Quantitative Measure of Cytotoxicity of Anticancer Drugs and Other Agents.

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Quantitative measure of cytotoxicity of anticancer drugs and other agents

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**Article info**

Received 2 April 2008
Available online 18 June 2008

Keywords:
Cytoxicity
Respiration
Phosphorescence
Oxygen consumption

**Abstract**

Many anticancer drugs act on cancer cells to promote apoptosis, which includes impairment of cellular respiration (mitochondrial O2 consumption). Other agents also inhibit cellular respiration, sometimes irreversibly. To investigate the sensitivity of cancer cells to cytoxins, including anticancer drugs, we compare the profiles of cellular O2 consumption in the absence and presence of these agents. Oxygen measurements are made at 37°C, using glucose as a substrate, with [O2] obtained from the phosphorescence decay rate of a palladium phosphor. The rate of respiration k is defined as −d[O2]/dt in a sealed container. Different toxins produce different profiles of impaired respiration, implying different mechanisms for the drug-induced mitochondrial dysfunction. The decrease in the average value of k over a fixed time period, I, is proposed as a characteristic value to assess mitochondrial injury. The value of I depends on the nature of the toxin, its concentration, and the exposure time as well as on the cell type. Results for several cell types and 10 cytoxins are presented here.

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Although the process of drug-induced tumor cell death remains poorly understood, many anticancer agents induce apoptosis through similar mechanisms such as the release of cytochrome c from mitochondria into cytosol and the consequent activation of caspasases. The activated caspases then attack the permeabilized mitochondria, disrupt the mitochondrial electron transport chain, collapse the mitochondrial inner membrane potential, and diminish the mitochondrial structural integrity [1,2]. Interferences in the apoptotic scheme reduce cancer cells’ sensitivity to therapy [3,4]. Because most anticancer drugs and other cytotoxic agents impair mitochondria directly or indirectly, their cytotoxicity can be easily assessed by measuring their effects on mitochondrial respiration. It is proposed here that measurement of cellular respiration in the presence and absence of an agent is a useful way to characterize cytotoxicity. In the case of an anticancer drug, this measurement becomes an index of the cell’s sensitivity to treatment by the drug.

A variety of agents are studied here. The anthracycline antibiotic doxorubicin intercalates with DNA and produces DNA breaks by stimulating topoisomerase II cleavable complex formation [5]. In the cell, the quinone moiety of doxorubicin is reduced to the semi-quinone radical, generating reactive oxygen species that directly damage cell organelles and induce apoptosis [6]. Dactinomycin is an important anticancer chromopeptide that intercalates between DNA base pairs and inhibits transcription [7]. Platinum (Pt) compounds, including cisplatin, carboplatin, oxaliplatin, and nedaplatin, bind to DNA and promote apoptosis [8]. The latter three Pt agents differ from cisplatin (the parent compound) in the different ligands occupying the Pt coordination spheres. These structural variations yield unique antitumor activity and toxicity profiles such as the reactivity with DNA and induction of apoptosis [8]. Transplatin, the geometric isomer of cisplatin, is expected to be less cytotoxic than cisplatin. Tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide) is a promising agent that is effective in hypoxic tumor environments [9]. The activated compound results from a two-electron reduction of tirapazamine, which is readily reoxidized to its parent form in aerobic conditions [10]. A highly cytotoxic hydroxyl radical (HO) is released and produces DNA breaks, chromosomal aberrations, and eventually cell death [9–11]. Not considered as an anticancer drug, cyclosporine A (CsA) is an important immune suppressor. Its cellular targets include cyclophilin and calmodulin, modulating calcium fluxes through mitochondrial ion channel [12,13]. CsA decreases the possibility of mitochondrial transition permeability (MTP) induced by Ca2+ accumulation in the cytosol [14,15]. In addition, it blocks Ca2+ efflux from mitochondria, causing calcium flood in the mitochondrial matrix that induces serious cytotoxicity and further injures mitochondrial respiration [16]. Finally, caffeine is known to activate Ca2+ channels on the plasma membrane and endoplasmic reticulum, rapidly accumulating Ca2+ in neighboring mitochondria, and this may impair their function. Caffeine also potentiates the cytotoxicity of anticancer drugs.

The purpose of this work is to define a characteristic cytotoxicity parameter that will be useful in pharmacology and toxicology. The parameter is the extent of inhibition of respiration, where inhibition is defined in terms of the ratio of the average rates of...
respiration in the presence and absence of the cytotoxic agent. Both rates must be measured on cells from the same batch because rates depend on previous history and other uncontrollable factors. It is expected that the rate of respiration in the presence of the agent will depend on the agent’s nature, its concentration, and the incubation time, as well as on the cell type, so that the inhibition will depend on all of these factors. For simplicity, the experiments discussed in this article are limited to two cell types: Jurkat and HL-60.

Respiration rate is obtained from the decrease in dissolved oxygen concentration in a closed vessel. Dissolved oxygen concentration is measured from the decay rate of phosphorescence from a palladium phosphor, present in the solution, using a homemade instrument [17–20]. In previous studies, we used this instrument to explore the mitochondrial perturbation during anticancer therapy in vitro as well as enzyme reactions involving O2 [21–24]. The phosphorescence method of oxygen analysis has several advantages compared with electrochemical and other methods. It is totally noninvasive and does not affect the system being measured because no oxygen is consumed by the measurement. It allows measurement of [O2] down to nanomolar concentrations. It can be used continuously for long periods of time with no deterioration (as for electrodes) or loss of accuracy, so that slow processes such as decrease in respiration rates can be followed. Furthermore, it requires only a single calibration, with no necessity to check the instrument during or after long runs.

