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In Vivo Development and *In Vitro* Characterization of a Subclone of Murine P388 Leukemia Resistant to Bis(diphenylphosphine)ethane

GLENN D. HOKE,[1] FRANCIS L. MCCABE, LEO F. FAUCETTE, JOAN O'LEARY BARTUS, CHIU-MEI SUNG, BRUCE D. JENSEN,[2] J. RICHARD HEYS, GLENN F. RUSH, DAVE W. ALBERTS, RANDALL K. JOHNSON, and CHRISTOPHER K. MIRABELLI[1]

SmithKline and French Research and Development Laboratories, King of Prussia, Pennsylvania 19406

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SUMMARY

Bis(diphenylphosphine)ethane (DPPE) and its gold coordination complexes have demonstrated antitumor activity in transplantable tumor models. This report describes the development of a P388 cell line (P388/DPPE^c) that is resistant to DPPE and its analogues and the *in vitro* characterization of the cross-resistance of this subline to various antitumor and cytotoxic agents. The P388/DPPE tumor cell line was developed by serial transplantation in DPPE-treated mice. Resistance to DPPE was phenotypically stable. The P388/DPPE subline was cross-resistant to DPPE analogues and metal coordination complexes of DPPE. In addition, P388/DPPE cells were resistant to several mitochondrial uncouplers, including rhodamine-123, tetraphenylphosphonium, and carbonylcyanide-*p*-trifluro-methoxyphenyl hydrazone. P388/DPPE cells were less capable of sequestering and retaining ^{123}Rh than were sensitive (P388/S) cells. Exposure to $Au(DPPE)_2^+$, a gold complex of DPPE with increased antitumor activity, resulted in a depletion of cellular ATP; the depletion was more rapid in the sensitive than the resistant cells. The rate of mitochondrial respiration, as measured by $^{14}CO_2$ evolution from [6-^{14}C]glucose, was greater in P388/S than in P388/DPPE. As with that evidenced for ^{123}Rh, the cellular uptake of radiolabeled DPPE was decreased in P388/DPPE^c cells. The results suggest that the basis for the resistance of this cell line may be an alteration in mitochondrial membrane potential. These data and the striking cross-resistance of P388/DPPE to mitochondrial uncouplers support the hypothesis that mitochondria may be one target involved in the cytotoxic or antitumor activities of these compounds. Mitochondria may also be causally related to the cytotoxic or antitumor activities, in that DPPE may be concentrated in cells via the presence of the inner mitochondrial membrane potential. Thus, P388/DPPE cells can serve as a tool to screen for and evaluate drugs that rely on affecting mitochondrial function, either mechanistically or causally, for their antitumor efficacy.

Several gold-containing complexes have been reported to display antitumor activity in *in vivo* rodent tumor models (1-3). Structure-activity relationships have been defined for the coordinating ligands, and one of these ligands, DPPE (Table 1), was shown to possess antitumor activity that was approximately equivalent to that of its gold(I) or gold(III) coordination complex (4). DPPE administered at its MTD (50 μmol/kg) to mice bearing P388 leukemia produced 107 ± 4% ILS, whereas the $Au(DPPE)_2^+$ complex exhibited similar activity (ILS, 98 ± 4%) at a much lower MTD of 7 μmol/kg (4). The clonogenic capacity of cultured B16 melanoma cells following a 2-hr exposure to DPPE or $Au(DPPE)_2^+$ complex demonstrated a monoexponential survival curve, with an IC_{50} of 60 or 6 μM, respectively. Thus, the $Au(DPPE)_2^+$ complex was 7- to 10-fold more potent than the DPPE ligand on the basis of IC_{50} (*in vitro* cytotoxicity) or MTD *in vivo*.

Further evaluation suggested that, not only were the gold(I) and gold(III) complexes of DPPE more potent cytotoxic agents than DPPE, but Cu(II) complexes also were more potent than the ligand, whereas Pt(II), Ag(I), Ni(II), and Pd(II) coordination complexes had either marginal or no activity (5, 6). Because DPPE itself has *in vitro* and *in vivo* antitumor activity, it has been suggested that coordination of DPPE to metals may result in the accelerated delivery and increased concentration of DPPE at relevant biological targets (5, 6).

The antitumor properties of DPPE and its gold coordination complexes may be mediated by chromatin damage, which can lead to inhibition of gene replication, transcription, and translation (7). $Au(DPPE)_2^+$ has been shown to induce DNA strand breaks and DNA-protein cross-links in cells (7). The DNA-protein cross-links appear to be the more relevant lesion at

[1] Present address: ISIS Pharmaceuticals, Carlsbad, CA.
[2] Present address: Zynaxis Cell Science, Inc., Malvern, PA.

ABBREVIATIONS: DPPE, 1,2-bis(diphenylphosphino)ethane; MTD, maximum tolerated dose; TPP^+, tetraphenylphosphonium; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; ILS, increase in life span; PBS, phosphate-buffered saline.

cytotoxic concentrations of $Au(DPPE)_2^+$, because strand breaks were evident only at supralethal concentrations.

To define more clearly the biochemical pharmacology of these antitumor complexes and to provide a cell system to evaluate related compounds, a DPPE-resistant subline of P388 leukemia (P388/DPPE) was developed by serial passage of tumor cells in mice treated with DPPE, using established procedures (8). In this paper, we describe the development of this resistant cell line and its pattern of cross-resistance to other cytotoxic and antitumor compounds, as well as studies to define the mechanism underlying the resistant phenotype.

Experimental Procedures

Materials. Phosphine ligands were purchased from Strem Chemicals, and metal complexes of these ligands were synthesized by SmithKline and French Laboratories (King of Prussia, PA). Rhodamine-123 was obtained from Molecular Probes, Inc. (Eugene, OR). Cisplatin, vinblastine, Cremaphor, *N,N*-dimethylacetamide, 2-mercaptoethanol, and tetrazolium salts were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium, fetal calf serum, penicillin, and streptomycin were obtained from GIBCO (Grand Island, NY). Noble agar was purchased from DIFCO (Detroit, MI). The ATP monitoring kit was obtained from LKB Instruments (Gaithersburg, MD). [6-^{14}C]Glucose was purchased from New England Nuclear (Boston, MA). Doxorubicin was from the Drug Synthesis Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). All other reagents were obtained from commercial sources and were of the highest possible grade.

