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Biochemical Pharmacology 66 (2003) 977–987
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Immediate effects of anticancer drugs on mitochondrial oxygen consumption

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Received 21 February 2003; accepted 1 June 2003

Abstract

The evolving role of mitochondria as a target for many anticancer drugs (e.g. platinum-based compounds, alkylating agents and anthracyclines) prompted us to investigate their immediate effects on the mitochondrial respiratory chain. For this purpose, we used a phosphorescence analyzer that measures [O_2] in solution. The [O_2] of solutions containing an appropriate substrate and various cell lines, tumors from patients or beef heart submitochondrial particles (SMPs) declined almost linearly ($r > 0.99$) as a function of time, indicating that the kinetics of cellular oxygen consumption were zero order. Rotenone inhibited respiration, confirming that oxygen was consumed by the respiratory chain. Exposure to a clinically relevant concentration of cisplatin (5 μM at 37° for 1–3 hr) had no effect on the respiration in cells or in SMP. Higher cisplatin concentrations (10–99 μM at 37° for 1–3 hr) produced <25% inhibition. Incubations with 4-hydroperoxycyclophosphamide (50–100 μM at 37° for 1 hr) inhibited oxygen consumption in SMP (~70% inhibition at 50 μM) and in cells (~30% inhibition at 50 μM). Incubations (37° for 1 hr) of SMP with doxorubicin (25–100 μM) and daunorubicin (25–100 μM) had no inhibitory effect on the respiration. By contrast, incubations (37° for 1 hr) of cells with doxorubicin (5–20 μM) and daunorubicin (2–20 μM) produced significant inhibition. We conclude that cisplatin does not directly damage the energy converting mechanism of mitochondria. On the other hand, comparable exposures to alkylating agents and anthracyclines produce immediate and dose-dependent impairment of cellular respiration.

Keywords: Cisplatin; 4-Hydroperoxycyclophosphamide; Anthracyclines; Mitochondria; Cellular respiration; Apoptosis

1. Introduction

The mitochondria are a major producer of cell energy. This vital organelle plays a central role in cellular integrity, and many cellular poisons may target it [1]. Some toxins can directly damage mitochondria, producing a rapid adenosine 5′-triphosphate depletion and non-apoptotic cell death (necrosis). Others may invoke indirect injury (e.g. loss of cytochrome *c*) via activation of the apoptotic pathway [2]. The newly recognized role of mitochondria in mediating chemotherapy-induced apoptosis [3] prompted the studies described here. The three leading classes of anticancer drugs (platinum-based compounds, alkylating agents and anthracyclines) are investigated for their immediate effects on the mitochondrial respiratory chain.

Cisplatin, *cis*-diamminedichloroplatinum (II), exerts its antitumor activity by binding to cellular DNA [4]. When the drug enters the cell, it passes through the cytosol, enters the nuclear envelope, binds to nitrogen atoms on the bases of DNA and promotes cell death by apoptosis [5]. The maximum serum concentration (C_{max}) of cisplatin in patients is ~5–10 μM and the half-life ~25–35 min [6,7]. The side effects of cisplatin include nephropathy, neuropathy and hearing loss.

Cyclophosphamide [*cis*-(±)-2-(bis(2-chloroethyl)amino)tetrahydro-2-oxide-2*H*-1,3,2-oxazaphosphorine], on the

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Abbreviations: [O_2], oxygen concentration; C_{max}, maximum concentration; τ, lifetime of phosphorescence; Pd phosphor, palladium derivative of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin; 4OOH-CP, 4-hydroperoxycyclophosphamide; NADH, β-nicotinamide adenine dinucleotide; ADP, adenosine 5′-diphosphate; BSA, bovine serum albumin; dH_2O, distilled deionized water; PBS, phosphate-buffered saline; FBS, fetal bovine serum; SMP, submitochondrial particles; PBMC, peripheral blood mononuclear cells; Pt, platinum; *k*, zero-order rate constant.

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doi:10.1016/S0006-2952(03)00418-0

other hand, is a potent alkylating agent [8]. This drug requires *in vivo* activation by the hepatic microsomal cytochrome P-450 system. The resulting 4-hydroxylated metabolite degrades to phosphoramide mustard (the active component) and acrolein (α,β-unsaturated aldehyde). The reaction mechanism of phosphoramide mustard [*N*,*N*-bis-2-(2-chloroethyl)phosphorodiamidic acid, R–N(CH_2–CH_2–Cl)$_2$] involves generating the intermediate phosphoramide aziridinium ion, which rapidly reacts with cellular nucleophiles, including those on the DNA and proteins. The reaction of acrolein (CH_2=CH–CHO) is via nucleophilic addition at the β-carbon atom [9]. The C_{max} of plasma cyclophosphamide varies with the dose [10]. Peak phosphoramide mustard plasma levels of ∼50–100 μM are reported in patients receiving 60 or 75 mg kg^{-1} (∼1.8–2.2 g m^{-2}) of the drug [11]. The side effects of cyclophosphamide include hematopoietic suppression, cardiac and lung toxicities, gonadal atrophy, hemorrhagic cystitis and induction of cancer (e.g. acute leukemia). *In vitro* systems employ 4OOH-CP, which spontaneously degrades to the reactive alkylating species [12]. 4OOH-CP is used to purge hematopoietic cells prior to autologous bone marrow transplantation.

Anthracycline antibiotics (e.g. doxorubicin and daunorubicin) exhibit a wide spectrum of antitumor activity. Their mechanism of action is still not fully understood. These agents are well known to intercalate with DNA. Moreover, inside the cell, their quinone moieties are reduced to semiquinone radical species, which participate in electron-accepting and -donating reactions, generating superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). These reactive metabolites directly damage DNA, proteins and cell membranes [13]. Anthracyclines also target the sarcoplasmic reticulum, disturb intracellular Ca^{2+} homeostasis [14] and induce apoptosis [15–17]. The C_{max} of plasma doxorubicin following a 30 mg m^{-2} intravenous bolus dose is ∼3 μM. However, cellular levels are ∼30–100-fold higher than that of the plasma [18]. On the other hand, the C_{max} of plasma daunorubicin following a 45 mg m^{-2} intravenous bolus dose is ∼0.4 μM, but cells also accumulate much higher levels [19]. Both drugs have long terminal half-lives (∼30–40 hr). Their side effects include irreversible cardiomyopathy, hematopoietic suppression, mutagenesis and carcinogenesis.