**Materials and methods**

**Reagents**

Pd(II) complex of meso-tetra-(4-sulfonatophenyl)-tetraphenylporphyrin (sodium salt, palladium [Pd] phosphor) was obtained from Porphyrin Products (Logan, UT, USA). Its solution (2.5 mg/ml = 2 mM) was prepared in distilled water (dH2O) and stored at −20 °C in small aliquots.

Doxorubicin HCl (3.45 mM) was purchased from Bedford Laboratories (Bedford, OH, USA). Dactinomycin (actinomycin D, MW 194.19) and remaining reagents were purchased from Sigma-Aldrich. It allows measurement of [O2] down to nanomolar (MW 194.19) and remaining reagents were purchased from Sigma-Aldrich.

Human promyelocytic leukemia (HL-60) and T-cell lymphoma (Jurkat) cells were purchased from American Tissue Culture Collection (Manassas, VA, USA). The cells were cultured in medium plus 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. Tu183 cells were obtained from Edward J. Shillitoe (State University of New York, Upstate Medical University). They were derived from a squamous cell carcinoma of the tonsil and were highly resistant to therapy. The cells were cultured in Dulbecco’s modified Eagle’s medium nutrient mixture F-12 (Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.2% primosin. For harvesting, the cells were incubated at 37 °C in 2.5 ml of 0.05% (w/v) trypsin plus 0.53 mM ethylenediaminetetraacetic acid (EDTA) for 5 min and then collected. Cell counts and viabilities were determined by light microscopy using a hemocytometer under standard trypan blue staining conditions.

Cells were suspended at 0.5 to 1.0 × 10⁶ cells/ml in medium plus 2 μM Pd phosphor and 0.5% fat-free bovine serum albumin. For each condition, 1.0 ml of the cell suspension was placed in a 1-ml glass vial (8-mm clear vials, Krazek Scientific, Albany, NY, USA). The vial was sealed with a crimp top aluminum seal (using a Wheaton hand crumper, Fisher Scientific). Mixing was accomplished with the aid of parylene-coated stirring bars (1.67 × 2.01 × 4.8 mm, V&P Scientific, San Diego, CA, USA).

**O2 measurement**

The O2 detection system was built to measure the phosphorescence of Pd phosphor as described previously [21,22]. DASYlab (Measurement Computing, Norton, MA, USA) was used for data acquisition. The data were analyzed by a C++ language computing program [25] that calculated phosphorescence lifetime (τ) and decay constant (1/τ). Because oxygen quenches the phosphorescence, the decay rate 1/τ is a linear function of [O2] as

\[
\frac{1}{\tau} = \frac{1}{\tau_0} + k [O_2]
\]

where 1/τ₀, the decay rate in the absence of oxygen, was 10,087 ± 156 s⁻¹ and the value of the quenching rate constant, k, was 96.1 ± 1.2 μM⁻¹ s⁻¹ [21].

**Index of respiration inhibition**

Here we propose an index for the efficiency with which a chemical agent inhibits cellular respiration. The index can be calculated from a graph of [O2] versus t when [O2] is measured in a closed container because the negative of the slope, −d[O2]/dt, is the respiration rate k (considered as a zero-order rate constant). For cells not treated by cytotoxic agents, k is a constant, so that [O2] is a linear function of time,

\[
[O_2] = [O_{2,0}] - kt.
\]

The initial concentration of oxygen, [O2]₀, can be calculated from the Henry’s law constant for O2, assuming that the solution is saturated with air ([O2]₀ is 225 μM for 37 °C). For cells treated with most drugs, the value of k is time dependent; that is, [O2] does not decrease linearly with time. Therefore, we propose to calculate the average value of k over a fixed time interval from experimental measurements of [O2] versus t,

\[
\langle k \rangle = \frac{1}{t_f - t_i} \int_{t_i}^{t_f} k dt,
\]
and use \( k \) as a measure of cytotoxicity. Because \( k = -\frac{d[O_2]}{dt} \),
\[
(2) \quad (k) = \frac{[O_2]_i - [O_2]_f}{t_i - t_0}
\]

Because of the fluctuations in measured \( [O_2] \), one cannot simply use the measured \( [O_2] \) values at the end points of the interval to calculate \( k \). Rather, one should use all of the measured values in the calculation of \( k \).

To do this, we fit all of the measurements to a suitable analytic function and calculate \( [O_2] \) at the end points from the parameterized function. A simple and convenient fitting function is a second-order polynomial, \( [O_2] = a + bt + ct^2 \). This is appropriate because it includes the linear function as a special case, \( \gamma = 0 \). With this function, \( k = -\beta + 2\gamma t \) and \( (k) = -\beta - \gamma(t_1 + t_0) \). It is expected that the quadratic will be concave upward, corresponding to a gradual decrease in respiration rate, so that \( \beta \) will be negative and \( \gamma \) will be positive. More generally, a power series, \( [O_2] = a + bt + ct^2 + dt^3 \ldots \), can be adopted. If the parameters \( c \) and \( d \) are small, \( [O_2] \) versus \( t \) will be a line; if not, it will be curved. The cubic term makes it possible to represent a \( k \) that is nonmonotonic in time. Although we have not found any cases where the fit is improved significantly by going past the \( t^2 \) term, this must be checked under specific circumstances. The average value of \( k \) for the cubic is
\[
(3) \quad (k) = \frac{t_i^4 - t_i^2}{t_i^4 - t_i^2} k dt = -\frac{1}{t_i - t_0} \int (b + 2ct + 3dt^2) dt
\]
\[
= -b - c(t_i + t_0) - d(t_i^2 + t_0^2 + t_i^2).
\]

For all toxin-treated conditions, we choose \( t_i = t_0 \) as a fixed time interval. Invariably, \( t_i \) is longer than the preceding incubation time by the time required for the sample preparation. In our experiments, this time can vary from a few minutes to a half-hour, depending on the number of sampling conditions, and is different for different samples. However, this does not affect the calculation of \( k \) given that \( (k) \) is an average over the same time period for all samples.

