

Phenotype-Based Probabilistic Analysis of Heterogeneous Responses to Cancer

Drugs and Their Combination Efficacy

Natacha Comandante-Lou¹, Mehwish Khaliq^{1,2}, Divya Venkat³, Mohan Manikkam¹ and Mohammad Fallahi-Sichani^{1,2,4}

¹ Department of Biomedical Engineering, University of Michigan Medical School, Ann Arbor, MI, USA

² Program in Cancer Biology, University of Michigan Medical School, Ann Arbor, MI, USA

³ Department of Biochemistry, University of Michigan Medical School, Ann Arbor, MI, USA

⁴ Department of Dermatology, University of Michigan, Ann Arbor, MI, USA

Running Title:

Probabilistic analysis of cancer drug efficacy and interactions

Address correspondence to:

Mohammad Fallahi-Sichani

Address: 1150 W. Medical Center Dr. Building MSRB II, Room 2560B, Ann Arbor, MI 48109

ORCID ID: 0000-0003-0917-3525

Email: fallahi@umich.edu

Tel/Fax: (734) 647-2263

Abstract

Cell-to-cell variability generates subpopulations of drug-tolerant cells that diminish the efficacy of cancer drugs. Efficacious combination therapies are thus needed to block drug-tolerant cells via minimizing the impact of heterogeneity. Probabilistic models such as Bliss independence are developed to evaluate drug interactions and their combination efficacy based on probabilities of specific actions mediated by drugs individually and in combination. In practice, however, these models are often applied to conventional dose-response curves in which a normalized parameter with a value between zero and one, generally referred to as fraction of cells affected (f_a), is used to evaluate the efficacy of drugs and their combined interactions. We use basic probability theory, computer simulations, time-lapse live cell microscopy, and single-cell analysis to show that f_a metrics may bias our assessment of drug efficacy and combination effectiveness. This bias may be corrected when dynamic probabilities of drug-induced phenotypic events, i.e. induction of cell death and inhibition of division, at a single-cell level are used as metrics to assess drug efficacy. Probabilistic phenotype metrics offer the following three benefits. First, in contrast to the commonly used f_a metrics, they directly represent probabilities of drug action in a cell population. Therefore, they deconvolve differential degrees of drug effect on tumor cell killing versus inhibition of cell division, which may not be correlated for many drugs. Second, they increase the sensitivity of short-term drug response assays to cell-to-cell heterogeneities and the presence of drug-tolerant subpopulations. Third, their probabilistic nature allows them to be used directly in unbiased evaluation of synergistic efficacy in drug combinations using probabilistic models such as Bliss independence. Altogether, we envision that probabilistic analysis of single-cell phenotypes complements currently available assays via improving our understanding of heterogeneity in drug response, thereby facilitating the discovery of more efficacious combination therapies to block drug-tolerant cells.

Author Summary

Resistance to therapy due to tumor cell heterogeneity poses a major challenge to the use of cancer drugs. Cell-to-cell variability generates subpopulations of drug-tolerant cells that diminish therapeutic efficacy, even in populations of cells scored as highly sensitive based on drug potency. Overcoming such heterogeneity and blocking subpopulations of drug-tolerant cells motivate efforts toward identifying efficacious combination therapies. The success of these efforts depends on our ability to distinguish how heterogeneous populations of cells respond to individual drugs, and how these responses are influenced by combined drug interactions. In this paper, we propose mathematical and experimental frameworks to evaluate time-dependent drug interactions based on probabilistic metrics that quantify drug-induced tumor cell killing or inhibition of division at a single-cell level. These metrics can reveal heterogeneous drug responses and their changes with time and drug combinations. Thus, they have important implications for designing efficacious combination therapies, especially those designed to block or overcome drug-tolerant subpopulations of cancer cells.