2. Materials and methods

2.1. Chemicals

The Pd derivative of Pd phosphor (sodium salt, $M_\gamma \sim 1300$) was purchased from Porphyrin Products, Inc.; a high flux LED-based illuminator with red (peak, 630 nm) visible emission (OTL630A-5-10-66-E) was purchased from Opto Technology, Inc.; cisplatin (1 mg mL^{-1}, ∼3.3 mM in 154 mM NaCl) was obtained from American Pharmaceutical Partners; 4OOH-CP (D-18864, M_γ 293.1) was obtained from ASTA Medica AG; doxorubicin HCl (M_γ 579.99; ∼3.45 mM solution) was obtained from GensiaSicor Pharmaceuticals; daunorubicin HCl (M_γ 563.99; ∼8.87 mM solution) was obtained from Bedford Laboratories; NADH, rotenone, L-ascorbic acid, ascorbate oxidase, ADP, NaH_2PO_4, Na_2HPO_4, EDTA, and fatty acid-free BSA were purchased from Sigma–Aldrich; ethyl alcohol (200 proof; USP grade) was purchased from Pharmco Products; PBS w/o calcium or magnesium was purchased from BioWhittaker; FBS, dH_2O (water for injection quality water), and RPMI-1640 medium (pH 7.15) without L-glutamine were purchased from Mediatech.

2.2. Solutions

The Pd phosphor was dissolved in dH_2O (2.5 mg mL^{-1}, ∼2.0 mM) and stored in the refrigerator. NADH (1.0 M) and ADP (0.1 M) solutions were made in dH_2O and stored at −20°. Rotenone (1.0 mM) was dissolved in absolute ethanol and used fresh; ethanol had no noticeable effect on cellular respiration. L-Ascorbic acid (10 mM), ascorbate oxidase (1.0 unit μL^{-1}) and 4OOH-CP (∼10 mM) solutions were made in dH_2O immediately prior to use [20]. The Pd phosphor solution contained 2 μM Pd phosphor, 90 mg BSA, 50 mM glucose, 5 mM ADP, 5 mM Na_2HPO_4 and 2.55 mL RPMI (final volume, 3.0 mL and final pH, ∼7.5). The mixture was freshly made in a 30-mL quartz tube and continuously stirred at room temperature for 1–2 hr before use.

2.3. SMP

Measurements with SMP were included in these experiments in order to demonstrate a direct effect (or absence of an effect) of the various toxic agents on the mitochondrial respiratory chain. SMP were prepared from beef heart mitochondria [21]. SMP (one mg per condition) were suspended in 1.0 mL of 10 mM Tris–Cl (pH, 8.0), 25 mM sucrose. The mixtures were incubated (37° for 1 or 3 hr) with and without the toxic agent (cisplatin, 4OOH-CP, doxorubicin, daunorubicin or rotenone). At the end of the incubation period, the particles were collected by centrifugation (23,000 *g* at 4° for 30 min), washed with 1.0 mL of the same buffer and suspended in 1.0 mL of the Pd phosphor solution (with three passages of the pestle through a glass-Teflon homogenizer). Approximately 150 μL of each suspension was diluted to 0.5 mL with the Pd phosphor solution, transferred to a vial and placed in the instrument. Oxygen consumption was initiated with the addition of 50 mM NADH. The sample was then sealed with 50 μL of mineral oil and the measurements continued until oxygen was totally depleted. The k (in μM min^{-1}) was calculated as the rate of decay of [O_2] from ∼225 to ∼50 μM. This linear portion of the curve had r value >0.99. The k value prior to the addition of NADH was near zero.

2.4. Cells

The human T-cell lymphoma cell line Jurkat was maintained, in a fully humidified atmosphere containing 5% CO_2 at 37°, at a density ~5×10^5 cells per mL in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 μg mL^{-1} streptomycin, 100 IU mL^{-1} penicillin and 2.0 mM L-glutamine. PBMC were prepared from whole blood of a volunteer as described [22]. An ovarian cancer specimen from a patient with recurrent tumor was collected immediately following tumor resection and processed as described [23]. HL60 leukemia and ovarian cancer cells were maintained in cultures as above and harvested at near confluence. The cells were examined and counted on the Coulter Z2 model (Beckman Coulter, Inc.) as described [24].

The use of volunteer blood and ovarian tumor specimen was approved by the institutional review board (State University of New York, Upstate Medical University) for the protection of human subjects. Informed consent was obtained from the patient with the ovarian tumor.

2.5. DNA–platinum (Pt) adducts

HL60 cells were suspended in 5.0 mL RPMI medium and incubated at 37° for 1 hr with and without 33 μM cisplatin. The cells were then recovered by centrifugation, washed with PBS and suspended in 10 mM Tris–Cl (pH 8.0), 10 mM EDTA. DNA was extracted and the DNA–Pt adducts were determined on the atomic absorption spectrometer as described [23].

2.6. Incubation of cells with the cytotoxic agents

Incubations were carried out in RPMI medium at 37°. In a typical experiment, the cytotoxic agent (cisplatin, 4OOH-CP, doxorubicin, daunorubicin or rotenone) was added to the cell suspension and the mixture was incubated for 1 or 3 hr. The cells were then recovered by centrifugation, washed with PBS or RPMI and suspended in 0.5 mL of the Pd phosphor solution. The cell suspension was placed in a 0.8-mL tube, sealed with ~50 μL mineral oil and placed in the instrument. Mixing was achieved with a ceramic stirring bar. When $[O_2]$ reached ~50 μM, the cells were collected by centrifugation, resuspended in a fresh Pd phosphor solution and the measurements repeated. The k value (μM min^{-1}) was calculated as the rate of decay of $[O_2]$ from ~225 to ~50 μM. This linear portion of the curve had r value of >0.99. The presence of ADP in the Pd phosphor solution had no effect on the rate of cellular respiration. Each experiment included a rotenone condition (5–50 μM; rotenone inhibits complex I of the respiratory chain).

2.7. Instrument and analysis

Respiration was measured at room temperature in sealed vials containing SMP or cells suspended in 0.5 mL of the Pd phosphor solution, and stirred continuously with the aid of a glass-endorsed magnetic stirring bar. The substrate for the cells was glucose, while NADH served as substrate for SMP. $[O_2]$ in the suspension was determined as a function of time with the aid of the phosphorescence probe Pd(II) *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin, essentially as described [25–29]. The phosphorescence decay of the probe is closely characterized by a single exponential and the phosphorescence decay time (τ) was inversely proportional to the $[O_2]$ since oxygen quenches phosphorescence [28].

The absorbance of the samples at the wavelengths of excitation and emission was less than 0.1. Samples were exposed to light flashes (10 s^{-1}) from a pulsed light-emitting diode array with peak output at 625 nm (OTL630A-5-10-66-E, Opto Technology, Inc.). Emitted phosphorescent light was detected by a Hamamatsu photomultiplier tube (#928) after first passing through a wide-band interference filter centered at 800 nm. The amplified phosphorescence decay was digitized at a rate of 1 MHz by a 20 MHz A/D converter (ComputerBoards, Inc.). Two hundred and fifty samples were collected from each decay curve and the data from 10 consecutive decay curves were averaged for calculation of τ. $[O_2]$ was calculated using the following equation:

$$\frac{\tau^o}{\tau} = 1 + \tau k_q [O_2] \quad (1)$$

where τ, is the lifetime in the presence of oxygen; τ^o is the lifetime in the absence of oxygen; and k_q is the second-order oxygen quenching rate constant for the Pd phosphor in the presence of BSA (4.1×10^8 M^{-1} s^{-1}) [27].