After the calculation of \( k \) from fitting of the experimental data to a polynomial, we propose to calculate an index, \( I \), defined as
\[
(4) \quad I = 1 - \frac{(k)}{(k)_{0}}
\]
where \( (k) \) is the average value of negative slope in the toxin-treated condition and \( (k)_{0} \) is the average value of negative slope in the untreated condition. The meaning of \( I \) is the average inhibition of cellular respiration due to the toxin treatment during the period when measurements are made. If \( I \) depends on dosage \( D \), experimentally determined \( I \) values can be fitted to the function
\[
(5) \quad I = \frac{D}{A + D} \cdot I_{\text{max}}
\]
where \( I_{\text{max}} \) and \( A \) are constants for a particular drug and cell line. When drug dosage reaches the concentration \( A \), \( I \) equals half-maximal inhibition (\( I_{\text{max}} \)) of cellular respiration.

When a polynomial is used to fit experimental \([O_2] \) for \( t_i < t < t_0 \), it must be remembered that the polynomial has no validity outside this region (e.g., \([O_2] \) certainly does not increase rapidly for later \( t \) as a quadratic does). Because the polynomial cannot be used to extrapolate to \( t = 0 \), it may be necessary to use the initial \([O_2] \) (e.g., \( 225 \mu M \) at \( 37^\circ C \)). This information is added to the experimental points before fitting.

Results

Dactinomycin and doxorubicin

We first evaluated the impact of a prolonged exposure to dactinomycin or doxorubicin on cellular respiration. HL-60 cells were suspended in medium, 2 \( \mu M \) Pd phosphor, and 0.5% bovine serum albumin with 10 \( \mu M \) dactinomycin (plus signs) or without drug (open circles). Time zero corresponds to the addition of dactinomycin. The cells were then placed in the \( 37^\circ C \) incubator. Aliquots (1.0 ml each) of untreated and treated cells were taken periodically for \([O_2] \) measurement, performed every 30 s. Linear fits were made to the data for each condition (dashed lines for untreated cells, solid lines for treated cells). (B) Effect of combined dactinomycin plus doxorubicin on cellular respiration. Jurkat cells (0.5 \( \times 10^6 \) cells/ml) were suspended in medium, 2 \( \mu M \) Pd phosphor, and 0.5% albumin without drug addition (open circles) or with the addition of 20 \( \mu M \) dactinomycin (open squares), 20 \( \mu M \) doxorubicin (open triangles), or 10 \( \mu M \) dactinomycin + 10 \( \mu M \) doxorubicin (filled diamonds). Time zero corresponds to the addition of drugs. Each vial was taken sequentially (every 60 s) for respiration measurement. Linear fits were made to the data for each condition (dashed lines for cells untreated or treated with dactinomycin only, solid lines for cells treated with doxorubicin or both drugs). (C) Effect of dactinomycin on cellular respiration. Jurkat cells (0.5 \( \times 10^6 \) cells/ml) were suspended in medium, 2 \( \mu M \) Pd phosphor, and 0.5% albumin without drug addition (open circles) or with the addition of 10 \( \mu M \) dactinomycin (filled triangles) or 20 \( \mu M \) dactinomycin (filled diamonds). \([O_2] \) was measured alternately every minute on the three conditions. Linear fits to the data set for untreated cells are dashed lines, and quadratic fits to dactinomycin data sets are solid lines.
was found that [O2] decreased linearly with time,
samples of untreated and treated cells were run. For each run, it 
treated cells that was run for approximately 50 min. Thereafter, 
samples of untreated and treated cells were run. For each run, it 
responds to the addition of dactinomycin or the beginning of 
termination (open circles) or with the addition of 20 µM dactinomycin plus 10 µM doxorubicin (filled diamonds) (Fig. 1B). Time zero corresponds to the drug addition (beginning of incubation), 

The values of k for the two measurements on untreated cells, at 0 and 210 min, were 1.42 and 1.99 µM O2/min, respectively ($r^2 = 0.998$ and 0.991, respectively). The increase in respiration rate with time 
requires fewer cells per condition, allowing lower rates of O2 con-
sumption and, thus, longer measurement times that may permit 
detection of deviations from linearity in [O2] versus t. Based on 
the above experience, we adopted the latter protocol in most of 
our experiments.

We previously reported [21] that [O2] versus t plots for cells treated with doxorubicin were actually well fit by two lines; that is, k was constant and equal to the value for untreated cells for approximately 150 min and then decreased to a lower value. The results of Fig. 1B may show this discontinuity in slope. However, this does not have a major effect on our conclusions given that the k values from linear fits, reported above, are average values for the full time of measurement. In contrast to doxorubicin, dactinomycin produced nonlinear [O2] versus t plots that were concave upward; that is, k decreased gradually with time [22]. Therefore, dactinomycin data for long periods of time should be analyzed using a polynomial model.

