a proton translocator like DNP (Fig. 9). However, in potassium acetate Au(DPPE)$_2^+$ did induce swelling in the presence and the absence of valinomycin. Differing responses to Au(DPPE)$_2^+$ in these two buffers may be explained, in part, by the relative solubility coefficients for the two complexes, Au(DPPE)$_2^+$, NO$_3^-$, and Au(DPPE)$_2^+$-acetate$^-$ (44). For these compounds the solubility coefficients in 10% ethanol are 0.03 and 0.3 (mg·ml$^{-1}$), respectively. Thus, addition of Au(DPPE)$_2^+$ to a buffer containing NO$_3^-$ may result in a relatively insoluble complex that cannot be taken up by mitochondria. The swelling induced by Au(DPPE)$_2^+$ in potassium acetate in the absence of valinomycin may be explained by increased permeability of the inner membrane to monovalent cations (K$, H^+$, etc.). However, if increased permeability to cations occurs this permeability change would not be extensive since increased influx of OAA was not observed (Fig. 8). With increased cation permeability the ionization of acetate, HOAc to H$^+$ and OAc$^-$, would not increase the internal concentration of protons. These internal protons could exchange with K$^+$ in an electrogenic process, and the net effect would be increased potassium acetate inside mitochondria which would support passive swelling. During Au(DPPE)$_2^+$-induced passive swelling in potassium acetate in the absence of valinomycin, addition of DNP resulted in inhibition of swelling and a return to a rate of swelling similar to that induced by DNP alone (Fig. 11). These results support Au(DPPE)$_2^+$-induced exchange of external K$^+$ for internal H$^+$ since DNP would effectively translocate protons out of the mitochondria. Translocation of these protons would dissipate the exchange mechanism induced by Au(DPPE)$_2^+$ and prevent K$^+$ uptake by the mitochondria.

A release of divalent cations by Au(DPPE)$_2^+$ could also lead to the perturbations in mitochondrial function observed for this compound. Au(DPPE)$_2^+$ may induce proton permeability by causing release of Mg$^{2+}$ or other divalent cations from mitochondria. Mg$^{2+}$ has been suggested to be involved in maintenance of mitochondrial membrane integrity (46, 47) and its removal may induce permeability changes that result in dissipation of the membrane potential. This consideration is presently under investigation.

Au(DPPE)$_2^+$ does not appear to cause an increased permeability of the inner membrane by a mechanism analogous to heavy metal cations (48, 49). These cations (Cu$^{2+}$, Hg$^{2+}$, etc.) appear to bind to sulfhydrols of inner membrane proteins and alter the permeability to anions and cations. The gold moiety of Au(DPPE)$_2^+$ does not undergo reactions with sulfhydrols (11). Also, diethiothreitol had no effect on Au(DPPE)$_2^+$-induced dissipation of membrane potential (data not shown). Increased permeability does not result in Ca$^{2+}$ release via these permeability changes since Ca$^{2+}$ efflux in Au(DPPE)$_2^+$-treated mitochondria is partially inhibited by ruthenium red (Fig. 4).

D. Picken, personal communication.
Mitochondrial Dysfunction induced by Gold Complexes of Bidentate Phosphines

unipporter (21, 48). Thus, efflux of Ca\(^{2+}\) induced by Au(DPPE)\(^{2+}\) is the result of decreased membrane potential and not efflux through a permeabilized inner membrane or activation of the Ca\(^{2+}/2H^+\) antiporter. Ca\(^{2+}\) efflux mediated by the Ca\(^{2+}/2H^+\) antiport mechanism has been related to oxidation/hydration of pyridine nucleotides (40, 53). Exposure of mitochondria to Au(DPPE)\(^{2+}\) did not result in extensive oxidation/hydration of pyridine nucleotides (Fig. 6) which is consistent with Au(DPPE)\(^{2+}\)-induced Ca\(^{2+}\) efflux via reversal of the unipporter.