***In vivo* establishment of the P388/DPPE subline.** Parental P388 leukemia cells (P388/S) were maintained by serial intraperitoneal passage in syngenic DBA/2 mice, according to standard screening protocols (9). The DPPE-resistant subline (P388/DPPE) was developed by serial intraperitoneal passage of 1×10^6 cells in C57BL/6 × DBA/2 mice (hereafter called $B6D2F_1$), which were treated intraperitoneally with DPPE at 32 mg/kg/day on days 1 and 5, as previously described (6). Cells were serially transplanted when ascites became evident. Stability of resistance to DPPE was assessed by serial passage of the resistant line in mice without drug treatment (8). DPPE dose-response studies were conducted every second transplant generation up to and including the 17th generation and at generations 22, 26, and 32, using the P388/S and P388/DPPE sublines. DPPE was evaluated at five dose levels (4–64 mg/kg, intraperitoneally, with each dose being half of the next higher dose) on days 1 and 5. Each experiment included three groups of six mice as untreated controls, a titration (10^6 to 10^0 cells/animal) of eight mice/inoculum concentration, and a positive control (cisplatin at 6 and 3 mg/kg, intraperitoneally, on days 1 and 5) for both sublines.

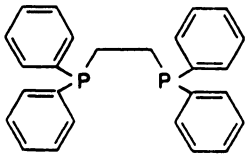
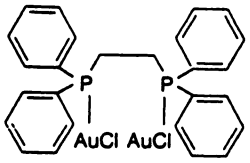
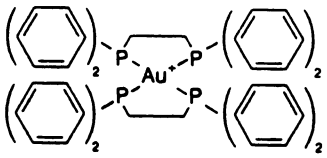
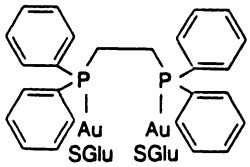
The median survival time, ILS (percentage of increase in median survival time relative to untreated controls), growth characteristics, drug-induced cell killing, and sensitivity or cross-resistance of the resistant line were assessed (10–11). P388/DPPE was judged to be resistant to DPPE or any other drug if there was a ≥3-log difference in the net change in tumor burden at the end of treatment for P388/S and P388/DPPE. No cross-resistance was assumed if there was a <2-log difference in the net change in tumor cell burden following therapy. Partial resistance was ascribed to a compound that produced a 2–3-log difference in cells surviving therapy for the two cell lines.

***In vitro* cloning and maintenance of P388 tumor cell sublines.** P388/S and P388/DPPE tumors were maintained by serial intraperitoneal passage in syngenic DBA/2 and $B6D2F_1$ mice, respectively, as described above. On day 7, ascites cells from each line were aseptically

TABLE 1

Cross-resistance between gold complexes to P388/DPPE *in vivo*

Resistance was assigned for a ≥1 log difference in the net change in tumor burden at the end of treatment. Treatment was the MTD, intraperitoneally, on days 1 and 5.

Structure	ILS at MTD	
	P388/S	P388/DPPE
	%	
Ethyl$_3$-AuSGlu(acetyl)$_4$	70	68 (S)*
Auranofin		
	60	23 (R)
DPPE		
	60	0 (R)
DPPE(AuCl)$_2$		
	80	32 (PR)
$Au(DPPE)_2^+$		
	55	18 (R)
DPPE(AuSGlu)$_2$		

* S, sensitive; R, resistant; PR, partial resistance.

removed, washed twice by centrifugation in PBS,[3] and resuspended in RPMI 1640 medium supplemented with 20% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 μM 2-mercaptoethanol, and 0.6% noble agar. Following 6–7 days of incubation (37° and 95% O_2/5% CO_2), macroscopic colonies were visible. Cloning efficiency, under these conditions, for both tumors was assessed to be 0.5 to 1.0%. Individual colonies were removed and grown as suspension cultures in medium minus the 0.6% agar. Individual cultures of P388/S^c (sensitive to DPPE) and P388/DPPE^c (insensitive to DPPE) cells were resuspended in 95% fetal calf serum, 5% dimethyl sulfoxide, at a cell density of 1×10^7 cells/ml, and were stored in liquid nitrogen. All *in vitro* experiments were conducted utilizing those cultures of the clones that had undergone fewer than 30 passages in tissue culture.

Colony formation assay. P388/S^c and P388/DPPE^c cells were

[3] PBS, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}, purchased from GIBCO (Grand Island, NY); Krebs-Henseleit buffer, 118.1 mM NaCl, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.9 mM $CaCl_2$, 23.8 mM $NaHCO_3$, 25 mM 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid (pH 7.4).

treated with various concentrations of DPPE, or other compounds, for 2 hr at 37° in an *in vitro* cloning medium (RPMI 1640-supplemented with 20% fetal calf serum). Treated cells were then diluted (1:200) into *in vitro* cloning medium and incubated for 7 days to permit development of macroscopic colonies. Colonies were stained with 0.1% tetrazolium salts for 24–48 hr and then evaluated with a Biotran III automatic count totalizer (New Brunswick Scientific Co., Edison, NJ), as previously described (6). Cloning efficiency for each tumor subline was 2–3%. Data are expressed as the logarithm of surviving fraction (cloning efficiency of drug-treated samples/cloning efficiency of controls) versus drug concentration. Growth inhibition assays were performed in suspension cultures by incubation of cells (1×10^6/ml) with DPPE or other agents for 72 hr at 37° in *in vitro* cloning medium minus noble agar. Cell growth was determined by assessment of viability using trypan blue exclusion.

Uptake of labeled DPPE and $Au(DPPE)_2^+$ by P388/S^c and P388/DPPE^c. P388/S^c and P388/DPPE^c cells (1×10^6/0.5 ml of *in vitro* cloning medium minus noble agar) were incubated with several concentrations of [^{14}C]DPPE (11 mCi/mmol) at 37° for various times (up to 60 min). Following incubation, cells were diluted with 5 ml of ice-cold PBS, washed, and pelleted by centrifugation (5 min at 1000 × *g*). The final cell pellet was suspended in 5 ml of H_2O and 10 ml of scintillation fluor for liquid scintillation counting.