The phosphorescence-based oxygen analyzer was in our hands superior to the conventional oxygen electrode system in that it provided an accurate, sensitive and reproducible quantitation of $[O_2]$. Measurements in this study were performed over periods up to 60 min, thus ensuring cell viability. However, much longer measurement times, necessitated by the availability of scarce material, can easily be managed with the phosphorescence-based device but would be more difficult with an oxygen electrode. Measurements on re-aerated cell suspensions showed that the probe was stable and did not interfere with cell function [27].

Stoichiometric titration of the oxygen with ascorbate in the presence of ascorbate oxidase ($2\,\text{ascorbate} + O_2 \rightarrow 2\,\text{dehydroascorbate} + 2H_2O$) was done as described [29]. The drift of the Pd phosphor solution without cells or SMP was 0.6 μM min^{-1} at $[O_2]$ of ~250 μM, 0.2 μM min^{-1} at $[O_2]$ of ~125 μM, 0.8 μM min^{-1} at $[O_2]$ of ~75 μM, and 1.3 μM min^{-1} at $[O_2]$ of ~50 μM.

3. Results

3.1. PBMC oxygen consumption

Oxygen consumption in PBMC (~5×10^7 cells per condition) incubated (at 37° for 1 hr) in the presence

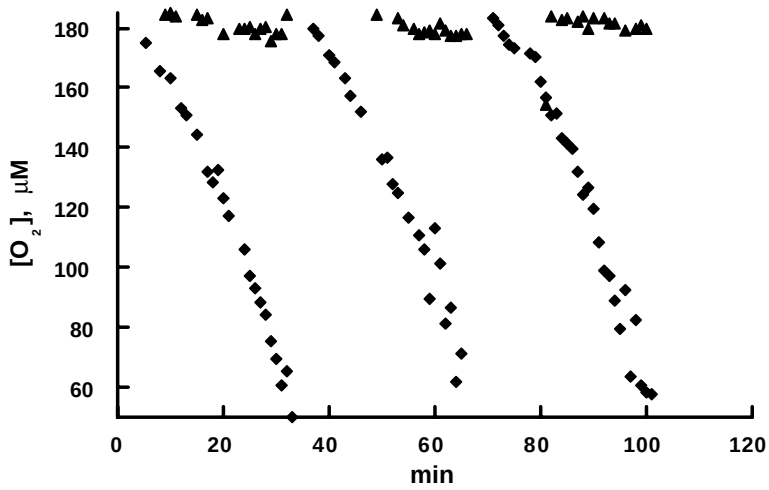

Fig. 1. PBMC oxygen consumption. PBMC ($\sim 5 \times 10^7$ cells per condition) were incubated at 37° for 1 hr with (triangles) and without (diamonds) rotenone (33 μM). The cells were then suspended in the Pd phosphor solution and placed in the instrument. When [O_2] reached ~50 μM, the cells were collected by centrifugation, suspended in a fresh Pd phosphor solution and re-placed in the instrument. The k values for the cells incubated without rotenone were 4.5 μM min^{-1} ($r > 0.99$) and with rotenone 0.5 μM min^{-1}.

and absence of rotenone (33 μM) is shown in Fig. 1. The k value in the absence of rotenone was 4.5 μM min^{-1} ($r > 0.99$) and in the presence of rotenone 0.5 μM min^{-1}. Thus, rotenone inhibited ~90% of cell respiration. In this experiment, the measurements were repeated three times in order to show that the Pd phosphor solution did not impair the ability of the cells to respire on glucose (Fig. 1).

3.2. Effect of cisplatin on SMP oxygen consumption

Figure 2 shows the effect of incubation (at 37° for 3 hr) with 25 μM cisplatin on SMP oxygen consumption. Oxygen consumption prior to the addition of NADH was negligible. The k value for the SMP incubated with no addition (circles) was 28.2 μM min^{-1} ($r > 0.98$), with

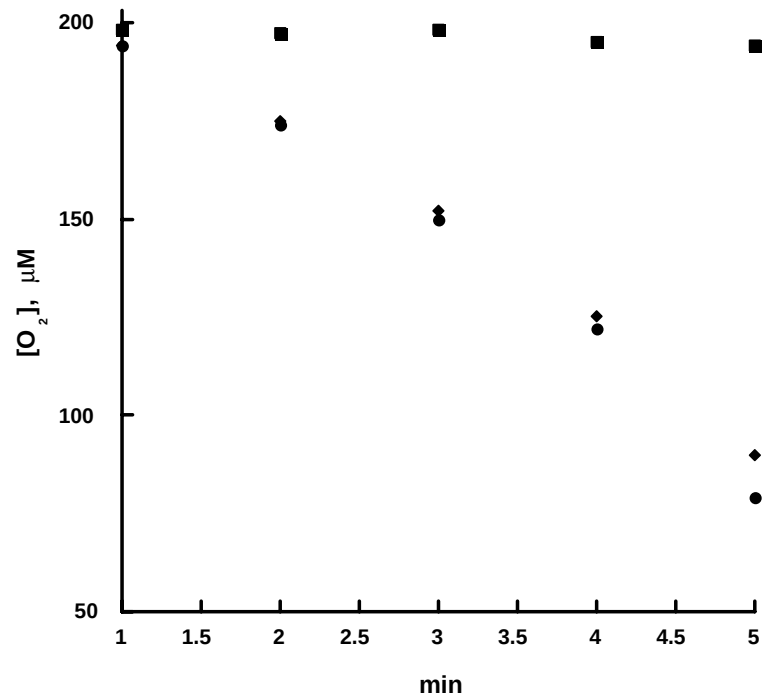

Fig. 2. Effect of cisplatin on SMP oxygen consumption. SMP (1.0 mg per condition) were incubated at 37° for 3 hr without and with cisplatin (25 μM) or rotenone (50 μM). Oxygen consumption was started by the addition of 50 mM NADH. The k value for the SMP incubated with no addition (circles) was 28.2 μM min^{-1} ($r > 0.99$), with cisplatin (diamonds) 25.8 μM min^{-1} ($r > 0.98$) and with rotenone (squares) 1.0 μM min^{-1}. Oxygen consumption prior to the addition of NADH was negligible.