The results of the structure-activity relationship comparisons suggest that the mitochondrial effects are shared by other analogs within this class of compounds and that their respective potencies may be modulated via their ability to permeate mitochondria. With increasing size or possible steric interactions of the ligands in membranes, the ability of these compounds to dissipate membrane potential is reduced (for example Au(DPPE)\(^{3+}\) compared with Au(DPPEB)\(^{2+}\) in Table I). With the benzyl bridged compounds, increasing the charge of the gold from +1 to +3 results in a greater potency. Thus uptake by mitochondria also appears to be related to the cationic properties of these compounds. The targeting of Au(DPPE)\(^{3+}\) to mitochondria may occur in a manner analogous to the cytotoxic compounds Rho 123 and tetraphenylphosphonium; both have been shown to concentrate in mitochondria due to their lipophilic-cationic properties (4). Thus, the ability of mitochondria to concentrate Au(DPPE)\(^{3+}\) may be related, in part, to the cytotoxic properties of the drug. According to this model, dissipation of the membrane potential via Au(DPPE)\(^{3+}\) results from increased permeability to selective cations. Disruption of the membrane potential results in Ca\(^{2+}\) efflux and disruption of ATP synthesis probably leading toward cell death.

Rho 123 has been shown to be retained in certain carcinoma-derived cell lines longer than in nontransformed cultured cell lines (49). The increased sensitivity of the carcinoma cell lines to the cytotoxic effects of Rho 123 have been correlated with increased retention of the drug by the cells. The basis for Rho 123 cytotoxicity has been reported to be a result of impairment of ATP synthesis by mitochondria (4). Au(DPPE)\(^{3+}\) may act by a functionally similar mechanism, in that Au(DPPE)\(^{3+}\) is targeted to mitochondria and disrupts ATP synthesis. Rho 123 has been shown to inhibit ATP synthesis by direct inhibition of the Fo-F1 ATPase (51). Au(DPPE)\(^{3+}\)-induced disruption of ATP synthesis may not be a result of direct effects upon the mitochondrial ATPase in a manner analogous to Rho 123. Au(DPPE)\(^{3+}\)-induced collapse of the membrane potential, via increased proton permeability, would act to uncouple oxidation from phosphorylation and inhibit ATP synthesis. Thus, the basis for the antitumor activity of Au(DPPE)\(^{3+}\) may be the uptake and retention by tumor cells relative to other tissues and organs of the tumor bearing animal. This uptake would be facilitated by the electrogenic potentials at the plasma and mitochondrial membranes. Studies by Chen et al. (2) have suggested that certain tumor cell types demonstrate an increased mitochondrial membrane potential compared to nontransformed cells. This hypothesis may be tested by determining if the relative sensitivities of a variety of murine tumors to the antitumor effects of Au(DPPE)\(^{3+}\) are associated with the respective mitochondrial membrane potential of these tumors. In addition, this effect of Au(DPPE)\(^{3+}\) produces mitochondrial function may provide a mechanistic explanation for the hepatic and cardiac lesions exhibited by dogs and rats upon exposure to the drug (12). These two organs, liver and heart, are highly aerobic and possess high numbers of mitochondria/cell relative to other organs.

In addition to mitochondria, nuclear chromatin appears to be a cellular target for Au(DPPE)\(^{3+}\) as evidenced by the production of DNA single-strand breaks and DNA-protein cross-links in tumor cells (50). The relationship of the effects localized in the mitochondria and nucleus to one another and their respective impact on the anti-tumor or toxic properties of the compound is unclear. The delineation of these cause and effect relationships is currently under investigation.

Finally, the potent and rapid effect of Au(DPPE)\(^{3+}\) as an uncoupler of oxidative phosphorylation suggests that this compound may be a useful tool for modulating cellular ATP levels. Our results demonstrate that Au(DPPE)\(^{3+}\) is more potent than DNP and can deplete cellular ATP levels to the same extent as DNP (53). Thus Au(DPPE)\(^{3+}\) may provide an appropriate and effective mechanism for depleting cellular ATP levels for studying biochemical pathways such as ADP-ribosylation and cellular events such as reduced energy charge and their respective consequences on the actions of drugs and cellular viability.

