An unbiased metric of antiproliferative drug effect *in vitro*

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*In vitro* cell proliferation assays are widely used in pharmacology, molecular biology, and drug discovery. Using theoretical modeling and experimentation, we show that current metrics of antiproliferative small molecule effect suffer from time-dependent bias, leading to inaccurate assessments of parameters such as drug potency and efficacy. We propose the drug-induced proliferation (DIP) rate, as an alternative, time-independent metric.

Evaluating antiproliferative drug activity on cells *in vitro* is a widespread practice in basic biomedical research1–3 and drug discovery4–6. Typically, quantitative assessment relies on constructing dose–response curves7 (Supplementary Note and Supplementary Fig. 1). Briefly, a drug is added to a cell population over a range of concentrations, and the effect on the population is quantified with a metric of choice8. The de facto standard metric is the number of viable cells 72 h after drug addition4,6,8,9. Since this is a single-time-point measurement, we refer to it as a 'static' drug effect metric. The data is then fit to the Hill equation10, a four-parameter log-logistic function, to produce a sigmoidal dose–response curve that summarizes the relationship between drug effect and concentration. Parameters extracted from these curves include the maximum effect ($E_{\text{max}}$), half-maximal effective concentration ($EC_{50}$), half-maximal inhibitory concentration ($IC_{50}$), area under the curve (AUC), and activity area (AA)4,6,8,9 (Supplementary Fig. 1 and Supplementary Table 1). These are useful for quantitatively comparing various aspects of drug activity across drugs and cell lines.

We contend that dose–response curves constructed using standard metrics of drug effect can result in erroneous and misleading values of drug–activity parameters, skewing data interpretation. This is because these metrics suffer from time-dependent bias: i.e., the metric value varies with the time point chosen for experimental measurement. We identify two specific sources of time-dependent bias: (i) exponential growth and (ii) delays in drug effect stabilization. The former can lead to erroneous conclusions (e.g., that a drug is increasing in efficacy over time), while the latter requires shifting the window of evaluation to only include data points after stabilization has been achieved (Supplementary Fig. 2).

To overcome this problem of bias, we propose as an alternative drug effect metric the drug-induced proliferation (DIP) rate11,12, defined as the steady-state rate of proliferation of a cell population in the presence of a given concentration of drug. Using related approaches, we previously quantified clonal fitness12 and heterogeneous single-cell fates11 within cell populations responding to perturbations. Here, we show that DIP rate is an ideal metric of antiproliferative drug effect because it naturally avoids the bias afflicting traditional metrics, it is easily quantified as the slope of the line on a plot of the doubling of cell populations versus time (Supplementary Fig. 2), and it is interpretable biologically as the rate of regression or expansion of a cell population.

To theoretically illustrate the consequences of time-dependent bias in standard drug effect metrics, we constructed a simple mathematical model of cell proliferation that exhibits the salient features of cultured cell dynamics in response to drug (Online Methods, Supplementary Note, Supplementary Fig. 3, and Supplementary Table 2). The model assumes that cells experience two fates, division and death, and that the drug modulates the difference between the rates of these two processes, i.e., the net rate of proliferation. Drug action may occur immediately or gradually over time, depending on the chosen parameter values. In all cases, a stable DIP rate is eventually achieved and, when calculated over a range of drug concentrations, a sigmoidal dose–response relationship emerges (Supplementary Note and Supplementary Fig. 3).

We model three scenarios: treatment of a fast-proliferating cell line with a fast-acting drug (Fig. 1a), treatment of a slow-proliferating cell line with a fast-acting drug (Fig. 1b), and treatment of a fast-proliferating cell line with a delayed-action drug (Fig. 1c). In each case, we generate simulated growth curves in the presence of increasing drug concentrations (Fig. 1, columns 1 and 2) and from these produce static dose–response curves by taking cell counts at single time points between 12 h and 120 h (Fig. 1, column 3). As expected, in each scenario the shape of the dose–response curve varies depending on the time of measurement. Consequently, parameter values ($EC_{50}$ and AA) extracted from these curves also vary (Fig. 1, columns 4 and 5). Similar results are obtained for an alternative drug effect metric proposed by the U.S. National Cancer Institute's Developmental Therapeutics Program13 (Supplementary Note and Supplementary Fig. 4).