Table 1
Rate constants (k) for oxygen consumption in SMP in the presence and absence of cisplatin

Addition	k ($\mu M\ min^{-1}\ mg^{-1}$)	
	1-hr incubation at 37°	3-hr incubation at 37°
None	285 ± 70 (100%)	151 ± 71 (100%)
Cisplatin (5 μM)	281 ± 73 (99%)	156 ± 78 (103%)
Cisplatin (10 μM)	236 ± 4 (83%)	166 ± 45 (110%)
Cisplatin (20 μM)	240 ± 111 (84%)	132 ± 31 (87%)
Cisplatin (25 μM)	236 ± 111 (83%)	121 ± 49 (80%)
Cisplatin (50 μM)	228 ± 5 (80%)	–
Rotenone (50 μM)	2 ± 1 (<1%)	2 ± 1 (<1%)

The k values (mean ± SD, N = 2) were set equal to the slopes of $[O_2]$ vs. time curves.

cisplatin (diamonds) 25.2 $\mu M\ min^{-1}$ ($r > 0.99$) and with 50 μM rotenone (squares) 1.0 $\mu M\ min^{-1}$. Thus, cisplatin produced ~9% inhibition and rotenone ~97% inhibition.

Control experiments in the absence of inhibitors showed that the respiration decreased more after a 3-hr incubation than after a 1-hr incubation (Table 1). Nevertheless, the incubations with 5 μM cisplatin (therapeutic concentration of the drug) produced no effect on oxygen consumption. Incubations with higher drug concentrations (≥10 μM) produced ≤20% decrease in the k values. Rotenone (50 μM) produced near complete inhibition (Table 1).

3.3. Effect of cisplatin on cellular oxygen consumption

HL60 leukemia cells were incubated (at 37° for 3 hr) with and without cisplatin (33–99 μM). The results are summarized in Table 2. Cisplatin at concentrations ≤66 μM had no effect on the rate of cellular oxygen consumption, whereas 99 μM cisplatin produced ~17% inhibition. In these experiments, rotenone (50 μM) inhibited ~90% of the cellular respiration. In a parallel experiment, the amount of DNA–Pt adducts in cells incubated (37° for 3 hr) with 33 μM cisplatin was determined. The number of DNA–Pt adducts was 156 Pt molecules per 10^6 nucleotides. Thus, cellular respiration was unaffected by a 3-hr incubation with 33 μM cisplatin despite adequate DNA platination by the drug.

Table 2
HL60 leukemia cell oxygen consumption in the presence and absence of cisplatin

Cisplatin			
0 μM	33 μM	66 μM	99 μM
1.05 ± 0.15	1.11 ± 0.15	1.05 ± 0.07	0.87 ± 0.07

HL60 leukemia cells were incubated for 3 hr at 37° with and without cisplatin (33–99 μM). The k values (mean ± SD, N = 3) are expressed in $\mu M\ min^{-1}\ mg^{-1}$ (dry cell pellet), $r > 0.99$.

A similar lack of effect of cisplatin on Jurkat cell respiration was observed. Jurkat cells, in RPMI medium supplemented with 10% FBS, were incubated (37° for 3 hr) with and without 25 μM cisplatin. The k value for the cells incubated without cisplatin was 3.95 $\mu M\ min^{-1}$ ($r > 0.99$) and with cisplatin 3.68 $\mu M\ min^{-1}$ ($r > 0.99$) (Fig. 3).

We also investigated the effect of cisplatin on oxygen consumption in ovarian cancer cells (~7×10^6 cells maintained in a short-term culture) from a patient with recurrent tumor (Fig. 4). The k values without addition were

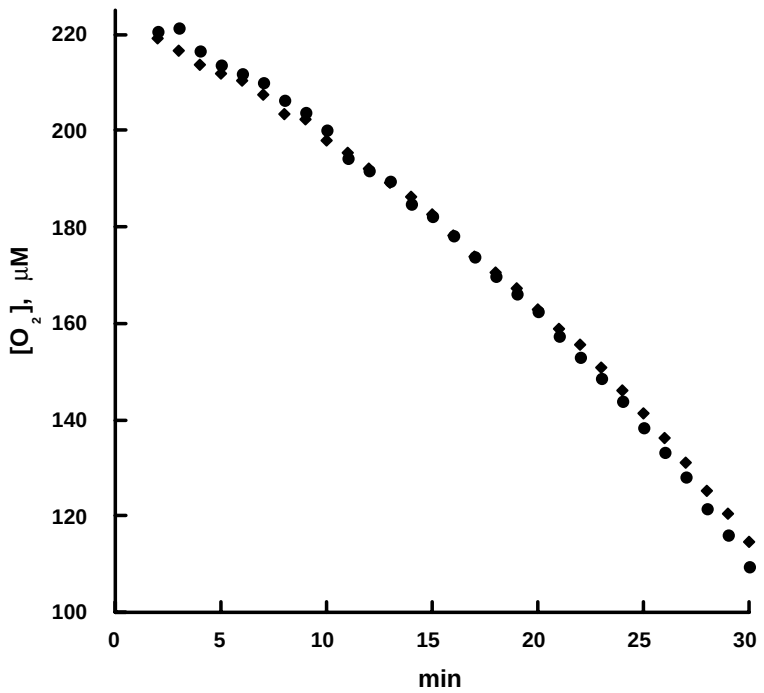

Fig. 3. Effect of cisplatin on Jurkat cell oxygen consumption. Jurkat cells were suspended in RPMI medium (containing 10% FBS) and incubated at 37° for 3 hr with (diamonds) and without (circles) 25 μM cisplatin. The cells were then collected by centrifugation, suspended in the Pd phosphor solution and placed in the instrument. The k value for the cells incubated without cisplatin was 3.95 $\mu M\ min^{-1}$ ($r > 0.99$) and with cisplatin 3.68 $\mu M\ min^{-1}$ ($r > 0.99$).