**Table 1**  
Values of k for cisplatin-treated and untreated cells and $r^2$ values

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell type and concentration</th>
<th>Drug</th>
<th>Concentration (µM)</th>
<th>Incubation time (min)</th>
<th>k (µM O2/min), untreated</th>
<th>k (µM O2/min), treated</th>
<th>Ratio of k values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HL-60, 10^6/ml</td>
<td>Dactinomycin</td>
<td>10</td>
<td>90</td>
<td>1.660</td>
<td>0.846</td>
<td>0.510</td>
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<tr>
<td>2</td>
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<td>Dactinomycin</td>
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<td>150</td>
<td>1.830</td>
<td>0.642</td>
<td>0.350</td>
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<td>10</td>
<td>270</td>
<td>2.150</td>
<td>0.639</td>
<td>0.300</td>
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<tr>
<td>4</td>
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<td>Dactinomycin</td>
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<td>50</td>
<td>0.929</td>
<td>0.732</td>
<td>0.790</td>
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<tr>
<td>5</td>
<td>Jurkat, 0.5 x 10^6/ml</td>
<td>Doxorubicin</td>
<td>20</td>
<td>50</td>
<td>0.929</td>
<td>0.833</td>
<td>0.900</td>
</tr>
<tr>
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<td>Jurkat, 0.5 x 10^6/ml</td>
<td>Dactinomycin + doxorubicin</td>
<td>10</td>
<td>50</td>
<td>0.929</td>
<td>0.826</td>
<td>0.890</td>
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<tr>
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<td>Jurkat, 0.5 x 10^6/ml</td>
<td>Dactinomycin</td>
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<td>30</td>
<td>1.252</td>
<td>0.898</td>
<td>0.717</td>
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<td>1.252</td>
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<td>1.252</td>
<td>0.128</td>
<td>0.102</td>
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<td>0.886</td>
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<td>0.559</td>
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<td>Doxorubicin</td>
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<td>Doxorubicin</td>
<td>20</td>
<td>180</td>
<td>0.435</td>
<td>0.223</td>
<td>0.513</td>
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</table>

(10^6 cells/ml) were suspended in medium, 2 µM Pd phosphor, and 0.5% bovine serum albumin without or with 10 µM dactinomycin. The suspensions were then placed in the 37 °C incubator. Aliquots (1.0 ml each) of untreated and treated cells were taken periodically for [O2] measurement, with [O2] being determined every 30 s. Measured [O2] is shown as a function of time in Fig. 1A (open circles for untreated cells, plus signs for treated cells). Time zero corresponds to the addition of dactinomycin or the beginning of incubation. First, a sample of untreated cells was run for approximately 75 min, and then it was replaced by a sample of drug-treated cells that was run for approximately 50 min. Thereafter, the three measurements on treated cells (Fig. 1A) were 0.846, 0.642, and 0.639 µM O2/min ($r^2 = 0.993, 0.986, and 0.986$), respectively. The increase in respiration rate with time 
requires fewer cells per condition, allowing lower rates of O2 con-
sumption and, thus, longer measurement times that may permit detection of deviations from linearity in [O2] versus t. Based on 
the above experience, we adopted the latter protocol in most of 
our experiments.

We previously reported [21] that [O2] versus t plots for cells treated with doxorubicin were actually well fit by two lines; that is, k was constant and equal to the value for untreated cells for approximately 150 min and then decreased to a lower value. The results of Fig. 1B may show this discontinuity in slope. However, this does not have a major effect on our conclusions given that the k values from linear fits, reported above, are average values for the full time of measurement. In contrast to doxorubicin, dactinomycin produced nonlinear [O2] versus t plots that were concave upward; that is, k decreased gradually with time [22]. Therefore, dactinomycin data for long periods of time should be analyzed using a polynomial model.

**Fig. 1C** shows [O2] versus t for 0.5 x 10^6 Jurkat cells/ml under the same conditions as for Figs. 1A and 1B. Open circles represent
untreated cells, filled triangles represent cells treated with 10 μM dactinomycin, and filled diamonds represent cells treated with 20 μM dactinomycin. All of the data for untreated cells were well fit to a line ($r^2 = 0.994$) as expected. The slope was $-1.252 \mu M/\text{min}$, and the $y$ intercept was 214.7 μM. The linear fit for 10 μM drug-treated cells is $[O_2] = 227.7 \mu M - (0.420 \mu M/\text{min})t$ ($r^2 = 0.982$); for 20 μM drug-treated cells, $[O_2] = 198.6 \mu M - (0.674 \mu M/\text{min})t$ ($r^2 = 0.899$). Obviously, both poor linear fits show that curvature is important, so that quadratic fits (shown as solid lines) are a big improvement. For 10 μM drug-treated cells, the quadratic is $[O_2] = 221.0 \mu M - (1.088 \mu M/\text{min})t + (0.002 \mu M/\text{min})^2 t^2$ ($r^2 = 0.998$), so that $\beta = -1.088 \mu M/\text{min}$ and $\gamma = 0.002 \mu M/\text{min}^2$. For 20 μM drug-treated cells, $[O_2] = 259.6 \mu M - (1.033 \mu M/\text{min})t + (0.002 \mu M/\text{min})^2 t^2$ ($r^2 = 0.972$). We take $t_1 = 30, 90, 150$, and 210 min and $t_1 - t_0 = 60$ min for all four cases. Then ($k$) is calculated as $-\beta - \gamma t_1$ for all eight cases, giving the results in Table 1 (lines 7–14). For each $t_1$, the inhibition is greater for the higher drug concentration; for each drug concentration, the inhibition increases with time.

The same method was employed to analyze the next experiments on doxorubicin-treated cells. Altered $O_2$ consumption profiles due to treatment with doxorubicin at various doses are shown in Fig. 2A. Here $0.5 \times 10^6$ Jurkat cells/ml were treated with 0 μM doxorubicin (open circles), 3 μM doxorubicin (open triangles), 5 μM doxorubicin (open squares), 10 μM doxorubicin (filled triangles), 20 μM doxorubicin (filled diamonds), and 40 μM doxorubicin (filled squares). For all drug-treated conditions, $[O_2]$ was measured out to 200 min. The point at $t = 0, [O_2] = 225 \mu M$, calculated for air-saturated dH2O, was added to the measured points. As shown in Fig. 2A, the $O_2$ consumption profiles for doxorubicin-treated conditions were fitted to quadratic forms, and the profile for untreated cells was fitted to a line (constant $k$ value). The value of $k$ for untreated cells, the negative of the slope of the best-fit line, is 1.144 μM/Min ($r^2 = 0.988$).