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In contrast, as DIP rate is the slope of a line, it is independent of measurement time. Using it as the drug effect metric gives a single dose–response curve (Fig. 1, columns 3 and 6) and single values of the extracted drug-activity parameters (Fig. 1, columns 4 and 5).

As a first confirmation of our theoretical findings, we subjected triple-negative breast cancer cells (MDA-MB-231) to the metabolic inhibitors rotenone (Fig. 2a) and phenformin (Fig. 2b). Using fluorescence microscopy time-lapse imaging11,12,14 (Online Methods), we quantified changes in cell number over time for a range of drug concentrations. For both drugs, we observed a rapid stabilization of the drug effect (<24 h delay) and stable exponential proliferation thereafter, reminiscent of the growth dynamics of the theoretical cell lines treated with fast-acting drugs (Fig. 1a,b). We generated dose–response curves from these data using the standard static effect metric and DIP rate for various drug exposure times. Consistent with our theoretical results, the shape of the static-based dose–response curve strongly depended on the time point at which cell counts were taken, an illustration of time-dependent bias. The DIP rate, on the other hand, was free of time-dependent bias and produced a single dose–response curve in both cases.

These DIP-rate-based dose–response curves produce interesting insights (Fig. 2a,b). For example, they indicate that while rotenone is much more potent than phenformin (EC$_{50}$ ≈ 8.5 nM versus 25 µM), phenformin is more effective ($E_{\text{max}}/E_0$ ≈ 0.1 versus 0.1). The static dose–response curves can discriminate the ordering of potencies (rotenone >> phenformin) but not the ordering of efficacies: i.e., the static drug effect metric obscures the crucial fact that at saturating concentrations phenformin is cytotoxic (cell populations regress) while rotenone is partially cytostatic (cell populations continue to expand slowly). This information is critical to studies assessing drug mechanism of action. This example illustrates the perils of biased drug effect metrics and the ability of DIP rate to produce reliable dose–response curves from which accurate quantitative and qualitative assessments of antiproliferative drug activity can be made.

To illustrate the confounding effects that a delay in the stabilization of the drug effect can have, we examined single-cell-derived clones of the lung cancer cell line PC9, which is known to be hypersensitive to erlotinib15, an epidermal growth factor receptor (EGFR) kinase inhibitor. Consistent with our previous report11, three drug-sensitive PC9-derived clones (DS3, DS4, and DS5) each responded to 3 µM erlotinib with nonlinear growth dynamics over the first 48–72 h, followed by stable exponential proliferation thereafter (Fig. 2c). These dynamics are reminiscent of those for the theoretical fast-proliferating cell line with a delayed-action drug (Fig. 1c). Because of the delay in drug action, all three clones had nearly identical population sizes 72 h after drug addition for all concentrations considered. The static 72-h metric thus produces essentially identical dose–response curves for all clones (Supplementary Fig. 5). In contrast, dose–response curves based on DIP rate make a clear distinction between the clones in terms of their long-term response to drug: i.e., erlotinib is cytotoxic (negative DIP rate) for two of the clones but partially cytostatic (positive DIP rate) for the other (Fig. 2c).

We then investigated the effects of erlotinib and lapatinib (a dual EGFR/human EGFR 2 (HER2) kinase inhibitor) on HER2-positive breast cancer cells (HCC1954; delay ~48 h; Fig. 2d). In each case, DIP-rate-based dose–response curves produced EC$_{50}$ values more than five-fold larger than their static counterparts; i.e., by the static drug effect metric the drugs appeared significantly
Figure 2 | Experimental illustration of time-dependent bias in dose–response curves for drug-treated cancer cells. Shown are population growth curves (log, scaled) and derived (static- and/or DIP-rate-based) dose–response curves for (a) MDA-MB-231 triple-negative breast cancer cells treated with rotenone; (b) MDA-MB-231 cells treated with phenformin; (c) three single-cell-derived drug-sensitive (DS) clones of the EGFR-mutant-expressing lung cancer cell line PC9 treated with erlotinib; and (d) HCC1954 HER2-positive breast cancer cells treated with erlotinib and lapatinib.