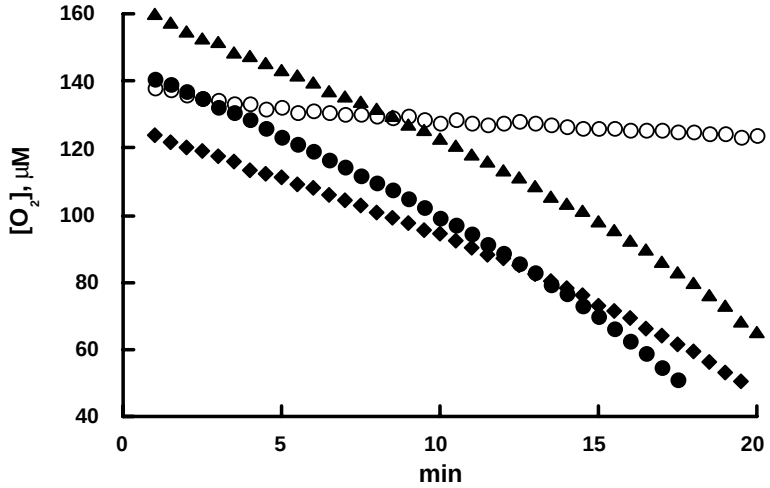

Fig. 4. Effect of cisplatin on ovarian cancer cell oxygen consumption. Ovarian cancer cells ($\sim 7 \times 10^6$ cells maintained in a short-term culture) from a patient with recurrent tumor were suspended in the Pd phosphor solution and placed in the instrument (closed circles). When $[O_2]$ was ~50 μM, the cells were suspended in a fresh Pd phosphor solution and re-placed in the instrument (triangles). At the end of the run, the cells were incubated (at 37° for 30 min) with 33 μM cisplatin, suspended in a fresh Pd phosphor solution and placed in the instrument (diamonds). At the end of the run, the cells were incubated (at 37° for 15 min) with 33 μM rotenone, then suspended in a fresh Pd phosphor solution and placed in the instrument (open circles). The k value for the first run (no addition) was 5.3 μM min^{-1} ($r > 0.99$), the second run (no addition) 5.0 μM min^{-1} ($r > 0.99$), the third run (33 μM cisplatin) 3.9 μM min^{-1} ($r > 0.99$) and the fourth run (33 μM rotenone) 0.6 μM min^{-1} ($r > 0.96$). Thus, cisplatin produced ~22% inhibition and rotenone ~88%.

5.3 μM min^{-1} (closed circles, $r > 0.99$) and 5.0 μM min^{-1} (triangles, $r > 0.99$). The k value with the addition of cisplatin (33 μM at 37° for 30 min) was 3.9 μM min^{-1} (diamonds, $r > 0.99$) and rotenone (33 μM) 0.6 μM min^{-1} (open circles, $r > 0.96$). Thus, cisplatin produced ~22% inhibition and rotenone ~88%.

3.4. Effect of 4OOH-CP on SMP oxygen consumption

One-hr incubation of SMP with 4OOH-CP produced dose-dependent inhibition of the mitochondrial respiratory chain (Fig. 5). Respiration was inhibited by ~51% with 25 μM 4OOH-CP (diamonds), ~69% with 50 μM 4OOH-CP (squares), ~81% with 75 μM 4OOH-CP (triangles) and ~96% with 100 μM (right triangles). The latter inhibition is similar to that induced by rotenone (open triangles).

3.5. Effect of 4OOH-CP on cellular oxygen consumption

The effect of 4OOH-CP on cellular mitochondrial oxygen consumption was investigated in Jurkat cells. Incubations (at 37° for 1 hr) with 5–20 μM 4OOH-CP had no effect on cellular respiration. By contrast, 1-hr incubation with 50 μM 4OOH-CP produced ~33% inhibition, with 75 μM 4OOH-CP ~37% and with 100 μM 4OOH-CP ~44% (Fig. 6). In another experiment, 1-hr incubation with 50 μM 4OOH-CP produced ~46% inhibition ($r > 0.99$), with 100 μM 4OOH-CP ~73% ($r > 0.99$), with 5 μM rotenone ~73% and with 50 μM rotenone ~94%.

We then investigated the effect of 4OOH-CP on cellular respiration in HL60 leukemia cells. HL60 cells were suspended in 15 mL RPMI medium and divided into three equal aliquots. The cells were incubated (37° for 1 hr) with and without 100 μM 4OOH-CP or 50 μM rotenone. The cells were then collected by centrifugation, suspended in the Pd phosphor solution and placed in the instrument. At the end of the runs, the cells were suspended in a fresh Pd phosphor solution and the measurements were repeated. The k for the cells incubated without addition (triangles) was (mean ± SD, N = 3) 5.4 ± 0.4 μM min^{-1} ($r > 0.99$), with 100 μM 4OOH-CP (diamonds) 2.4 ± 0.2 μM min^{-1} ($r > 0.99$), and with 50 μM rotenone (circles) 0.9± 0.2 μM min^{-1} ($r > 0.96$) (Fig. 7). Thus, the incubation with 100 μM 4OOH-CP inhibited ~50% of the cellular oxygen consumption and rotenone ~83%.

3.6. Effects of doxorubicin and daunorubicin on SMP oxygen consumption

The effect of doxorubicin (25–100 μM at 37° for 1 hr) on SMP respiration is shown in Fig. 8. The rates of respiration in SMP incubated with the drug were increased by ~32–62%. Rotenone, on the other hand, produced over 95% inhibition. Very similar results were obtained in SMP incubated with daunorubicin (25–100 μM at 37° for 1 hr). SMP that were exposed to doxorubicin (50 μM for 1 hr at 37°) retained their sensitivity to rotenone (50 μM for 1 hr at 37°).

3.7. Effects of doxorubicin and daunorubicin on cellular oxygen consumption

The effect of doxorubicin on Jurkat cell oxygen consumption is shown in Fig. 9. Incubation (at 37° for 1 hr) with 5 μM doxorubicin produced ~30% inhibition ($r > 0.99$), with 10 μM doxorubicin ~33% ($r > 0.99$)

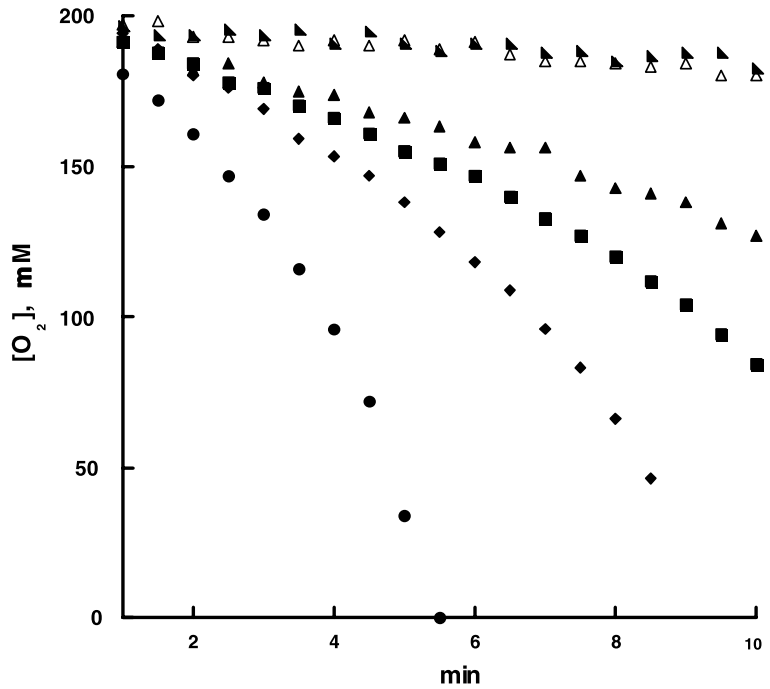

Fig. 5. Effect of 4OOH-CP on SMP oxygen consumption. SMP (1.0 mg per condition) were incubated at 37° for 1 hr without and with 4OOH-CP (25–100 μM) or rotenone (50 μM). Oxygen consumption was started by the addition of 50 mM NADH. The k value for the SMP incubated with no addition (circles) was 36.5 μM min^{-1} ($r > 0.97$), 25 μM 4OOH-CP (diamonds) 17.9 μM min^{-1} ($r > 0.98$), 50 μM 4OOH-CP (squares) 11.3 μM min^{-1} ($r > 0.98$), 75 μM 4OOH-CP (triangles) 6.9 μM min^{-1} ($r > 0.99$), 100 μM 4OOH-CP (right triangles) 1.5 μM min^{-1} ($r > 0.97$), and with 50 μM rotenone (open triangles) 1.7 μM min^{-1} ($r > 0.98$).