For cells treated with 3, 5, 10, 20, or 40 μM doxorubicin, least-squares fitted to quadratics, the values of the linear and quadratic coefficients $\beta$ and $\gamma$ were as follows: for 3 μM drug, $\beta = -1.504$ and $\gamma = 0.0022$ ($r^2 = 0.996$); for 5 μM drug, $\beta = -1.341$ and $\gamma = 0.0016$ ($r^2 = 0.998$); for 10 μM drug, $\beta = -1.420$ and $\gamma = 0.0022$ ($r^2 = 0.988$); for 20 μM drug, $\beta = -1.681$ and $\gamma = 0.0037$ ($r^2 = 0.988$); for 40 μM drug, $\beta = -1.406$ and $\gamma = 0.0026$ ($r^2 = 0.979$). Values of $k$ were then calculated from the quadratic forms, with $t_1 = 30, 90, 150$, and 210 min and $t_1 - t_0 = 60$ min, as for dactinomycin. The values are given in Table 1 for $t_1 = 90$ and 150 min only. This is sufficient because if a single quadratic is used to fit all of the data for $[O_2]$ versus $t$, ($k$) will be a linear function of $t_1$ so long as $t_1 - t_0$ is kept constant.

Calculated inhibitions (values of $I$) are plotted in Fig. 2B. It is noted that results for 40 μM doxorubicin are less reliable than results for other concentrations because $d[O_2]/dt$ becomes zero (100% inhibition) before the time of measurement, which is 200 min. Also shown in Fig. 2B is the best fit ($r^2 = 0.933$) of $l$ to Eq. (5). The best values for $l_{max}$ and $A$ are 0.735 and 3.27 μM, respectively. We also fitted to Eq. (5) with $l_{max} = 1$ (i.e., 100% inhibition) and found the best value of $A$ (8.45 M). However, the low value of $r^2$ (0.817) indicated that the latter fitting was not suitable.

The results presented so far indicate that dactinomycin is approximately twice as potent as doxorubicin for Jurkat cells; that is, to get comparable inhibition of respiration, doxorubicin must be used at twice the concentration of dactinomycin. We next investigated the effect of these two drugs on HL-60 cell respiration. We incubated 0.5 × 10^6 HL-60 cells/ml in open containers without or with 10 μM dactinomycin or 20 μM doxorubicin. A 1.0-ml aliquot for untreated and treated cells was taken every 150 min for O2 measurement. Fig. 2C shows the results for untreated cells (open circles), 10 μM dactinomycin-treated cells (filled triangles), and 20 μM doxorubicin-treated cells (filled squares). The incubation time for each set of measurements is the time of the first measurement (i.e., 30 min for the first three runs and 180 min for the second three runs). Because each set of measurements was over a limited time, the $k$ values were obtained from the best linear fits. At $t = 30$ min, $k = 0.645 \mu M O_2/\text{min}$ for untreated cells ($r^2 = 0.993$), 0.559 μM $O_2/\text{min}$ for dactinomycin-treated cells ($r^2 = 0.989$), and 0.503 μM $O_2/\text{min}$ for doxorubicin-treated cells ($r^2 = 0.989$); at $t = 180$ min, $k = 0.435 \mu M O_2/\text{min}$ for untreated cells ($r^2 = 0.996$), 0.228 μM $O_2/\text{min}$ for dactinomycin-treated cells ($r^2 = 0.967$), and 0.223 μM $O_2/\text{min}$ for doxorubicin-treated cells ($r^2 = 0.903$). Thus, after 30 min incubation, dactinomycin inhibited HL-60 cell respira-

![Fig. 2.](Image 337x201 to 537x581)

tion by 13.3%, whereas doxorubicin inhibited respiration by 22.0%; after 180 min incubation, dactinomycin inhibited cell respiration by 47.6%, whereas doxorubicin inhibited respiration by 48.8%. Thus, dactinomycin is twice as potent as doxorubicin for HL-60 cells as well as for Jurkat cells.

There is an apparent decrease in the respiration rate for untreated cells with time \((k = 0.645\) at 30 min and 0.435 at 180 min), possibly due to problems with maintenance of the cell culture. Day-to-day variations are likely even more important than intraday variations. This underlines the importance of always comparing measured \(k\) for treated cells with \(k\) for untreated cells from the same batch measured over the same time period, as we did here.

Pt compounds

We next investigated the effects of Pt compounds on the respiration of Jurkat cells. Fig. 3A shows results of one experiment in which 10\(^6\) cells/ml were untreated (open circles) or were treated with 20 \(\mu\)M cisplatin (filled squares), carboplatin (filled triangles), oxaliplatin (filled diamonds), or nedaplatin (filled circles) or with 10 mM NaCN (crosses). The values of \(k\) were obtained from the best linear fits and were as follows (means ± standard deviations from measurements. Fig. 3B shows the results for untreated cells (open circles) and 40 \(\mu\)M cisplatin-treated cells (filled squares), with the dashed lines being the best linear fits for untreated cells. The

\[
\begin{align*}
1.54 ± 0.05 \mu M \text{ O}_2/\text{min} & \text{ for cisplatin-treated cells} \\
1.66 ± 0.05 \mu M \text{ O}_2/\text{min} & \text{ for cisplatin-treated cells} \\
1.61 ± 0.07 \mu M \text{ O}_2/\text{min} & \text{ for nedaplatin-treated cells} \\
0.27 ± 0.05 \mu M \text{ O}_2/\text{min} & \text{ for the cyanide-treated condition} \quad (r^2 > 0.660).
\end{align*}
\]