Data for a and b are from single experiments with technical duplicates; data in c are from individual wells for two experiments containing technical duplicates (growth curves) and from a single experiment with technical duplicates (dose–response curves); data in d are sums of technical duplicates from a single experiment (growth curves) and mean values (circles) with 95% confidence intervals (gray shading) on the log-logistic model fit (dose–response curves; \( n = 4, 6 \) for erlotinib and lapatinib, respectively). Dashed gray lines indicate y-axis values of 0. Red dashed lines indicate EC50 values.

more potent that they actually were. Taken together with the PC9 results (Fig. 2c), these data illustrate the importance of accounting for delays in drug action when assessing antiproliferative drug activity, and they further emphasize the ability of the DIP rate metric to produce accurate drug-activity parameters and qualitative conclusions about drug-response dynamics.

Within the last several years, a number of studies have been published reporting drug responses for hundreds of cell lines derived from various cancer types, and organ sites and similar data sets. Raw data are available for the responses of over 1,000 cancer cell lines to a panel of 24 drugs in the Cancer Cell Line Encyclopedia (CCLE) and for the responses of over 1,200 cell lines to 140 drugs in the Genomics of Drug Sensitivity in Cancer (GDSC) project. These data are largely based on 72-h cell counts, a metric that we have shown contains time-dependent bias.

To investigate bias in these data sets, we treated four BRAFV600E- or BRAFV600E-expressing melanoma cell lines with various concentrations of the BRAF-targeted agent PLX4720, an analog of vemurafenib. We produced experimental growth curves (Fig. 3a) and static- and DIP-rate-based dose–response curves (Fig. 3b), and we extracted IC50 values for each cell line and compared these to IC50 values obtained from the CCLE and GDSC data sets (Fig. 3c). In all cases, our IC50 values corresponded closely to the value from at least one of the public data sets. While in three cases the static- and DIP-rate-based IC50 values corresponded within an order of magnitude, in one case (A375), they differed by nearly two orders of magnitude. This discrepancy can be traced to a period of complex, nonlinear dynamics (brief regression followed by rebound) observed for this cell line between 24 h and 72 h post drug addition (Fig. 3a). This result is particularly intriguing because it shows that, based on DIP rate, this cell line is not much different than the other three cell lines in terms of drug sensitivity. Using the biased static drug effect metric, however, one would be led to the incorrect conclusion that it is significantly more sensitive. It is likely that cases like this abound within and other similar data sets and this likelihood illustrates the critical need for new antiproliferative drug effect metrics.