Introduction

In pre-clinical studies, potentially effective drug combinations are usually identified based on evidence of synergy [1–4]. In the case of cancer drugs, synergistic interactions are typically assessed on the basis of bulk cell population measurements, such as relative viability (normalized cell count) and net growth rate inhibition, and their variations with drug dose and combination [5–9]. The benefit of drug combination is then evaluated based on whether using two drugs together improves the potency (via minimizing the dose) or efficacy of treatment (via enhancing the effect) as compared with using either of the drugs alone [10–16]. The benefit of drug combination with respect to efficacy and potency, however, may be decoupled [10], as each metric encodes distinct information about cellular response to a drug

[17]. Variations in potency are often explained by differences in target engagement (e.g. physicochemistry of drug-target interaction), concentration of drug available to cells (e.g. drug uptake and efflux), or existence of pathway redundancy (e.g. presence of a secondary oncogenic driver), among others [18]. Thus, a more potent drug combination enables engaging the target and achieving the desired effect in a cell population by using lower doses of treatment [19,20]. Efficacy, on the other hand, refers to the maximum response achievable using tolerable doses of a drug. A more efficacious drug or drug combination engages a larger proportion of cells [21,22]. Previous systematic studies have revealed that variation in cancer drug efficacy is associated with the extent of cell-to-cell variability in drug response [17,23], although such heterogeneity is not directly scored in most pre-clinical drug response assays.

Cell-to-cell variability may generate subpopulations of drug-tolerant cells that diminish cancer drug efficacy [24–30]. An example of such heterogeneity is observed following the emergence of adaptive resistance or selection of resistant sub-clones even in populations of cells that are scored as highly responsive based on drug potency (e.g. EC_{50} measurements) in routine 3 to 5-day assays. In such cases, while more than half (often as many as 90-99%) of cells may respond to treatment (depending on time and dose), the remaining cells give rise to a drug-insensitive subpopulation of survivors that may stay quiescent or divide slowly in the presence of drug [31]. Although not obvious from the most commonly used potency measurements, the emergence of such survivors limits therapeutic efficacy, leading to residual cells from which drug-resistant clones may eventually arise and drive disease progression [32–35]. Overcoming such heterogeneity in drug response and eradicating subpopulations of drug-tolerant cells provide a strong motivation for identifying more efficacious combination therapies [36]. A key step toward this goal is the ability to distinguish how heterogeneous populations of cells respond to individual drugs in short-term assays, and how these responses are influenced by combined drug interactions. However, the standard way in which drugs or their combinations are screened using

normalized population assays obscures single-cell and subpopulation effects that likely play a major role in diminishing the therapeutic efficacy [21,37].

Focusing on efficacy, the benefit of drug combination in a heterogeneous population of cells may arise either from its cooperative inhibitory effect on target cells [22], or simply from the increased probability of cells being sensitive to any of the constituent drugs [38]. In both cases, the overall phenotypic consequences of drug interactions may be assessed in cell culture experiments based on null models of non-interaction [3]; synergistic efficacy is typically concluded when the observed combinatorial effect exceeds the expected effect from a given null model. The most commonly used model, Bliss independence, evaluates interactions based on the probability theory for statistically independent drug actions [16]. In cancer treatment, two basic phenotypic events affected by drug action are cell death and division. The effect of a drug on an individual cell changes the probability of its survival or division within a given time interval. However, current application of the Bliss independence typically uses fraction of cells affected (f_a), a number between zero and one defined based on relative viability or net growth rate inhibition normalized to an untreated control at a fixed timepoint, as drug effect [3,7]. We argue that this commonly used approach leads to a bias in the estimation of both drug efficacy and combination effectiveness in heterogeneous cell populations, especially when the ultimate goal is to block or eradicate small subpopulations of drug-tolerant cells. This is because f_a quantities are not equal to the time-dependent probabilities at which cell death or inhibition of cell division are induced by a drug.

In this paper, we discuss evaluating time-dependent drug responses based on probabilistic metrics that quantify drug-induced tumor cell killing and inhibition of division at a single-cell level. Using these phenotype metrics, we re-evaluate criteria for statistical independence of drug interactions based on probability theory. Experimentally, phenotype metrics are measured using time-lapse live cell

microscopy via monitoring cells engineered to express fluorescent reporters for nucleus identification (to distinguish live versus dead cells) and cell cycle progression (to score division events). As a proof of concept, we evaluate the performance of the metrics in two BRAF-mutant melanoma cell lines exposed to a range of targeted drugs which have been tested or proposed to be studied in combination with standard of care BRAF and MEK kinase inhibitors. Dynamic measurements of the phenotype metrics reveal distinctive responses of melanoma cells to drug combinations that may not be distinguishable when assessed based on conventional assays. This is because these metrics deconvolve differential degrees of drug effect on tumor cell killing versus inhibition of division, which are not necessarily correlated across various drug treatments and their combinations. Furthermore, these metrics increase the sensitivity of short-term drug response assays to cell-to-cell heterogeneities and thus the presence or emergence of drug-tolerant subpopulations, which are typically overlooked in conventional drug response assays.