Thus, the average rate of cellular respiration under treatment of different Pt drugs was 1.58 ± 0.07 \(\mu\)M \(\text{O}_2/\text{min}\), which was the same as that for individual drugs within statistical error. Furthermore, the rate of respiration for drug-treated cells was not statistically different from the rate for untreated cells within statistical error. The plots for the drug-treated cells showed no systematic deviation from linearity, confirming that none of the drugs inhibited respiration during the 100 min when \([\text{O}_2]\) was measured. In contrast, the addition of NaCN inhibited cellular respiration 82.5% during the 100 min incubation. On performing the same experiments with all of the Pt compounds but changing the cell line to Tu183 and HL-60 cells, similar results were observed; that is, there was no inhibition occurring in Pt drug-treated cell respiration after approximately 2 h exposure.

The next experiment was performed to confirm and extend the above observations. Jurkat cells (0.5 × 10\(^6\) cells/ml) were incubated without or with 40 \(\mu\)M cisplatin. Aliquots (1.0 ml each) from untreated and treated samples were taken every 180 min for \(\text{O}_2\) measurement. Fig. 3B shows the results for untreated cells (open circles) and 40 \(\mu\)M cisplatin-treated cells (filled squares), with the dashed lines being the best linear fits for untreated cells. The

\[
\begin{align*}
1.50 ± 0.06 \mu M \text{ O}_2/\text{min} & \text{ for carboplatin-treated cells} \\
1.53 ± 0.06 \mu M \text{ O}_2/\text{min} & \text{ for oxaliplatin-treated cells} \\
1.61 ± 0.07 \mu M \text{ O}_2/\text{min} & \text{ for nedaplatin-treated cells} \\
0.27 ± 0.05 \mu M \text{ O}_2/\text{min} & \text{ for the cyanide-treated condition} \quad (r^2 > 0.660).
\end{align*}
\]
Cells treated with 20 l close to each other, confirming that the measurements are consistent for untreated cell samples (open or filled circles, connected by lines) stay similar to that for untreated cells up to 17 h and then decreased. It is clear that cellular O2 consumption rates between treated and untreated cells for up to 6 h continuous exposure to 40 l 20 l 40 l of Table 2. Values of k for cisplatin-treated and untreated cells and r^2 values

<table>
<thead>
<tr>
<th>Condition</th>
<th>k (µM O2/min)</th>
<th>12.0 h exposure</th>
<th>13.5 h exposure</th>
<th>14.5 h exposure</th>
<th>15.5 h exposure</th>
<th>16.5 h exposure</th>
<th>17.5 h exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated 1</td>
<td>1.80</td>
<td>(0.962)</td>
<td>2.19</td>
<td>(0.972)</td>
<td>2.05</td>
<td>(0.963)</td>
<td>2.33</td>
</tr>
<tr>
<td>Untreated 2</td>
<td>1.59</td>
<td>(0.974)</td>
<td>2.00</td>
<td>(0.951)</td>
<td>2.11</td>
<td>(0.985)</td>
<td>2.22</td>
</tr>
<tr>
<td>20 µM cisplatin</td>
<td>1.68</td>
<td>(0.987)</td>
<td>1.81</td>
<td>(0.979)</td>
<td>2.32</td>
<td>(0.977)</td>
<td>2.36</td>
</tr>
<tr>
<td>40 µM cisplatin</td>
<td>1.36</td>
<td>(0.992)</td>
<td>1.07</td>
<td>(0.947)</td>
<td>1.54</td>
<td>(0.966)</td>
<td>1.45</td>
</tr>
<tr>
<td>20 µM transplatin</td>
<td>1.61</td>
<td>(0.969)</td>
<td>1.81</td>
<td>(0.989)</td>
<td>2.54</td>
<td>(0.972)</td>
<td>2.41</td>
</tr>
<tr>
<td>40 µM transplatin</td>
<td>1.80</td>
<td>(0.951)</td>
<td>2.06</td>
<td>(0.989)</td>
<td>2.08</td>
<td>(0.961)</td>
<td>2.29</td>
</tr>
</tbody>
</table>

Note: r^2 values are in parentheses.

k values were as follows: at t = 0 min, 0.666 µM O2/min for untreated cells (r^2 = 0.987) and 0.690 µM O2/min for cisplatin-treated cells (r^2 = 0.992); at t = 180 min, 0.572 µM O2/min for untreated cells (r^2 = 0.983) and 0.580 µM O2/min for cisplatin-treated cells (r^2 = 0.991); at t = 440 min, 0.354 µM O2/min for untreated cells (r^2 = 0.990) and 0.343 for cisplatin-treated cells (r^2 = 0.936). Here again, the respiration rates in untreated cells decline as time elapses. However, there is no statistically significant difference in cellular O2 consumption rates between treated and untreated cells for up to 6 h continuous exposure to 40 µM cisplatin.

In the next series of experiments, we incubated Jurkat cells (0.5 × 10^6 cells/ml) suspended in medium (containing 2 µM Pd phosphor and 0.5% bovine serum albumin) with 20 or 40 µM cisplatin for 12 h. At the same time, a culture of the same cells was maintained with no cisplatin added and, as a further control, a culture of the same cells was incubated with 20- or 40-µM concentrations of transplatin. After 12 h, aliquots (1.0 ml each) of untreated and treated samples were taken periodically for O2 measurement. To check reproducibility, duplicate samples were taken of the untreated cells. Oxygen measurements were stopped and a new set of aliquots was taken when [O2] in a sample of untreated cells reached 50 µM. This almost always meant a new set every hour. Results are shown in Fig. 3C.