Determination of cellular ATP concentration. Cellular ATP concentrations in suspensions of P388/S^c and P388/DPPE^c cells (1×10^6 cells/ml in *in vitro* cloning medium minus noble agar) exposed to various concentrations of DPPE and $Au(DPPE)_2^+$, at 37° under 95% O_2/5% CO_2, were determined. At appropriate times, 1-ml cell suspensions were assayed using the method (luciferin-luciferase) of Farber and Young (12), as previously described (13), using a LKB Wallac luminometer (LKB Instruments, Inc., Gaithersburg, MD). Simultaneously, cell viabilities were determined by trypan blue exclusion.

$^{14}CO_2$ evolution from [6-^{14}C]glucose. Mitochondrial respiration was estimated by the evolution of $^{14}CO_2$ from [6-^{14}C]glucose, as previously described (14). P388/S^c or P388/DPPE^c cells (2×10^6 cells/ml in Krebs-Henseleit[3] medium without glucose and supplemented with 1.2 mM $CaCl_2$) were preincubated for 15 min in a shaking water bath at 37° under 95% O_2/5% CO_2. Glucose (1 mM) containing 0.5 μCi of [6-^{14}C]glucose (55 mCi/mmol) was added, and evolved $^{14}CO_2$ was trapped on alkaline filter paper. Following a 30-min incubation, cells were killed by addition of acid. The flasks were placed on ice for 60 min, after which the filters were removed, neutralized with HCl, and mixed in 10 ml of scintillation fluor for liquid scintillation counting. Data (mean ± standard deviation) are expressed as nmol of $^{14}CO_2$ liberated/flask.

Uptake and retention of rhodamine-123. P388/S^c or P388/DPPE^c cells were suspended at 10^7/ml in PBS supplemented with 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5.0 mM glucose. Rhodamine-123 was added to yield a final concentration of 10^{-6} M. The time course of dye uptake was monitored by flow cytometry periodically over 30 min. To monitor the rate of rhodamine-123 efflux, cells were centrifuged (400 × *g* for 5 min) and the cell pellets were resuspended in rhodamine-123-free medium. Retention was monitored at various time points by flow cytometry.

Flow cytometry. Flow cytometry measurements were performed on a Coulter EPICS 753 flow cytometer. Rhodamine-123 fluorescence was excited at 488 nm with the argon laser (500-mW output power). A 2.0 optical density neutral density filter was placed in front of the forward angle light scatter sensor. A 488-nm dichroic mirror, followed by a 488-nm bandpass filter, was used to collect the right angle light scatter signal at photomultiplier tube 4. A 515-nm interference filter and a 515-nm absorbance filter placed in series were used as laser-blocking filters. The rhodamine-123 and propidium iodide fluorescence signals were split by a 560-nm short pass dichroic mirror. A 525-nm bandpass and a 595-nm long pass interference filter were used to collect the rhodamine-123 and propidium iodide signals, respectively. Live/dead discrimination was based on forward and right angle light scatter, because propidium iodide exhibits a spectral overlap with rhodamine-123.

Results

***In vivo* development of resistance to DPPE.** After one transplant generation of exposure to DPPE *in vivo*, the treated subline demonstrated considerable resistance to the drug (Fig. 1). The degree of resistance increased during subsequent transplants in DPPE-treated mice, with complete resistance (≤25% ILS at the MTD) being evident by the 9th generation. After 11 transplant generations, a subline was transplanted and maintained in mice without DPPE treatment. This subline was still resistant after 22 transplant generations in untreated mice, indicating phenotypic stability of resistance to DPPE.

The *in vivo* growth kinetics of P388/S (sensitive) and P388/DPPE (resistant) cells, determined from parallel titrations in 10 experiments, are shown in Fig. 2. The doubling time was 11.6 hr for P388/S, compared with 15.4 hr for P388/DPPE. The TD_{50} (number of cells required to produce fatal disease in 50% of the mice) for both cell lines was one cell; intraperitoneal implantation of one cell resulted in leukemic death in 20 days for P388/S, compared with 24.5 days for P388/DPPE.

Cross-resistance and sensitivity of P388/DPPE to antitumor compounds. The sensitivities of P388/DPPE and P388/S to a variety of antineoplastic agents and gold-containing phosphine complexes were compared. Both tumor types were equally sensitive to cisplatin (Fig. 3). Cross-resistance was not observed for the DNA-binding agents doxorubicin or anthramycin (data not shown). The P388/DPPE tumor was partially cross-resistant to the tetrahedral gold(I) complex of DPPE, $Au(DPPE)_2^+$ (Fig. 3). The P388/DPPE tumor was not cross-resistant to auranofin, a monophosphine gold complex, but was resistant to various gold complexes of DPPE (Table 2).

***In vitro* selection, propagation, and chemosensitivity of cloned cell lines.** Clonally derived cell lines from P388/S and P388/DPPE tumors to be used for *in vitro* characterization were obtained by growth and selection in soft agar. Individual clones were isolated and established as suspension cultures. One culture each from the P388/S and P388/DPPE clones was randomly selected for further characterization and designated as P388/S^c or P388/DPPE^c, respectively. The two cell lines both had doubling times of 13.5 ± 1.5 hr and grew to approximately the same maximum cell density (2.5×10^6 cell/ml).