Acknowledgment—We wish to express our deep thanks to Dr. Stanley T. Crooke for his contributions to and his discussion concerning this work.

REFERENCES

1. Pederson, P. L. (1978) Prog. Exp. Tumor Res. 22, 190–274
2. Chen, L. B., Weiss, M. J., Davies, S., Bledary, R. S., Wong, J. R., Song, J., Terasaki, M., Shepherd, E. L., Walker, E. S., and Steele, G. D. (1985) Cancer Cells (Cold Spring Harbor) 3, 453–443
3. Chen, L. B., Belday, R., Weiss, M., Song, J., and Steele, C. (1986) Surg. Forum 37, 423–425
4. Chen, L. B., Lampidia, T. J., Bernal, S. D., Nakadakavure, K. K., and Summershayes, I. C. (1983) in Genes and Proteins in Oncogenesis (Weinstein, I. B., and Vogel, H. J., eds) pp. 369–377, Academic Press, New York
5. Abou-Khalil, W. H., Arimura, G. K., Yunis, A. A., and Abou-Khalil, S. (1986) Biochem. Biophys. Res. Commun. 137, 759–765
6. Modica-Napolitano, J. S., Weiss, M. J., Chen, L. B., and Apriilee, J. R. (1984) Biochem. Biophys. Res. Commun. 124, 717–723
7. Mirabelli, C. K., and Cooke, S. T. (1983) in Auranofin Proceedings of a SmithKline and French International Symposium (Capell, H. A., Coley, D. S., Manghani, K. K., and Morris, R. W., eds) pp. 17–29, Excerpta Medica, Amsterdam
8. Mirabelli, C. K., Johnson, R. K., Sung, C. M., Fauvette, L., Muirhead, K., and Cooke, S. T. (1985) Cancer Res. 45, 32–39
9. Mirabelli, C. K., Johnson, R. K., Hill, D. T., Fauvette, L., Girard, G., Kuo, G., Sung, C. M., and Cooke, S. T. (1986) J. Med. Chem. 29, 218–223
10. Johnson, R. K., Mirabelli, C. K., Fauchette, L. F., McCabe, L., Sutton, B. M., Bryan, D. L., Girard, G., and Hill, D. T. (1985) Proc. Am. Assoc. Cancer Res. 26, 254
11. Berners-Price, S. J., Mirabelli, C. K., Johnson, R. K., Mattern, M. R., McCabe, F. L., Sung, C. M., Mong, S. M., Sadler, P. J., and Cooke, S. T. (1986) Cancer Res. 46, 5486–5493
12. Rush, G. F., Alberts, D. W., Meunier, P., Leffler, K., and Smith, P. F. (1987) Toxicology 7, 59
13. Alberts, D. W., Smith, P. F., and Rush, G. F. (1987) Toxicologist 7, 62
14. Braustein, P., and Clark, R. J. H. (1973) J. Chem. Soc. Dalton Trans., 1845–1848
15. Johnson, D. and Lardy, H. (1967) Methods Enzymol. 10, 94–96
16. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
17. Akerman, K. E. O., and Wikstrom, M. K. F. (1976) FEBS Lett. 58, 191–197
18. Zanotti, A., and Azzone, G. F. (1980) Arch. Biochem. Biophys. 201, 235–265
19. Keidrick, M. C., Ratcliff, R. W., and Blaustein, M. P. (1977) Arch. Biochem. 83, 433–450
20. Lé-Quoc, K., and Lé-Quoc, D. (1982) Arch. Biochem. Biophys. 216, 639–651
Mitochondrial Dysfunction induced by Gold Complexes of Bidentate Phosphines

