Measuring Cytotoxicity: A New Perspective on LC₅₀

MING ZHANG¹, DOLLY AGUILERA², CHANDRA DAS², HERNAN VASQUEZ², PETER ZAGE², VIDYA GOPALAKRISHNAN² and JOHANNES WOLFF²

¹Department of Bioinformatics and Computational Biology and ²Division of Pediatrics, The University of Texas M.D. Anderson Cancer Center, Houston, TX, 77030, U.S.A.

Abstract. Background: Cytotoxicity in cell culture is typically expressed as LC₅₀, the concentration of a given agent which is lethal to 50% of the cells. This number is dependent on the incubation time with the agent. Previously, a two-term exponential model has been proposed to describe cell growth after a cytotoxic event: \( y(t) = k_1 \exp(-d_1 t) + k_2 \exp(d_2 t) \). A dose-response relationship was observed between the parameter \( k_2 \) in this formula and the concentration of the cytotoxic agent etoposide, in high-grade glioma cells was independent of incubation time. Materials and Methods: In order to test if the model can be applied more generally, DAOY medulloblastoma cells treated with MS275, a histone deacetylase inhibitor, were used. Results: The observed data fit the model well. Conclusion: The concentration at which \( k_2 \) is reduced by 50% is called KC₅₀. It provides a far better description of the cytotoxicity of an agent in a specific cell line than the traditional LC₅₀.

When describing the in vivo toxicity of a new agent, most people use the LD₅₀ (1, 2). By definition, LD₅₀ is the dose that kills half of the tested population in an animal model. Analogously, the most common way to describe cytotoxicity in cell culture is the LC₅₀, i.e., the concentration of a drug that kills half of the tested cells in culture (3, 4). However, unlike the LD₅₀, the LC₅₀ in cell culture is largely dependent on the time of incubation. For example, in some drugs, the area under the incubation time curve (AUC) is the same for different incubation times. In those circumstances, the LC₅₀ for a 24-h incubation period would be twice the LC₅₀ for a 48-h incubation. However, this is not the case for all chemotherapeutic agents (5). To make matters even more complicated, the LC₅₀ also depends largely on the time between the start of drug exposure and the measurement of the surviving cell number, often referred to as the recovery time or wash out period (5, 6). This makes LC₅₀ much less reliable than generally anticipated.

A large body of literature describes the research on drug cytotoxicity with different scenarios – a search of cytotoxicity on PubMed resulted in 54,147 entries as of August 2006. Mathematical modelling of cytotoxicity has also been an active research topic (7-9). Recently, an exponential model that describes drug cytotoxicity in cell culture (10) was proposed. The model was developed from experiments treating the human glioblastoma cell line U87 MG with varying concentrations of etoposide. The observed data could be explained by a simple formula with four parameters that acknowledges a cell sub-population that dies in response to drug exposure and a second sub-population that grows despite the presence of the drug. The second sub-population, described by two parameters, can be used to compute the cell-killing efficacy of a cytotoxic agent at any concentration.

It was hypothesized that this exponential model would also describe the toxicity in other cell types and of other treatments. The hypothesis was tested using human DAOY medulloblastoma cells and treatment with MS275, a histone deacetylase inhibitor. DAOY cells and MS275 were chosen because: (i) we wanted to determine if the two-term exponential model described previously for traditional cytotoxic agents in glioma can also hold true for epigenetic modifiers and another cell type, in this case medulloblastoma; and (ii) the experiments were also necessary as a part of preclinical evaluation possibly leading towards a clinical phase I study to treat children with relapsed medulloblastoma using MS275 in conjunction with other treatments. In more general terms, we defined KCₙ values, i.e., concentrations that kill \( n \) percentage of the tested cells. Moreover, KCₙ obtained from this approach is independent of the incubation time or recovery time. Thus, the model provides an alternative to LCₙ and, particularly, to LC₅₀.

Materials and Methods

Cell line. The DAOY human medulloblastoma cell line was obtained from the American Tissue Culture Collection (Manassas, VA, USA). This cell line was established from a 4-year-old Caucasian male with desmoplastic medulloblastoma and grows as a...
monolayer, attached as polygonal cells, with a median doubling time of 34 hours. These cells were maintained in DMEM from Invitrogen (Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA, USA), penicillin and streptomycin. The cells were incubated with 5% CO₂ at 37 degrees and plated in T-75 tissue-culture flasks. Adherent cells were put in subculture at 70% confluence for approximately 3-4 days.

Cytotoxic agent. MS275 (Axxora) was dissolved in 70% methanol. A 100 µM stock solution was prepared. Serial dilutions were performed in complete growth medium to achieve the desirable concentrations.