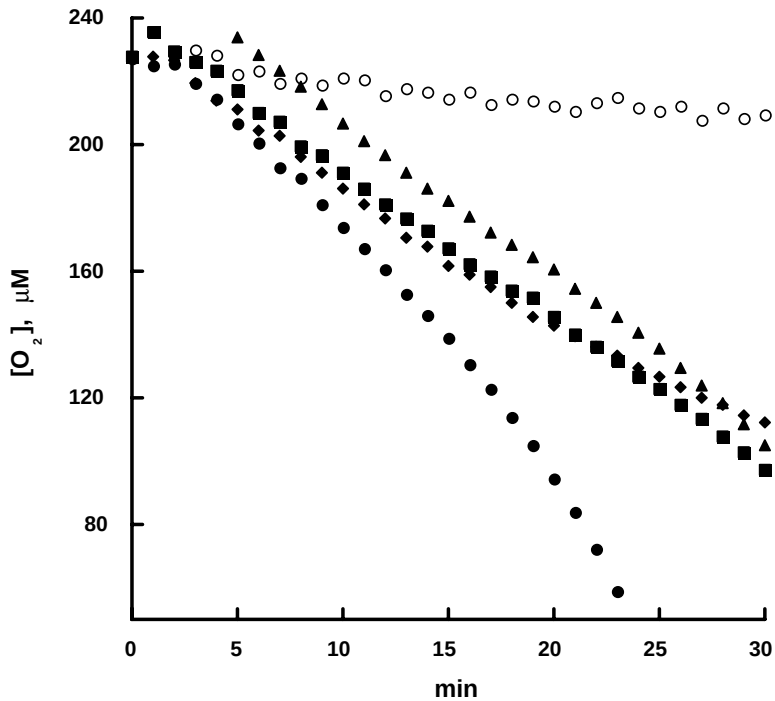

Fig. 6. Effect of 4OOH-CP on Jurkat cell oxygen consumption. Jurkat cells (~2.6×10^7 cells per condition) were suspended in RPMI medium (containing 10% FBS) and incubated at 37° for 1 hr without addition (closed circles) and with the addition of 50 μM 4OOH-CP (triangles), 75 μM 4OOH-CP (squares) or 100 μM 4OOH-CP (diamonds). The cells were then collected by centrifugation, washed with PBS, suspended in the Pd phosphor solution and placed in the instrument. The k value for the cells incubated with no addition was 7.3 μM min^{-1} ($r > 0.99$), with 50 μM 4OOH-CP 4.9 μM min^{-1} ($r > 0.99$), with 75 μM 4OOH-CP 4.6 μM min^{-1} ($r > 0.99$) and with 100 μM 4OOH-CP 4.1 μM min^{-1} ($r > 0.99$). The k value for the buffer alone (open circles) is 0.9 μM min^{-1}.

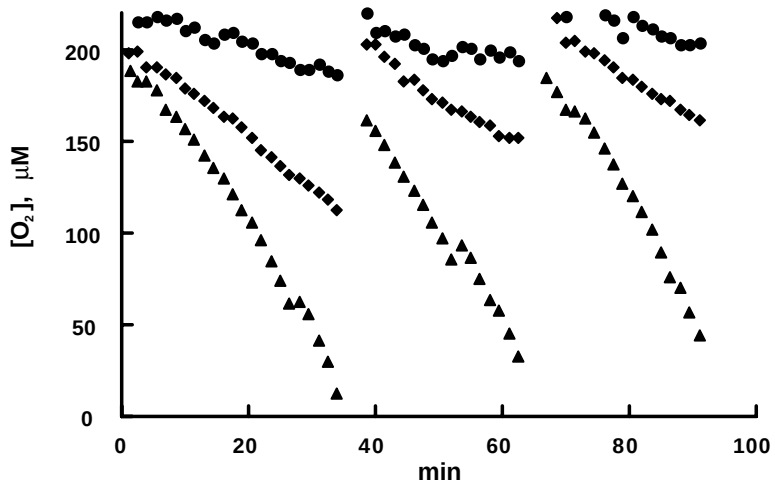

Fig. 7. Effect of 4OOH-CP on HL60 cell oxygen consumption. HL60 leukemia cells were incubated at 37° for 1 hr without (triangles) or with 100 μM 4OOH-CP (diamonds) or 50 μM rotenone (circles). The cells were then collected by centrifugation, suspended in the Pd phosphor solution and placed in the instrument. When oxygen was nearly depleted, the cells were re-suspended in fresh Pd phosphor solution and the measurements were repeated. The k value for the cells incubated with no addition was (mean ± SD, N = 3) 5.4 ± 0.4 μM min^{-1} ($r > 0.99$), with 100 μM 4OOH-CP 2.4 ± 0.2 μM min^{-1} ($r > 0.99$), and with 50 μM rotenone 0.9 ± 0.2 μM min^{-1} ($r > 0.96$).

and with 20 μM doxorubicin ~42% ($r > 0.99$). Moreover, incubations with 50–100 μM doxorubicin produced ~50% inhibition (data not shown). In this experiment, incubation with 50 μM rotenone (at 37° for 1 hr) produced 89% inhibition and with 5 μM rotenone ~73%.