The k values obtained from the best linear fits are shown in Table 2 and plotted versus sampling start time (which is equal to incubation time) in Fig. 3D. It is clear that k values in duplicate untreated cell samples (open or filled circles, connected by lines) stay close to each other, confirming that the measurements are consistent and repeatable. Overall, k values for untreated cells increase with time. The increase in k with time is ascribed to cell growth. Cells treated with 20 µM transplatin (open squares) or 40 µM transplatin (filled squares) show no difference from the untreated cells, suggesting that 18 h continuous exposure to transplatin has no inhibitory effect on cellular respiration.

For 20 µM cisplatin-treated cells, respiration rate remained similar to that for untreated cells up to 17 h and then decreased. This result agrees with a previous study on cisplatin-induced caspase activation in Jurkat cells, where caspase activity started at 14 h incubation with 20 µM cisplatin [23]. For 40 µM cisplatin-treated Jurkat cells, respiration rates appear to be significantly lower than those for untreated cells after 12 h, implying that the inhibition of mitochondrial O2 consumption occurred earlier for the higher concentration. The decrease in respiration is more marked after 15 h. The inhibition I may be calculated as 1 minus the ratio of k for treated cells to k for untreated cells (with the average of two values being used for untreated cells). Then, for the incubation times in Table 3, the values of I with 20 µM cisplatin are 0.01, 0.14, −0.12, −0.04, −0.23, and 0.35. For 40 µM cisplatin, I = 0.20, 0.48, 0.26, 0.36, 0.40, and 0.69. These results reflect a dose-dependent and time-dependent inhibition of cellular respiration by cisplatin, whereas there is no observable impairment by the trans-isomer.

Tirapazamine

We first studied the effect of tirapazamine in low concentrations (0–15 µM) on HL-60 cell respiration, with 0.5 × 10^6 cells/ml being incubated with 0, 1, 3, 5, 10, and 15 µM drug. Fig. 4A shows the respiratory profiles for untreated cells (open circles) and for cells treated with 1 µM tirapazamine (filled squares), 3 µM tirapazamine (filled triangles), 5 µM tirapazamine (filled diamonds), 10 µM tirapazamine (filled circles), or 15 µM tirapazamine (open squares) in 3 h exposure. Results for untreated cells were fitted to a line with a slope of −0.487 µM/min (best linear fitting, dashed line). Results for treated cells, which did not suggest curvature, were also fitted to lines. Quadratic fitting improves r^2 only in the fourth decimal place. The k values obtained from the best linear fits for 0, 1, 3, 5, 10, and 15 µM tirapazamine (r^2 = 0.995, 0.935, 0.986, 0.966, 0.974, and 0.991, respectively) are given in the first six lines of Table 3. There is no statistically significant trend in the values of (k/O2)/(kO2), although they are all significantly lower than unity. Thus, the effect of tirapazamine at these concentrations is too small to be accurately measured.

To study the effect of higher concentrations of tirapazamine on HL-60 cell respiration, 10^6 cells/ml were incubated with various concentrations of the drug for 3 h. Fig. 4B shows O2 consumption...
profiles for untreated cells (open circles) and for cells treated with 50 μM tirapazamine (filled squares), 100 μM tirapazamine (filled triangles), 200 μM tirapazamine (filled diamonds), 500 μM tirapazamine (filled circles), or 1 mM tirapazamine (open squares). Results for untreated cells were fitted to a line \(\text{O}_2 = 187.5 \mu M - (0.713 \mu M/\text{min})t\) \((r^2 = 0.949)\). Because of the low value of \(r^2\), results for untreated cells were fitted to a quadratic as with results for treated cells. The values of \(r^2\) were then 0.974 for no tirapazamine and 0.991, 0.987, 0.981, 0.971, and 0.951 for 50, 100, 200, 500, and 1000 μM tirapazamine, respectively.

Values of \(k\) were calculated according to Eq. (3) with \(d = 0\) and \(t = t_0 = 60\) min. The results are shown in Table 3, which gives \(k\) only for \(t = 90\) and 150 min. Because all O₂ consumption profiles were fitted to quadratic forms, \(k\) is a linear function of \(t\). For the calculation of \((k)/k_o\), the value of \((k)/k_o\) is taken from this set of runs, not the previous set. The inhibitions of cellular respiration \(I\) at 90 min incubation were 10.5, 16.9, 7.4, 19.6, and 37.3% for treatment with 50, 100, 200, 500, and 1000 μM tirapazamine, respectively. (Results for 90 min are used here because 90 min is in the middle of the range of data being fitted.) As shown in Fig. 4C, these data were fitted to Eq. (5); the best fit had \(I_{max} = 0.61\) and \(A = 751 \mu M\) \((r^2 = 0.760)\). Assuming that \(I_{max} = 1\) (i.e., 100% inhibition) led to a much worse fit with \(A = 1696 \mu M\) \((r^2 = 0.749)\).