MTT assay. Five thousand cells per well were seeded in a 96-well plate (B.D. Bioscience Falcon, Bedford, MA, USA). Cells were allowed to adhere for 24 h at 5% CO₂ and 37°C. Various concentrations of MS275, ranging from 0.03 µM to 20 µM were added to the cells. Incubation continued for different lengths of time (24, 48, 72, 96 and 144 h). Control cells were untreated cells. After incubation, the media and drug were removed and 3-(4,5 dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) at 100 µL per well was added for 1 h at 37°C, followed by 100 µL of lysis buffer (20% SDS, 30% phosphate-buffered saline (PBS) and 50% dimethylformamide). Absorbance was determined by spectrophotometry as 570 nm wavelength with a Spectramax plus 384 instrument (Molecular Devices, Sunnyvale, CA, USA) with the Softmax software (SoftMax, San Diego, CA, USA). Dose-response curves were obtained at different time points. All experiments were repeated 8 times.

Mathematical modeling. The previously developed model (10) describes post-treatment cell numbers from two separate processes: apoptosis of the first sub-population and cell proliferation of the second sub-population, which is unaffected by treatment. Both processes are described as exponentially time dependent. Accordingly, the number of post-treatment tumor cells is expressed by this formula:

\[ y(t) = k_1 \exp(-d_1 t) + k_2 \exp(d_2 t), \]  

where \( y(t) \) denotes tumor cell number at time \( t \). The parameters \( k_1, k_2, d_1 \) and \( d_2 \) have positive values. Parameter \( k_1 \) denotes the number of cells in the first sub-population (drug affected) at time 0, while \( k_2 \) represents the number of cells in the second sub-population (drug unaffected) at time 0. In this model, the negative exponent \(-d_1 t\) leads to the exponential decrease in cell number in the first sub-population; the positive exponent \(d_2 t\) leads to the exponential increase in cell number in the second sub-population.

The fitting process from Zhang et al. is also adopted here (10). That is, \( d_1 \) is determined first, using observed data from high concentrations and a single-term exponential model:

\[ y(t) = k_1 \exp(-d_1 t). \]  

Parameter \( d_2 \) is then determined similarly using observed data from low concentrations and a single-term exponential model:

\[ y(t) = k_2 \exp(d_2 t). \]  

Next, the resulting \( d_1 \) and \( d_2 \) values are held constant and used to fit the cell numbers from intermediate drug concentrations to Model [1], where only \( k_1 \) and \( k_2 \) are unknown.

The value of \( k_2 \) directly measures the drug toxicity. Thus, the obtained \( k_2 \) values and the drug concentrations are fitted to this sigmoidal model:

\[ k_2(x) = \frac{p_1}{1 + p_2 \exp(p_3 \log(x))} \]  

or

\[ k_2(x) = \frac{p_1}{1 + p_2 (x^{p_3})}, \]

where \( x \) denotes drug concentration and \( p_1, p_2, p_3 \) are positive parameters independent of drug concentrations.

The fitting procedure for each of the formulas was done using the "fit" function provided by the software MATLAB (Mathworks, Natick, MA, USA).

Results and Discussion

MS275 in DAOY. The human medulloblastoma cells tested here were sensitive to MS275 in a dose- and time-dependent manner. The effects of different drug concentrations on cell growth are shown in Figure 1. When MS275 concentrations were high (e.g., ≥2.5 µM), cell numbers did not recover by 144 h. When the concentrations were low (e.g., ≤0.325 µM), the cell numbers increased exponentially with time. This is consistent with the results of others who showed cytotoxic effects of other histone deacetylase inhibitors (12, 13) and effects on other medulloblastoma cell lines (14).

Determining the parameters. In order to calculate the value of \( d_1 \), the data from the two highest MS275 concentrations
(5 μM and 2.5 μM) were used and fitted to Model [2]. (The cell numbers from concentrations ≥10 μM were essentially zero, as almost all cells were killed by the drug.) The calculated value was $d_1 = 1.918 \times 10^{-2}$. In order to calculate the value of $d_2$, the data from the two lowest MS275 concentrations (0.038 μM and 0.075 μM) were used and fitted to Model [3]. The calculated value was $d_2 = 1.208 \times 10^{-2}$.

Using the values obtained for $d_1$ and $d_2$, the cell number data from all concentrations were fitted to Model [1]. The calculated values for $k_1$ and $k_2$ are shown in Table I and Figure 2. The original data could be fitted closely to the model as described above. The MS275 concentration correlated with $k_2$, but not with $d_1$ or $d_2$, which suggests that the rate of the cell death and growth rate after treatment was independent of the drug concentration.

The results derived from these data are similar to those from our previous work (10). Thus, this work presents further validation of the two-term model [1] for tumor cell numbers and the sigmoidal model [4] for cytotoxicity, despite the different cell types, cytotoxic agents, and exposure times used.