The effect of daunorubicin on HL60 cell oxygen consumption is shown in Fig. 10. HL60 leukemia cells were suspended in the Pd phosphor solution and placed in the instrument. When the oxygen was nearly depleted, the cells were collected and incubated (in PBS at 37° for 40 min) with ~18 μM daunorubicin. The cells were then washed with PBS, suspended in the Pd phosphor solution and replaced in the instrument. The k value for the cells incubated without addition was 5.6 μM min^{-1} and with daunorubicin

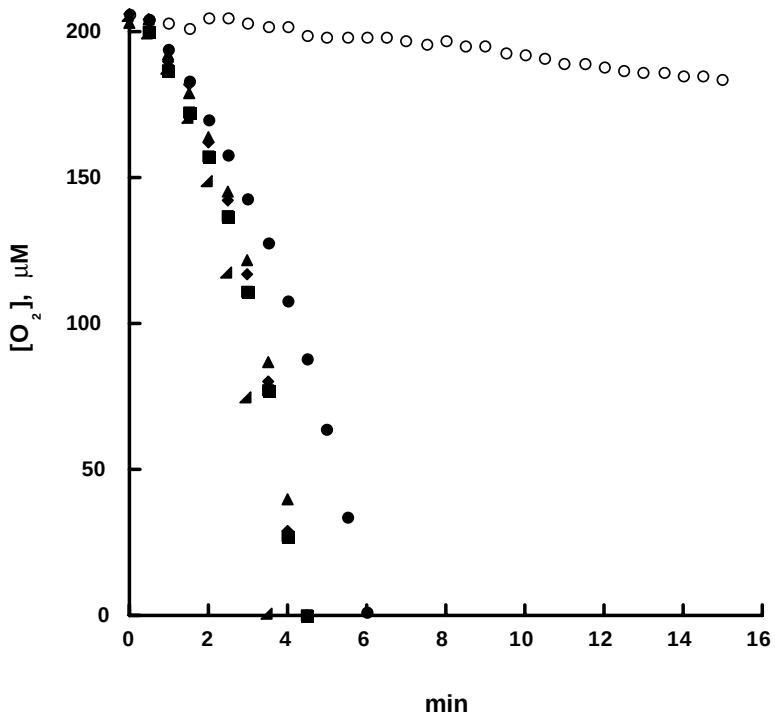

Fig. 8. Effect of doxorubicin on SMP oxygen consumption. SMP (1.0 mg per condition) were incubated at 37° for 1 hr without and with doxorubicin (25–100 μM) or rotenone (50 μM). Oxygen consumption was started by the addition of 50 mM NADH. The k value for the SMP incubated with no addition (closed circles) was 33.8 μM min^{-1} ($r > 0.97$), 25 μM doxorubicin (squares) 46.6 μM min^{-1} ($r > 0.96$), 50 μM doxorubicin (diamonds) 46.5 μM min^{-1} ($r > 0.96$), 75 μM doxorubicin (triangles) 44.7 μM min^{-1} ($r > 0.95$), 100 μM doxorubicin (right triangles) 54.7 μM min^{-1} ($r > 0.94$), and 50 μM rotenone (open circles) 1.4 μM min^{-1} ($r > 0.97$). Very similar results were obtained with SMP incubated (at 37° for 1 hr) with 25–100 μM daunorubicin.

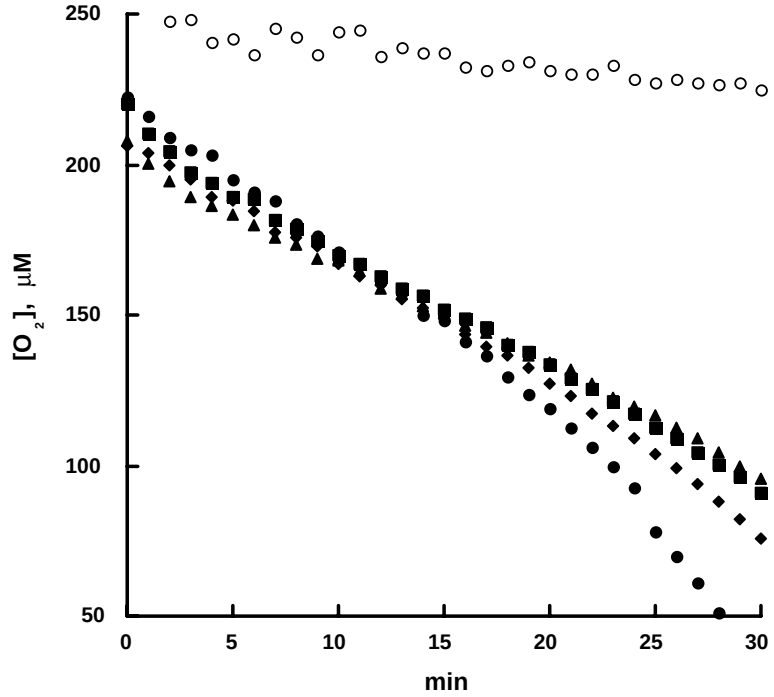

Fig. 9. Effect of doxorubicin on Jurkat cell oxygen consumption. Jurkat cells were suspended in RPMI medium (containing 10% FBS) and incubated at 37° for 1 hr without (closed circles) and with 5 μM (diamonds) doxorubicin, 10 μM doxorubicin (squares) or 20 μM doxorubicin (triangles). The cells were then collected by centrifugation, washed with PBS, suspended in the Pd phosphor solution and placed in the instrument. The k value for the cells incubated with no addition was 6.0 μM min^{-1} ($r > 0.99$), 5 μM doxorubicin 4.2 μM min^{-1} ($r > 0.99$), 10 μM doxorubicin 4.0 μM min^{-1} ($r > 0.99$) and 20 μM doxorubicin 3.5 μM min^{-1} ($r > 0.99$). The k value for the buffer alone (open circles) is 0.8 μM min^{-1}.

2.3 μM min^{-1}. Thus, incubation of the cells with 18 μM daunorubicin inhibited cellular respiration almost 60%.

The effect of daunorubicin on Jurkat cell oxygen consumption is shown in Fig. 11. Incubation (at 37° for 1 hr) with 2 μM doxorubicin produced ~22% inhibition ($r > 0.99$), with 10 μM doxorubicin ~31% ($r > 0.99$) and with 20 μM doxorubicin ~33% ($r > 0.99$).

4. Discussion

Despite the fact that many anticancer drugs may target mitochondria (directly or indirectly), the effects of these agents on cellular oxygen consumption (cellular respiration or oxidative phosphorylation) have not been extensively studied [1–3]. To address this question, a phosphorescence

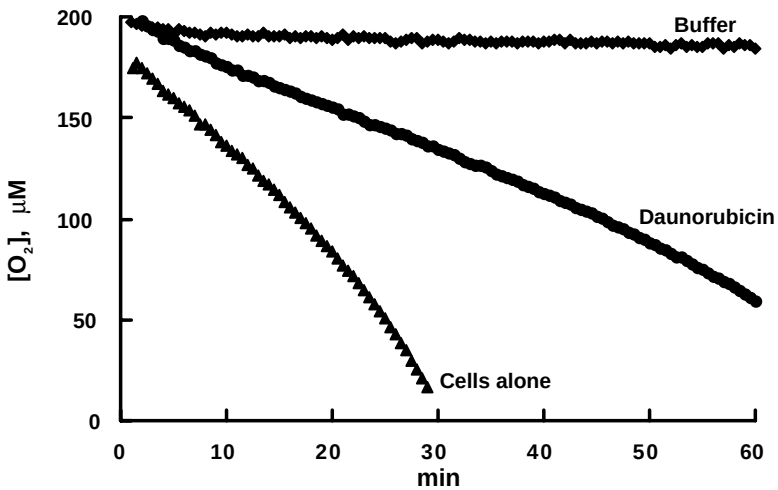

Fig. 10. Effect of daunorubicin on HL60 cell oxygen consumption. HL60 leukemia cells were suspended in the phosphor solution and placed in the instrument. When the oxygen was nearly depleted, the cells were collected by centrifugation, suspended in PBS and incubated (at 37° for 40 min) with ~18 μM daunorubicin. The cells were then washed with PBS, suspended in a fresh Pd phosphor solution and re-placed in the instrument. The k value for the cells incubated without addition was 5.6 μM min^{-1} and with the addition of daunorubicin 2.3 μM min^{-1}.