The sensitivity of the resistant and sensitive sublines to a variety of antineoplastic and cytotoxic agents was quantified by reduction of the clonogenic capacity in soft agar, as well as by inhibition of cell growth in suspension. As measured either by the soft agar colony formation assay (Fig. 4A) or by growth inhibition in suspension culture (Fig. 4B), the P388/S^c subline was approximately 10-fold more sensitive to the cytotoxic effects of DPPE than the P388/DPPE^c subline. To evaluate the cross-resistance of the P388/DPPE^c subline to DPPE analogues or other antitumor or cytotoxic agents, resistance ratios (P388/DPPE^c IC_{50} to P388/S^c IC_{50}) were determined by the growth inhibition assay (Table 2). Consistent with the findings for the uncloned tumors *in vivo*, P388/DPPE^c cells were not resistant to auranofin or doxorubicin. The P388/DPPE^c subline was not cross-resistant to vinblastine. The lack of cross-resistance of P388/DPPE^c to vinblastine and doxorubicin suggests that resistance to DPPE is unrelated to the P170 glycoprotein

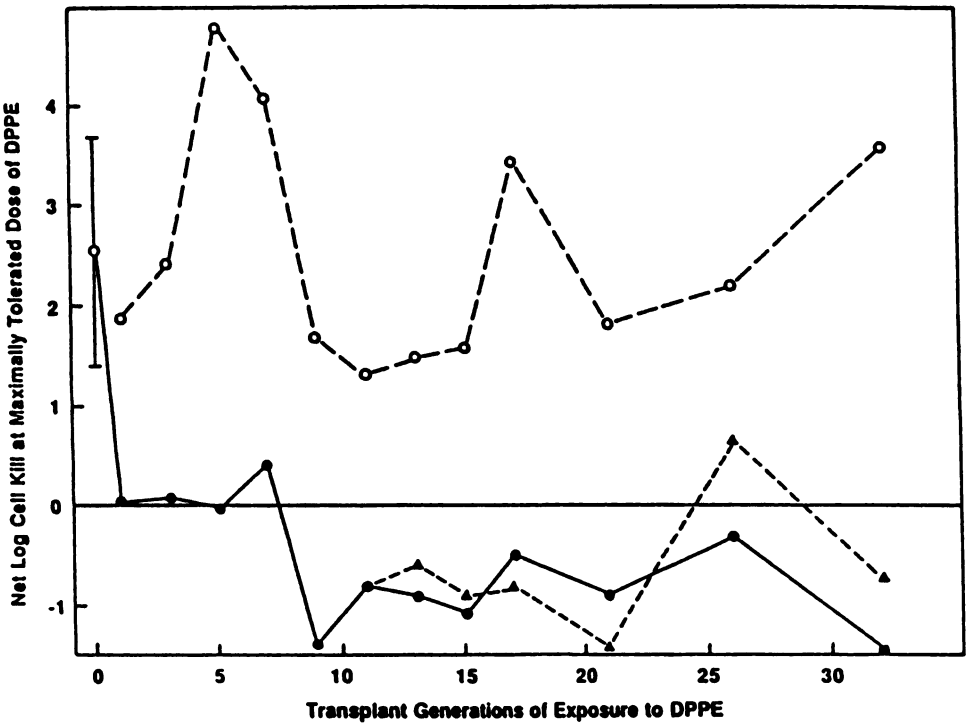


Fig. 1. Development and stability of resistance to DPPE in a subline of P388 leukemia. The DPPE-resistant subline was serially transplanted in mice, who received 32 mg/kg of body weight DPPE, intraperitoneally, on days 1 and 5 for 32 generations (●). After 11 transplant generations, a subline was serially transplanted without DPPE treatment (▲). Each *point* represents the response to a MTD of DPPE administered intraperitoneally on days 1 and 5 in groups of six mice bearing parental P388/S cells (○) or the resistant subline.

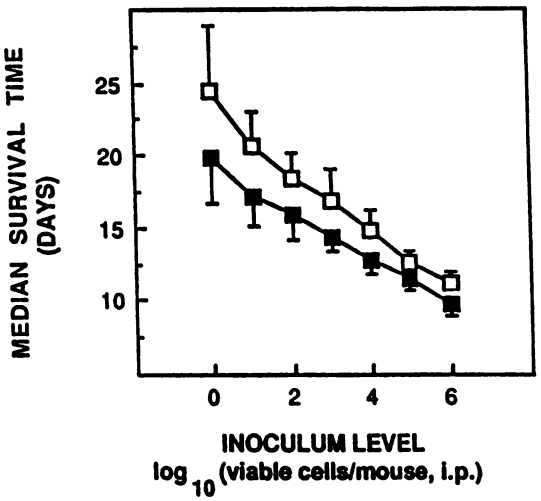


Fig. 2. Median survival time of B6D2F$_1$ mice bearing intraperitoneal P388/S (□) or P388/DPPE (■), as a function of cell inoculum. Each *point* represents the mean ± standard deviation for survival time, at each inoculum concentration, of 10 titrations (eight mice/group).

efflux pathway associated with pleiotropic drug resistance (15, 16). Although the tumors were equally sensitive to cisplatin *in vivo*, the P388/DPPE^c subline was about 3-fold more resistant to cisplatin *in vitro*.

Previous studies have suggested that the pharmacological activities of DPPE can be modulated by Cu^{2+} and that Cu^{2+}-chelated complexes of DPPE may be the active forms of DPPE *in vitro* and *in vivo* (6). In addition, the P388/DPPE^c cells were cross-resistant to three diphenylphosphine copper complexes tested (resistance ratios ranged between 5 and 10). The pattern of cross-resistance is not common to copper chelators, however, because neocuprine was equipotent against both cell types.

Surprisingly, the P388/DPPE^c subline was highly cross-resistant (resistance ratio, >180) to rhodamine-123, a lipophilic cationic inhibitor of mitochondrial oxidative phosphorylation that has been reported to have antitumor activity in some experimental tumor models (17–22). Two other uncouplers of mitochondrial oxidative phosphorylation, TPP^+ and FCCP, were also less cytotoxic in the P388/DPPE^c subline, with the resistance ratios being 50 and 11, respectively.

The cross-resistance of the cells to such structurally diverse uncouplers of oxidative phosphorylation suggests that there is an alteration in mitochondria in the P388/DPPE^c cells. However, other agents that affect mitochondrial function, oligomycin (an inhibitor of ATP synthetase), ionophores such as valinomycin and nigericin, or electron transport inhibitors (rotenone and antimycin A), were not significantly more toxic to the sensitive than to the resistant cells.