21. Moore, C. L. (1971) Biochem. Biophys. Res. Commun. 42, 298-305
22. Åkerman, K. E. O. (1978) Biochim. Biophys. Acta 502, 359-366
23. Cunarro, J., and Weiner, M. W. (1975) Biochim. Biophys. Acta 387, 234-240
24. Carafoli, E. (1979) FEBS Lett. 104, 1-5
25. Stucki, J. W., and Ineichen, E. A. (1974) Eur. J. Biochem. 48, 365-375
26. Pfeiffer, D. R., Schmid, P. C., Beatrice, M. C., and Schmid, H. H. O. (1979) J. Biol. Chem. 254, 11485-11495
27. Beatrice, M. C., Palmer, J. W., and Pfeiffer, D. R. (1980) J. Biol. Chem. 255, 8663-8671
28. Hofstetter, W., Muhlebach, T., Lotecher, H. R., Winterhalter, K. H., and Richter, C. (1981) Eur. J. Biochem. 117, 361-367
29. Wojtczak, L., Wojtczak, A. B., and Ernstner, L. (1984) Biochim. Biophys. Acta 191, 10-21
30. Haslam, J. M., and Krebs, H. A. (1968) Biochem. J. 107, 659-667
31. Brierley, G. P., (1974) Ann. N. Y. Acad. Sci. 227, 398-411
32. Brierley, G. P., Jurkowitz, M., Scott, K. M., and Merola, J. J. (1970) J. Biol. Chem. 245, 5404-5411
33. Henderson, P. J. F., McGivan, J. D., and Chappell, J. B. (1969) Biochem. J. 111, 521-535
34. Coll, K. E. Joseph, S. K., Cockey, B. E., and Williamson, J. R. (1982) J. Biol. Chem. 257, 8696-8704
35. Crompton, M. (1985) Curr. Top. Membr. Transp. 25, 231-276
36. Nicholls, D., and Crompton, M. (1980) FEBS Lett. 111, 261-268
37. Bernardi, P., Paradisi, V., Pozzan, T., and Azzone, G. F. (1984) Biochemistry 23, 1645-1651
38. Rossi, C. S., and Lehninger, A. L. (1964) J. Biol. Chem. 239, 3971-3980
39. Vercesi, A., Reynafarji, B., and Lehninger, A. L. (1978) J. Biol. Chem. 253, 6379-6385
40. Bellomo, G., Martino, A., Richelmi, P., Moore, G. A., Jewell, S. A., and Orrenius, S. (1984) Eur. J. Biochem. 140, 1-6
41. Riley, W. W., Jr., and Pfeiffer, D. R. (1985) J. Biol. Chem. 260, 12416-12425
42. Moore, G. A., Jewell, S. A., Bellomo, G., and Orrenius, S. (1983) FEBS Lett. 153, 289-292
43. Nicholls, D., and Åkerman, K. (1982) Biochim. Biophys. Acta 683, 57-88
44. Broekemeier, K. M., Schmid, P. C., Schmid, H. H. O., and Pfeiffer, D. R. (1985) J. Biol. Chem. 260, 105-113
45. Harris, E. J., Al-Shaikhaly, N., and Baum, H. (1979) Biochem. J. 182, 455-464
46. Brierley, G. P., Jurkowitz, M., Farequi, T., and Jung, D. W. (1984) J. Biol. Chem. 259, 14672-14678
47. Jung, D. W., and Brierley, G. P. (1986) J. Biol. Chem. 261, 6408-6415
48. Reed, K. C., and Bygrave, F. L. (1974) Biochem. J. 140, 143-155
49. Summerhayes, I. C., Lamoidis, T. J., Bernal, S. D., Nadakavukaren, J. J., Nadakavukaren, K. K., Shephard, E. L., and Chen, L. B. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5792-5796
50. Richter, C., and Frei, B. (1985) in Oxidative Stress (Sies, H., ed) pp. 221-241, Academic Press, Inc., London
51. Modica-Napolitano, J. S., and Aprollie, J. R. (1987) Cancer Res. 47, 4361-4365
52. Johnson, R. K., Jarrett, P. S., Mong, S. M., Sadler, P. J., Bartus, J. O., Crooke, S. T., Mirabelli, C. K., and Mattern, M. R. (1987) Proc. Am. Assoc. Cancer Res. 28, 316
53. George, M., Cherney, R. J., and Krishna, G. (1982) Toxicol. Appl. Pharmacol. 66, 349-360