In our previous work, the drug etoposide was removed after 24 h of treatment of the tumor cells. In this work, MS275 remained with the tumor cells during the whole course of incubation. This validates Model [1] and strongly suggests that the cells in the subpopulation described by the second term of the formula ($k_2 \exp(d_2 \tau)$) were not affected by the presence of drug and continued growing during exposure at the same speed.

### Table I. Calculated values for parameters $k_1$ and $k_2$ fitting the two-term exponential model for different drug concentrations.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>$k_1$</th>
<th>$k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.000 μM</td>
<td>5.040E-2</td>
<td>5.482E-7</td>
</tr>
<tr>
<td>2.500 μM</td>
<td>9.170E-2</td>
<td>1.938E-4</td>
</tr>
<tr>
<td>1.250 μM</td>
<td>1.462E-1</td>
<td>7.737E-3</td>
</tr>
<tr>
<td>0.625 μM</td>
<td>6.974E-2</td>
<td>5.902E-2</td>
</tr>
<tr>
<td>0.325 μM</td>
<td>2.742E-5</td>
<td>8.848E-2</td>
</tr>
<tr>
<td>0.150 μM</td>
<td>1.695E-8</td>
<td>1.133E-1</td>
</tr>
<tr>
<td>0.075 μM</td>
<td>2.237E-12</td>
<td>1.240E-1</td>
</tr>
<tr>
<td>0.038 μM</td>
<td>1.406E-10</td>
<td>1.321E-1</td>
</tr>
</tbody>
</table>

### Determining the relationship between parameter $k_2$ and the drug concentration.

The values of $k_2$ and drug concentrations were fitted to the sigmoidal model [4]. The coefficients are $p_1 = 0.127$, $p_2 = 4.168$ and $p_3 = 2.136$.

Therefore, the tumor cell number $y(t)$ can be modelled as a function of $t$ as in Model [1]. Particularly for low drug concentrations or for large $t$ values, $y(t)$ can be approximated by:

$$y(t) = k_2 \exp(0.01208 \times t)$$

where $t$ denotes time and $x$ denotes drug concentration.

These results suggest an alternative to the traditional LC$_{50}$ value. The LC$_{50}$ value depends on the point of time of observation: the LC$_{50}$ measurement at a single point of time may be higher if cells continue to die (early measurement) or lower if cells begin to recover (late measurement) (6). The $k_2$ in Model [1] provides a new measurement of drug efficacy, thereby avoiding this difficulty.

We define the term KC$_{50}$ as the concentration of the tested drug that results in a $k_2$ value that is 50% of the $k_2$ value in untreated cells. In the sigmoidal model [4], when the concentration of drug is zero (i.e., the control setting), $k_2 = p_1$. Thus, KC$_{50}$ is the concentration $x$ such that:

$$p_1/(1 + p_2 \cdot x^{p_3}) = p_1/2$$

That is,

$$KC_{50} = \exp(1/p_3 \cdot \log(1/p_2)).$$

The concept can be generalized to KC$_{n}$, (100-n)% of the $k_2$ value in untreated cells, where $n$ is any number between 0 and 100. KC$_{n}$ is the concentration $x$ such that:

$$p_1/(1 + p_2 \cdot x^{p_3}) = p_1 \times (100-n)/100.$$
\[ KC_n = \exp\left\{ \frac{1}{p_3} \log\left\{ \frac{n}{(100-n)/p_2}\right\} \right\}. \]

\[ (9) \]

That is, \( KC_n \) obtained in this fashion is independent of the incubation and recovery times: the effect of different incubation times is eliminated by \( p_1 \) and the effect of different recovery times has been taken into consideration by the second term in Model [1].

**Conclusion**

The numbers of tumor cells after cytotoxic treatment measured here fit well into the two-term exponential model [1]. The parameter \( k_2 \), which measures drug toxicity, can be computed using Model [4]. These results indicate that the rates of (logarithmic) cell growth and (logarithmic) cell death (i.e., the \( d_1 \) and \( d_2 \) values) are independent of drug treatment, whereas the killing effect of the drug (or cell survival, i.e., the \( k_2 \) value) has a sigmoidal relationship with drug concentration. Most importantly, the sigmoidal model [4] offers an alternative to the \( LC_{50} \) concept that is independent of both incubation and recovery times.

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**References**

3 Forget J, Pavillon JF, Menasria MR and Boeckene G: Mortality and \( LC_{50} \) values for several stages of the marine copepod *Tigriopus brevicornis* ( Muller) exposed to the metals arsenic and cadmium and the pesticides atrazine, carbofuran, dichlorvos, and malathion. Ecotoxicol Environ Saf 40: 239-244, 1998.
13 Li XN, Shu Q, Su JM, Perlaky L, Blaney SM and Lau CC: Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC. Mol Cancer Ther 4: 1912-1922, 2005.

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