Role of mitochondria in P388/DPPE^c resistance. To explore the role of mitochondrial alterations in the DPPE-resistant subline, uptake and retention of ^{123}Rh were explored. This drug was taken up by both sensitive and resistant cells, but uptake beyond the initial phase was slower in P388/DPPE^c cells (Fig. 5A). There was a dramatic difference in the ability of the two sublines to retain sequestered ^{123}Rh. Whereas the P388/S^c cells retained approximately 90% of the ^{123}Rh over 80 min in ^{123}Rh-free medium, the P388/DPPE^c cells exhibited rapid efflux of ^{123}Rh (Fig. 5B). By 80 min, P388/DPPE^c cells had lost approximately 75% of ^{123}Rh, with most of this efflux occurring during the first 10 min.

The ability of P388/S^c and P388/DPPE^c cells to sequester [^{14}C]DPPE is similar to their ability to take up ^{123}Rh (Fig. 6A). P388/S^c cells took up approximately 200 pmol of [^{14}C]DPPE/10^6 cells within 10 min, whereas 10^6 P388/DPPE^c cells accumulated approximately 160 pmol. However, the efflux of [^{14}C]DPPE from cells transferred into DPPE-free medium was approximately equal (Fig. 6B). Thus, the efflux of DPPE differs from the efflux of ^{123}Rh observed in Fig. 5.

If alteration of mitochondrial function was the basis of

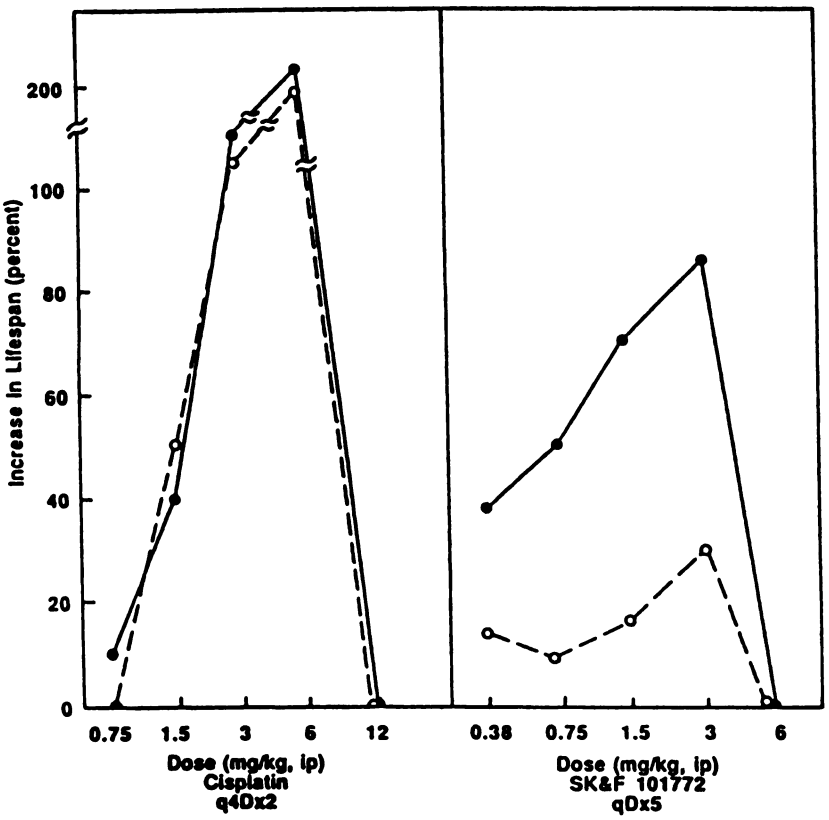


Fig. 3. Cross-resistance of P388/DPPE to $Au(DPPE)_2^+$ and lack of cross-resistance to cisplatin as a function of ILS. Cisplatin was administered at a MTD of 6 mg/kg of body weight, intraperitoneally, on days 1 and 5. $Au(DPPE)_2^+$ was administered at a MTD of 3 mg/kg, intraperitoneally, on days 1 thru 5. Each *point* represents the response of $B6D2F_1$ mice bearing 10^6 intraperitoneally implanted P388/S cells (○) or P388/DPPE cells (●).

resistance in the P388/DPPE^c subline, then alterations in adenine nucleotide metabolism might be evident. Upon exposure of cells to $Au(DPPE)_2^+$, both the P388/S^c and P388/DPPE^c sublines underwent a time- and dose-dependent decrease in cellular ATP concentration. In P388/S^c cells exposed to 75 μM $Au(DPPE)_2^+$, there was a rapid depletion of cellular ATP, with the lower limits of detection being reached within 60 min (Fig. 7A). In contrast, depletion of ATP in P388/DPPE^c cells occurred more gradually. At concentrations of $Au(DPPE)_2^+$ that produced a 90% depletion of cellular ATP in the P388/S^c subline, there was ≤50% depletion in P388/S^c cells (Fig. 7B). At high concentrations of $Au(DPPE)_2^+$ in the P388/DPPE^c cells, there was less depletion of cellular ATP than at 40 μM drug. This result was reproducible; however, the reason for this observation is not apparent. The basal concentration of ATP in the two sublines differed. The concentration of ATP in P388/S^c cells was 2.1 ± 0.1 nmol/10^6 cells, compared with 1.6 ± 0.3 nmol/10^6 cells in P388/DPPE^c cells (data not shown).

A comparison of the evolution of $^{14}CO_2$ from the metabolism of [6-^{14}C]glucose in the two sublines demonstrated that the turnover rate of the mitochondrial tricarboxylic acid cycle was different. P388/DPPE^c cells converted approximately 50% less glucose to CO_2 than did P388/S^c cells (3.3 ± 0.7 nmol of $^{14}CO_2$ compared with 7.3 ± 1.1 nmol $^{14}CO_2$, respectively, in 30 min) (Fig. 8). Exposure of the two sublines to 60 μM $Au(DPPE)_2^+$ decreased the production of $^{14}CO_2$ in both sublines. However, the drug inhibited $^{14}CO_2$ production in P388/S^c cells by 85% at 60 μM, as compared with only 48% inhibition in P388/DPPE^c cells.