CsA

To investigate the effect of CsA on mitochondrial O₂ consumption, we incubated Jurkat cells \((10^6\text{ cells/ml})\) with CsA and measured oxygen concentrations alternately on five conditions. As shown in Fig. 5A, the five conditions were as follows: without drug (open circles) and with 20 nM CsA (filled triangles), 50 nM CsA (filled diamonds), 200 nM CsA (filled circles), or 500 nM CsA (filled squares). Because there was no evident curvature in these plots, all O₂ consumption profiles were fitted to linear functions. Thus, for \([\text{CsA}] = 0, 20, 50, 200,\) and \(500\) nM, linear fits \((r^2 = 0.989, 0.985,\)
shown as a solid line and with $I = 1$ of CsA on cellular respiration was dose dependent. It is clear from these results that the inhibitory effect values became worse (0.950, 0.969, 0.967, 0.965, and 0.966, 0.984, 0.984, and 0.975, respectively) gave $k = 1.10, 1.03, 0.99, 0.89$, and 0.83 μM O$_2$/min, respectively. (By putting 225 μM as the initial oxygen concentration [O$_2$], and adopting quadratic fitting, $r^2$ values became worse (0.950, 0.969, 0.967, 0.965, and 0.966, respectively). It is clear from these results that the inhibitory effect of CsA on cellular respiration was dose dependent.

From the $k$ values obtained from the linear fitting, we calculated $I = 1 - k/k_o$. Results are shown in Fig. 5B, with the best fit to Eq. (5) shown as a solid line and with $I_{\text{max}} = 0.272$ and $A = 57.9$ nM ($r^2 = 0.994$). When $I_{\text{max}} = 1$ (i.e., 100% inhibition) was assumed, the best value of $A$ turned out to be 1150.3 nM, but $r^2 = 0.598$, so this fitting cannot be adopted.

Caffeine

We investigated the effect of pure caffeine (powder formulation without vehicle) on HL-60 cellular respiration. The cells (10$^6$ cells/ml) were suspended in medium plus 2 μM Pd phosphor and 0.5% bovine serum albumin and were incubated in the presence or absence of caffeine. (A) Caffeine concentrations were 0.1 mM (filled circles), 0.5 mM (open diamonds), 1.0 mM (open squares), and 2.0 mM (filled triangles). Best linear fits are shown for all four conditions. (B) Oxygen concentrations were measured for approximately 40 min with no caffeine present, at which point caffeine was injected. Results of two separate experiments are shown, with least-squares fits before and after caffeine injection. In the first experiment (open and filled circles with solid line for fits), caffeine concentration was 2.0 mM. In the second experiment (open and filled circles with solid line for fits), caffeine concentration was 3.0 mM. (C) Inhibition $I = 1 - k/k_o$ calculated from respiration rates derived from experiments in panels A and B. Shown is best-fit straight line for $l$ versus caffeine concentration.

Fig. 6. Effects of caffeine on cellular respiration. HL-60 cells (10$^6$ cells/ml) were suspended in medium, 2 μM Pd phosphor, and 0.5% bovine serum albumin and were incubated in the presence or absence of caffeine. (A) Caffeine concentrations were 0.1 mM (filled circles), 0.5 mM (open diamonds), 1.0 mM (open squares), and 2.0 mM (filled triangles). Best linear fits are shown for all four conditions. (B) Oxygen concentrations were measured for approximately 40 min with no caffeine present, at which point caffeine was injected. Results of two separate experiments are shown, with least-squares fits before and after caffeine injection. In the first experiment (open and filled circles with solid line for fits), caffeine concentration was 2.0 mM. In the second experiment (open and filled circles with solid line for fits), caffeine concentration was 3.0 mM. (C) Inhibition $I = 1 - k/k_o$ calculated from respiration rates derived from experiments in panels A and B. Shown is best-fit straight line for $l$ versus caffeine concentration.

Discussion

The use of respiratory monitoring is proposed here to assess the extent of drug-impaired cellular function and how it depends on parameters such as incubation time and dosage. To obtain respiration rates, we measure O$_2$ using phosphorescence decay (other methods could also be used [26,27]). Although different drugs attack different cellular targets such as mitochondria, DNA, and thiols, their inhibitory effects on the mitochondrial function sometimes appear to be similar. Clearly, answering the question of why a particular drug gives a specific cellular respiration pattern requires more understanding of the drug–cell interaction than is currently available. However, differences in oxygen consumption profiles (Figs. 1–6) indicate different mechanisms of action for dif-
different drugs. The diverse profiles make it important to characterize the drug effect by a single parameter such as the inhibition I.

To measure the extent of inhibition of mitochondrial oxygen consumption, various cell lines were exposed continuously to a variety of cytotoxic drugs. For testing, the cells and the cytotoxic compounds were sealed in a closed vessel that also contained growth medium, sufficient glucose (as a respiratory substrate), and the Pd phosphor. Untreated cells consume O₂ at a constant rate, whereas drug-treated cells exhibit a diversity of O₂ consumption profiles (linear or nonlinear).

Oxygen consumption is fit to a linear (Pt compounds and CsA) or quadratic (dactinomycin, doxorubicin, and tirapazamine) function of time. Fitting to a higher order, such as cubic or polynomial, can be applied on the condition that the fit is significantly improved (i.e., r² significantly increased). The rate of respiration k is the negative of the slope of a plot of [O₂] versus time as calculated from the fitting function. The time average respiration rate is proposed as a characteristic parameter. It is obtained by integrating k over a well-defined time interval. Then the inhibition I is calculated as D – (k)/k₀, where (k)/k₀ is the average value of k for cells not treated by drug. It is important to obtain (k) and (k)₀ from measurements on the same batch of cells. For the most meaningful comparison, the measurements of [O₂] should be done simultaneously (i.e. alternately) on treated and untreated cells.

When I has been measured for a series of drug concentrations D, it is convenient to fit the results to the two-parameter function, I = IₘₐₓD / (D + A). The value of I provides a simple and convenient characterization of drug cytotoxicity. (Other measures of drug cytotoxicity continue to be suggested[28–30].) It also helps to predict the dosing necessary to shut down respiration of malignant cells and, thus, may be useful in assessing the efficiency of anticancer drugs.

Acknowledgment

This work was supported by a fund from the Paige's Butterfly Run.

References