Current protocols for cell proliferation assays are based on informal ‘rules of thumb’, for example, counting cells after 72 h of treatment to ameliorate the impact of complex dynamics and delays in drug response. However, these de facto standards have no theoretical basis and, as demonstrated here, they suffer from time-dependent bias that leads to erroneous conclusions. In light of the widespread applications of cell proliferation assays in oncology, pharmacology, and basic biomedical science (for example, to assess activity of cytokines, cell surface receptors, altered signaling pathways, gene overexpression and silencing, or cell metabolic
adaptation to varied microenvironmental conditions), it is imperative that the quality of the metric for antiproliferative assays be improved. Toward this end, we have proposed DIP rate as a viable, unbiased alternative antiproliferative drug effect metric. DIP rate overcomes time-dependent bias by log-scaling cell count measurements to account for exponential proliferation and by shifting the time window of evaluation to accommodate lag in the action of a drug, changes that do not substantially alter experimental design (Supplementary Note and Supplementary Figs. 6–9). Moreover, DIP rate is an intuitive, biologically interpretable metric with a sound basis in theoretical population dynamics, and it faithfully captures, within a single value, the long-term effect of a drug on a cell population.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
D.R.T., L.A.H., P.L.F., and S.P.G. conceived and designed the study; D.R.T., L.A.H., and S.P.G. built the mathematical models and performed simulations; B.B.P., K.N.H., and P.L.F. acquired experimental data; D.R.T., L.A.H., P.L.F., and S.P.G. analyzed and interpreted the experimental data; C.F.L., D.R.T., L.A.H., P.L.F., S.P.G., and V.Q. wrote, reviewed, and/or revised the manuscript; and C.F.L., D.R.T., and V.Q. supervised the study.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Dose–response curve fitting. All drug-response data (theoretical and experimental) were fit with a four-parameter log-logistic function (Supplementary Note) using nonlinear least-squares regression21 within the R statistical programming environment (http://R-project.org). Fitting was performed using the “drm” function of the “drm” R package22. 95% confidence intervals for each parameter were obtained using the delta method assuming asymptotic variance21 as implemented within the “confint” function of the “stats” R package. EC50 is a fit parameter of the model. IC50 is the concentration at which Edrug = E0/2 (Supplementary Fig. 1 and Supplementary Table 1), independent of the value of Emax, and is obtained using the “ED” function of the “drc” R package. AA (Supplementary Fig. 1) is calculated as

\[
AA = -\sum_{i=1}^{N} \left( \frac{E_{\text{drug},i}}{E_0} - 1 \right) / N
\]  

where \(E_{\text{drug},i}\) is the value of the effect metric at the \(i\)th drug concentration and \(N\) is the total number of concentrations considered.

A simple two-state model of drug action on an exponentially proliferating cell population. We assume that cells can exist in two states, a ‘no-drug’ and a ‘drug-saturated’ state, and that cells in each state can experience two fates, division and death, with kinetic rate constants that are characteristic of the state, i.e., reflecting the effect of the drug (visual representation of the model is provided in Supplementary Fig. 3a). In the presence of drug, cells can transition from the no-drug to the drug-saturated state at a rate proportional to the concentration of drug. Reverse transitions occur at a rate independent of drug. If \(C\) is the number of cells in the no-drug state and \(C^*\) is the number of cells in the drug-saturated state, then the temporal dynamics of the drug-treated cell population are described by the following pair of coupled ordinary differential equations,

\[
d\frac{dC\text{ell}}{dt} = (k_{\text{div}} - k_{\text{death}} - k_{\text{on}}\text{Drug}) \times C\text{ell} + k_{\text{off}} \times C^*\tag{2}
\]

\[
d\frac{dC^*}{dt} = (k_{\text{div}} - k_{\text{death}} - k_{\text{off}}) \times C^* + k_{\text{on}} \times \text{Drug} \times \text{Cell}\tag{3}
\]

where \(k_{\text{div}}\) and \(k_{\text{death}}\) are the rate constants for cellular division and death, respectively, in the no-drug (drug-saturated) state; \(\text{Drug}\) is the drug concentration; \(k_{\text{on}}\) is the rate constant for the transition from the no-drug to the drug-saturated state; and \(k_{\text{off}}\) is the rate constant for the reverse transition.

At a given drug concentration (assumed to be constant; i.e., drug is not consumed, removed, or degraded), a population of cells will eventually reach a dynamic equilibrium in terms of the number of cells in each state. The effective DIP rate of a cell population is then the weighted average of the net proliferation rates (i.e., the difference between the division- and death-rate constants) of the two individual states (Supplementary Fig. 3b). With increasing drug concentration, the equilibrium shifts increasingly toward the drug-saturated state, asymptotically approaching 100% occupancy. The result is a sigmoidal dose–response relationship between DIP rate and drug concentration (Supplementary Fig. 3c,d). If the values of the rate constants governing the interconversion between the no-drug and drug-saturated state (\(k_{\text{on}}\) and \(k_{\text{off}}\)) are ‘large’ (effectively infinite), then the dynamic equilibrium between states is achieved immediately upon drug addition. This is known as the partial equilibrium assumption (PEA)23,24. Mathematically, the PEA asserts that

\[
k_{\text{on}} \times \text{Drug} \times \text{Cell} = k_{\text{off}} \times \text{Cell}^*\tag{4}
\]