Discussion

This report describes the development and biochemical characterization of a subline of the murine P388 lymphocytic leukemia tumor cell line that is resistant to DPPE. The cross-resistance pattern of the DPPE-resistant P388 subline suggests that cytotoxic resistance is based on alterations in energy metabolism. Cross-resistance of P388/DPPE with gold coordination complexes of DPPE was evident *in vivo* and *in vitro* with the P388/DPPE^c subline. The P388/DPPE^c subline was cross-resistant to several uncouplers of mitochondrial oxidative phosphorylation, such as TTP^+ and ^{123}Rh. Uptake by cells of both TTP^+ and ^{123}Rh is driven by the plasma membrane potential and targeted to mitochondria, due to the potential difference across the mitochondrial inner membrane (17). ^{123}Rh is taken up by isolated mitochondria only under "energized" conditions (18, 19). The cytotoxicity of ^{123}Rh has been related to its ability to inhibit mitochondrial oxidative phosphorylation and to disrupt energy metabolism (17–23). An inhibitor of glycolytic ATP production, 2-deoxyglucose, potentiates ^{123}Rh cytotoxicity, consistent with the relevance of inhibition of ATP production to ^{123}Rh cytotoxicity (22, 23).

$Au(DPPE)_2^+$-induced depletion of cellular ATP in P388/S^c cells was more extensive than the depletion in P388/DPPE^c cells. The IC_{50} for DPPE in B16 cells following a 1-hr exposure has been determined to be 60 μM (6), and for $Au(DPPE)_2^+$ an acute IC_{50} in P388 cells was 7 μM (7). Thus, the depletion of ATP in this study was determined at a concentration 10-fold greater than the IC_{50} for $Au(DPPE)_2^+$. Differences between the two sublines in ATP depletion suggest that the majority of metabolically derived cellular ATP in P388/S^c cells may be from mitochondrial oxidative phosphorylation, whereas that in P388/DPPE^c cells may be more dependent on glycolysis. The difference in basal concentrations of ATP in the two sublines suggests that P388/DPPE^c cells have a lower capacity for ATP synthesis than do P388/S^c cells. This reflects the fact that the

TABLE 2

***In vitro* resistance of P388/DPPE^c**

Compounds were tested at their IC_{50} concentrations, as described in Experimental Procedures, growth inhibition assays. Resistance ratios (rounded off to the nearest whole number) are calculated from IC_{50} of P388/DPPE^c/IC_{50} of P388/S^c. Resistance, >5; partial resistance, 3–5; and sensitive, ≤2. The analogue of DPPE, DPPV, contains a vinyl bond between the carbons. This double bond creates the *cis* and *trans* isomers.

Compound	IC_{50}[a]		Resistance ratio
	P388/S^c	P388/DPPE^c	
	μM		
Ligands/chelators			
DPPE	0.34	3.7	10 (R)
(*cis*)-DPPV	0.12	8.5	70 (R)
(*trans*)-DPPV	8	10	1 (S)
Neocuproine	0.14	0.2	1 (S)
Metal complexes			
$[Au(DPPE)_2]^+$lactate$^-$	0.18	1.3	7 (R)
$[Cu\text{-}(cis)\text{-}(DPPV)_2]^+Cl^-$	0.049	0.34	7 (R)
$[Cu\text{-}(DPPP)_2]^+Cl^-$	0.044	0.46	10 (R)
$Cu_2(DPPE)_3Cl_2$	0.085	0.42	5 (PR)
Auranofin	0.09	0.06	1 (S)
Antitumor compounds			
Doxorubicin	0.05	0.05	1 (S)
Vinblastine	0.04	0.05	1 (S)
Cisplatin	0.07	0.2	3 (PR)
Mitochondrial inhibitors			
Uncouplers			
TPP	0.04	20	50 (R)
FCCP	0.07	0.8	11 (R)
Rhodamine-123	0.55	>100	>180 (R)
ATPase inhibitor			
Oligomycin	0.0015	0.003	2 (S)
Ionophores			
Valinomycin	0.003	0.003	1 (S)
Nigericin	0.06	0.06	1 (S)
Electron transport chain inhibitors			
Rotenone	0.3	0.3	1 (S)
Antimycin	0.07	0.09	1 (S)

[a] Resistance determined with continuous exposure to drugs for 72 hr.