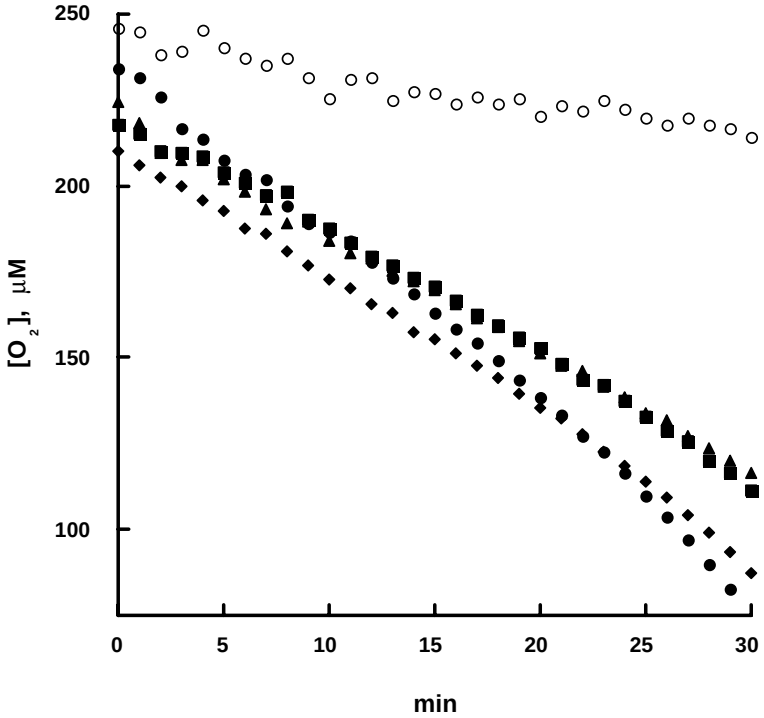

Fig. 11. Effect of daunorubicin on Jurkat cell oxygen consumption. Jurkat cells were suspended in RPMI medium (containing 10% FBS) and incubated at 37° for 1 hr without (closed circles) and with 2 μM (diamonds) daunorubicin, 10 μM daunorubicin (squares) or 20 μM daunorubicin (triangles). The cells were then collected by centrifugation, washed with PBS, suspended in the Pd phosphor solution and placed in the instrument. The k value for the cells incubated with no addition was 5.1 μM min^{-1} ($r > 0.99$), 2 μM daunorubicin 4.0 μM min^{-1} ($r > 0.99$), 10 μM daunorubicin 3.5 μM min^{-1} ($r > 0.99$) and 20 μM doxorubicin 3.4 μM min^{-1} ($r > 0.99$). The k value for the buffer alone (open circles) is 0.9 μM min^{-1}.

analyzer, as described [25–29], was used here to explore immediate effects of the commonly used cytotoxic drugs on mitochondrial oxygen consumption in human cells and in beef heart SMP.

Oxidative phosphorylation in human leukocyte (PBMC) and leukemic cell mitochondria is well described [30–32], and our studies demonstrate the integrity of mitochondrial function in these types of cells. The data in Fig. 1, showing oxygen consumption by PBMC mitochondria and inhibition by rotenone, support these reported observations.

Drug formulations normally given to patients are used throughout this study [33]. The effect of cisplatin on SMP oxygen consumption (Table 1 and Fig. 2) is similar to that on the cells (Table 2 and Figs. 3 and 4). Exposure to therapeutic cisplatin concentrations (~5–33 μM) had minimal immediate effect on the respiratory chain function (Tables 1 and 2 and Figs. 2–4), demonstrating lack of a direct impairment of mitochondrial oxygen consumption by Pt-compounds. Cisplatin, however, is known to induce apoptotic cell death, a process that leads to mitochondrial membrane leak, release of cytochrome *c* and activation of caspase 9 [5]. This delayed mitochondrial injury was demonstrated in our system by significant inhibition of Jurkat cell oxygen consumption at 24 and 48 hr following 3-hr exposure to 25 μM cisplatin (data not shown).

The effect of 4OOH-CP on SMP oxygen consumption (Fig. 5) is similar to its effect on cells (Figs. 6 and 7). This cytotoxic agent is highly reactive, producing a direct mitochondrial injury (Figs. 5–7). An acute mitochondrial insult of this kind may well contribute to the known adverse effects of alkylating agents.

In contrast, anthracyclines used in our *in vitro* system (thus lacking enzymatic drug activation) did not decrease SMP oxygen consumption (Fig. 8), but did inhibit respiration in cells (Figs. 9 and 10). Incubation of SMP with 50 mM NADH (a substrate for complex I of the respiratory chain) and doxorubicin did not change the results shown in Fig. 8 [13]. Thus, anthracycline-induced cellular respiratory chain damage (Figs. 9–11) appears to be indirect, perhaps mediated by effect of the drugs on the sarcoplasmic reticulum [14], by induction of cellular necrosis or by activation of the apoptotic pathways [15–17]. Alternatively, cellular reduction of the quinone moieties of the drugs to the semiquinone radical species may be necessary for mitochondrial injury [13,34].

We conclude that the mitochondria appear to be a common target for many anticancer drugs [1–3]. At high concentrations, anthracyclines and oxazaphosphorines rapidly impair cellular respiration, producing immediate cell death (lack of oxygen consumption, energy deprivation). Cisplatin, on the other hand, produces delayed mitochondrial dysfunction. These distinct effects of anticancer drugs on mitochondrial function may be relevant to their mechanism of action and to their adverse clinical responses.

Acknowledgments

We thank Dr. Paul T. Massa for the use of his cell culture facility. We also thank Bonnie Toms, Violeta Cuenca and Lydia Kaufman for supplying the HL60 and ovarian cancer cells. We appreciate the technical support of Mr. Andrew Holloway.

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