Under this assumption, an analytical solution for the total number of cells, \(C_{\text{ell}}(t) = C + C^*\), can be obtained as a function of time,

\[
\ln \frac{C_{\text{ell}}(t)}{C_{\text{ell}}(0)} = k_{\text{off}} \left( k_{\text{div}} - k_{\text{death}} + \text{Drug}(k_{\text{div}} - k_{\text{death}}^*) \right) t / k_{\text{on}} \tag{5}
\]

where \(C_{\text{ell}}(0)\) is the initial number of cells. All theoretical results shown in Figure 1a,b were obtained using equation 5. For the results in Figure 1c and Supplementary Figure 4, numerical integration of equations 2 and 3 was necessary since the values of \(k_{\text{on}}\) and \(k_{\text{off}}\) were set such that the PEA does not hold (Supplementary Table 2); i.e., there is a delay in the stabilization of the drug effect. Numerical integration was performed in R using the deSolve package25. For further details of the model, see Supplementary Note; for all parameter values used in this work, see Supplementary Table 2.

Cell lines. The PC9 cell line was originally obtained from W. Pao (Vanderbilt University). WM115 cells were from M. Herlyn (Wistar Institute). All other cell lines were obtained from the American Type Culture Collection (http://www.atcc.org). All cell lines are regularly tested for mycoplasma using a PCR-based method (MycoAlert, Lonza, Allendale, NJ) and any positive cultures are immediately discarded. Cell line authentication is provided by ATCC. Authenticity of PC9 and WM115 have not been verified.

Time-lapse fluorescence microscopic imaging. Time-lapse fluorescence microscopy of cells expressing histone H2B conjugated to monomeric red fluorescent protein (H2BmRFP) to facilitate automated image analysis for identifying and quantifying individual nuclei was performed as previously described11,12,14. Briefly, cells are engineered to express H2BmRFP using recombinant, replication-incompetent lentiviral particles and sorted for the highest 20% intensity. Cells are seeded at ~2,500 cells per well in 96-well imaging microtiter plates (BD Biosciences) and fluorescent nuclei are imaged using a BD Pathway 855 with a 20× objective in 3 × 3 montaged images per well at ~15 min intervals for 5 d. Alternatively, fluorescent cell nuclei are imaged twice daily using a Synentec Cellavista High End with a 20× objective and tiling of nine images. DIP-rate-based dose–response curves shown in Figure 2c were generated from a single experiment performed at the Vanderbilt High-Throughput Screening Core on a Molecular Devices ImageXpress using similar imaging parameters. The experiment had two technical replicates per condition and images were obtained at 0, 24, 48, 52, 56, 60, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, and 112 h after addition of erlotinib at each of eight different concentrations or dimethyl sulfoxide (DMSO) control.

Other statistical considerations and code availability. Estimates of DIP rate are determined within an experiment using the sum of
cells across all technical replicates at a given time point and obtaining the slope of a linear model of log2(cell number) ~time for time points greater than the observed delay. Minimum delay time is estimated by visual inspection of log-growth curves for the time at which they become approximately linear (for an automated method of estimating the stabilization time point, see Supplementary Note and Supplementary Figs. 6 and 7). All data analysis was performed in R (version 3.2.1, Supplementary Software) and all raw data and updated R analysis code is freely available at http://www.github.com/QuLab-VU/DIP_rate_NatMeth2016.

Publicly available data sets. Drug-response data were obtained from the Genomics of Drug Sensitivity in Cancer (GDSC) project4,9 website at ftp://ftp.sanger.ac.uk/pub/project/cancerrxgene/releases/release-5.0/gdsc_drug_sensitivity_raw_data_w5.zip and from the Cancer Cell Line Encyclopedia (CCLE)6 website at http://www.broadinstitute.org/ccle/ in the data file CCLE_NP24.2009_Drug_data_2015.02.24.csv (user login required).

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