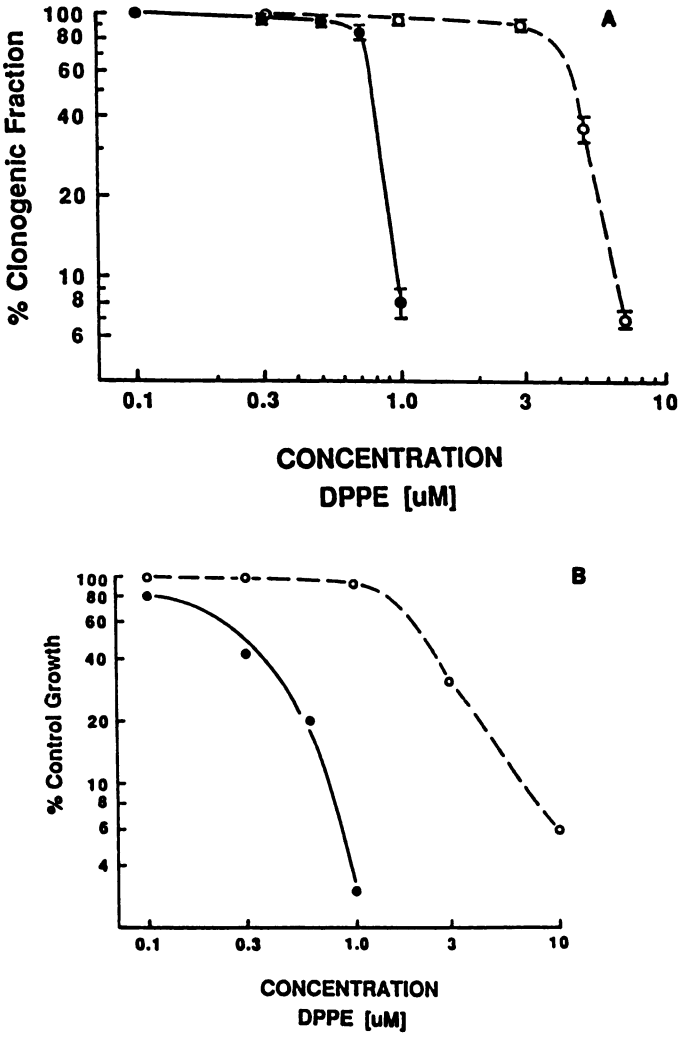


Fig. 4. Effect of DPPE on the *in vitro* clonogenic capacity (A) and inhibition of cell growth in suspension culture (B). P388/S^c or P388/DPPE^c cells were treated for 72 hr with DPPE, and the clonogenic capacity was assessed via colony formation (after 7 days) in soft agar, as described in Experimental Procedures. The percentage of clonogenic fraction represents the cloning efficiency of drug-treated samples/cloning efficiency of control samples. Data represent the mean ± standard deviation of three determinations for each concentration of DPPE. For the inhibition of cell growth, cells (1×10^6/ml of *in vitro* cloning medium) were exposed to DPPE for 72 hr, and viability was assessed via trypan blue exclusion, as described in Experimental Procedures. Data represent the mean of three experiments, expressed as the log percentage of control growth (viable DPPE-treated/viable control). ●, P388/S^c; ○, P388/DPPE^c.

ATP yield from glycolytic metabolism of 1 mol of glucose is 2 mol of ATP, whereas coupling of oxidative phosphorylation to glucose catabolism produces 36 mol of ATP (24). Comparison of the capacity to generate $^{14}CO_2$ from [6-^{14}C]glucose in the two sublines supports this difference in energy metabolism. The P388/S^c cells had a higher capacity for $^{14}CO_2$ evolution through mitochondrial catabolism, suggesting a greater reliance on oxidative phosphorylation. Exposure of the two sublines to $Au(DPPE)_2^+$ resulted in greater inhibition of $^{14}CO_2$ evolution in the P388/S^c cells. This increased ability of $Au(DPPE)_2^+$ to inhibit mitochondrial substrate utilization in P388/S^c cells is reflected in the rapid depletion of cellular ATP.

It has been demonstrated that tumor cells have varying capacities for glycolytic and mitochondrial production of ATP. The contribution by glycolysis may range from 10 to 50% of total ATP production (25). Some tumor cells have reduced numbers of mitochondria, whereas others have reduced mitochondrial acceptor control ratios (rate of state 3 respiration/rate of state 4 respiration) due to proton (or small cation) leakage into the mitochondrial inner matrix through the inner membrane. A number of factors are involved in determining whether tumor cells derive ATP predominantly from mitochondrial oxidation or rely in part on glycolysis. It appears that selection of the P388/DPPE^c subline may have been related to alterations in the cellular capacity for glycolytic ATP production.

Certain other compounds to which P388/DPPE^c was not cross-resistant, but that affect mitochondrial function (i.e., oligomycin, ionophores, and electron transport inhibitors), suggest that alterations in mitochondrial membrane potential may be an important determinant in resistance of P388/DPPE^c cells. Oligomycin is a specific inhibitor of mitochondrial ATP synthetase (26, 27). Both ^{123}Rh and oligomycin inhibit ATP synthesis in mitochondria, but the inhibition of mitochondrial ATPase by ^{123}Rh is directly related to depolarization of the inner membrane potential (19, 28, 29). Oligomycin is not charged at physiological pH and, thus, its inhibitory effect on mitochondrial ATPase is not dependent upon the presence of a membrane potential. Oligomycin inhibits the ATP-hydrolyzing capacity of mitochondrial ATP synthetase in the presence

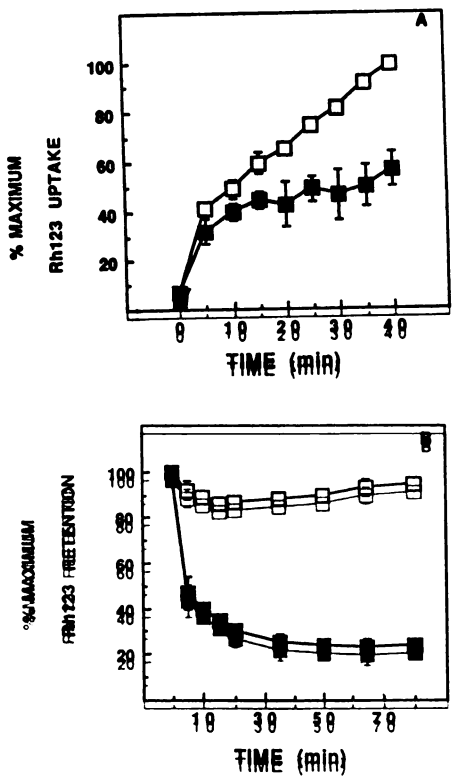


Fig. 5. Uptake (A) and retention (B) of rhodamine-123 by B388/S^c and B388/DPPE^c cells. Rhodamine-123 uptake and retention were determined as described in Experimental Procedures. B388/S^c (■) and B388/DPPE^c (□) data are presented as the mean ± standard deviation of triplicate determinations.

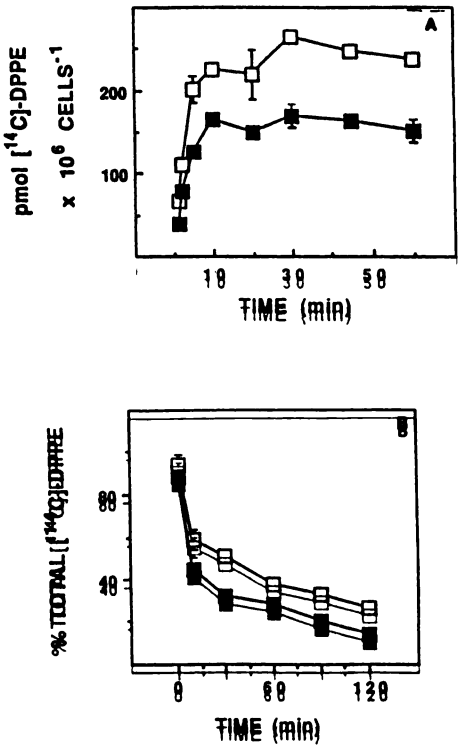


Fig. 6. Uptake (A) and retention (B) of [^{14}C]DPPE by B388/S^c and B388/DPPE^c cells. [^{14}C]DPPE (10 μM) was added to cells (1 × 10^6/ml) suspended in *in vitro* cloning medium, and uptake and retention of radiolabeled DPPE were determined as described in Experimental Procedures. B388/S^c (□) and B388/DPPE^c (■) data are expressed as the mean ± standard deviation (three experiments) of pmol/10^6 cells sequestered (A) or percentage of total retained (B) versus time.

of dinitrophenol, which dissipates the membrane potential (26). The reduced uptake and retention of ^{123}Rh and the reduced uptake of [^{14}C]DPPE by B388/DPPE^c cells suggest that a reduction in mitochondrial membrane potential of mitochondria in B388/DPPE^c may be the basis for resistance to DPPE and its metal-coordination complexes. The inability of B388/S^c cells to retain DPPE may reflect DPPE-induced dissipation of the mitochondrial inner membrane potential. This effect is not seen with ^{123}Rh, with which there is not dissipation of the inner membrane potential. Thus, in B388/S^c cells ^{123}Rh is retained while DPPE efflux occurs. The formation of DPPE-Cu^{2+} coordination complexes has been suggested to be responsible for the activity of DPPE in biological systems (6).

The cytotoxicity of $Au(DPPE)_2^+$ to isolated hepatocytes has been shown to be mechanistically related to disruption of mitochondrial function, resulting in inhibition of mitochondrial ATP synthesis and a rapid depletion of cellular ATP (30, 31). Due to the lipophilic and cationic properties of $Au(DPPE)_2^+$, the plasma and mitochondrial membrane potentials should tend to concentrate the drug inside the cells (31). Thus, $Au(DPPE)_2^+$ behaves like ^{123}Rh, in that both are concentrated in cells and disrupt mitochondrial ATP synthesis. The majority of cellular ATP in hepatocytes is generated by mitochondrial oxidative phosphorylation (32). $Au(DPPE)_2^+$-induced uncoupling of oxidative phosphorylation results in the rapid depletion of cellular ATP and hepatocyte lethality. The antitumor efficacy of DPPE and its gold coordination complexes may result from a similar effect of these compounds on energy metabolism in tumor cells. However, the role of mitochondria in the pharmacological activity or cellular toxicity of $Au(DPPE)_2^+$ and

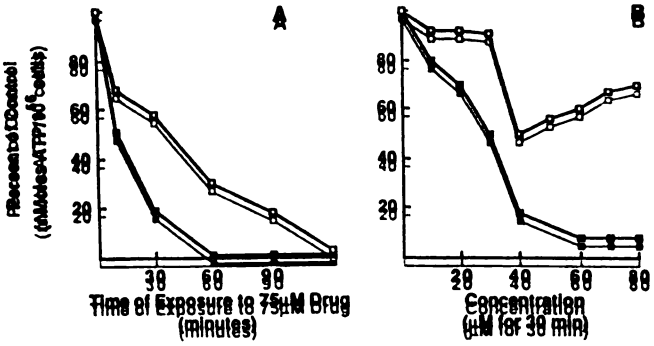


Fig. 7. Effect of $Au(DPPE)_2^+$ on the ATP concentration in B388/S^c and B388/DPPE^c cells. ATP concentrations were determined as described in Experimental Procedures. A, ATP depletion over time following exposure to 75 μM $Au(DPPE)_2^+$. B, ATP depletion following a 30-min exposure to various concentrations of $Au(DPPE)_2^+$. ■, B388/S^c; □, B388/DPPE^c.

DPPE in B388 cells may be mediated by increases in the concentrations of these compounds within cells, thus allowing access of these agents to other cellular targets (i.e., the nucleus). Increased concentrations of DPPE or its metal complexes within the cell may mediate the effects of these compounds on DNA (i.e., DNA-protein cross-linkages or DNA single-strand breaks) (7). With a reduction of the mitochondrial membrane potential in B388/DPPE^c cells, $Au(DPPE)_2^+$ may not attain sufficient intracellular concentrations to induce these nuclear DNA effects. The role of mitochondria in the activity of DPPE is supported by the striking level of cross-resistance to mitochondrial uncouplers.

The B388/DPPE^c subline should provide a useful tool for

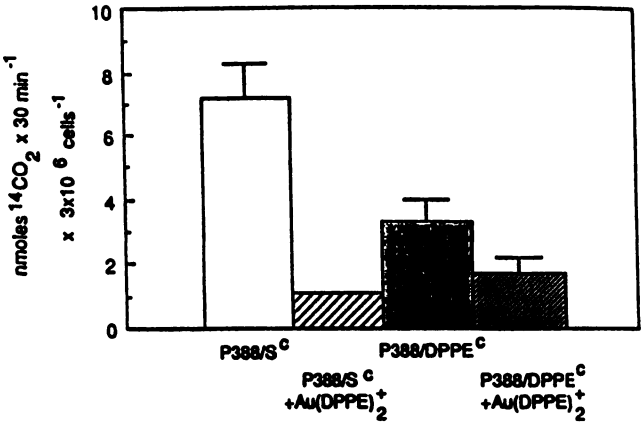


Fig. 8. Effect of $Au(DPPE)_2^+$ on the evolution of $^{14}CO_2$ following exposure of P388/S^c and P388/DPPE^c cells to [6-^{14}C]glucose. Determination of $^{14}CO_2$ evolution is described in Experimental Procedures. Data represent mean ± standard deviation (three experiments) and are expressed as nmol of CO_2 evolved following a 30-min incubation with radiolabeled glucose, in the absence or presence of 60 μM $Au(DPPE)_2^+$.

identifying new mitochondria-active antitumor agents. The P388/DPPE^c subline not only will be useful for screening other DPPE or metal-DPPE compounds but also should be useful in identifying other antitumor agents whose activity is related to mitochondria as targets.

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Send reprint requests to: Randall K. Johnson, Ph.D., SmithKline and French Laboratories, P. O. Box 1539, King of Prussia, PA 19406.