Results

Probabilistic description of drug-induced phenotypic events

We model the arrival of phenotypic events, including cell division and death, in a given cell population as independent non-stationary *Poisson* processes with time-varying rate constants (k_{event}). These rate constants are linked to the actual probabilities (P_{event}) with which such events occur in individual cells within a series of short time intervals (dt):

$$P_{event} = 1 - e^{-k_{event}dt} \approx k_{event}dt \quad (\text{Equation 1})$$

At a population level, the occurrence of these phenotypic events can be described by *Poisson* processes of which the time-dependent rates of occurrence are directly related to the probabilities of events at a single-cell level (Figure 1A). Therefore, the distribution of death and division events observed for a

population of N cells during a time period of Δt could be approximated using the following equation:

$$P\{N_{event}(t \rightarrow t + \Delta t) = x\} = \frac{(k_{event}(t)N\Delta t)^x}{x!} e^{-(k_{event}(t)N)\Delta t} \quad (\text{Equation 2})$$

where $N_{event}(t \rightarrow t + \Delta t)$ is the number of phenotypic events (death or division) occurring during the time interval between t and $t + \Delta t$.

Assuming negligible cell death in the absence of any treatment, the model describes the cytotoxic effect of a drug on a given cell by the probability with which it induces cell death per unit of time ($P_{death} = k_{death}dt$). The cytostatic effect of drug on a given cell is described by a conditional probability (P_{stasis}) with which it prevents the cell from dividing given that the same cell would have divided in the absence of drug with a probability of $P_{division}(no\ drug) = k_{division}(no\ drug)dt$. The relationship between the conditional probability P_{stasis} and the probability of cell division in the presence of drug ($P_{division} = k_{division}dt$) and their associated rate constants could be described as follows (see Materials and Methods for details):

$$P_{stasis} = 1 - \frac{P_{division}}{P_{division}(no\ drug)} = 1 - \frac{k_{division}}{k_{division}(no\ drug)} \quad (\text{Equation 3})$$

$$k_{stasis} = P_{stasis}k_{division}(no\ drug) \quad (\text{Equation 4})$$

The model provides a framework to simulate how dose-dependent responses in populations of cells vary with P_{death} per unit of time (h) and P_{stasis} using input parameters (k_{death} and k_{stasis}) that represent drugs with a wide range of cytotoxic and cytostatic effects. For each condition, the fraction of cells affected (f_a), defined based on changes in relative viability or net growth rate inhibition (using recently developed DIP and GR metrics [5,6]) normalized to an untreated control, are also derived as model outputs (Figure 1B, C). We compared f_a quantities with probabilistic measures of drug action (P_{death} and P_{stasis}) across a number of drug response simulations. Except for extreme cases such as when $P_{death} = P_{stasis} = 0$ (i.e. there is no drug) or when $P_{death} = 1$ (i.e. all cells dying within the first time interval), f_a quantities differed substantially from the probability with which drugs induced cell death or from the

probability with which they inhibited cell division (Figure 1D). f_a gives a closer estimate of the overall probability with which a drug induces either cell death or inhibits cell division ($P_{death \cup stasis}$), i.e. the probability of a cell being affected (Figure 1D). However, it still fails to accurately represent the probabilistic nature of drug action in cells. Together, simulation results suggest that using f_a for evaluating drug response or drug combination efficacy (such as in Bliss independence) may lead to inaccurate conclusions. Instead, we propose to use direct measures of probabilistic phenotype metrics (P_{death} and P_{stasis} or k_{death} and k_{stasis}) for such analyses.

Probabilistic rate constants capture time-dependent heterogeneities in phenotypic responses

Probabilistic rate constants are estimated based on the frequencies of occurrence of individual phenotypic events. These metrics are expected to exhibit high sensitivity in distinguishing cell-to-cell heterogeneities that lead to selection of small subpopulations of drug-tolerant cells. To test this hypothesis, we simulated drug treatment scenarios where the initial cell population consisted of heterogeneous subpopulations, in which a small fraction ($\omega \leq 5\%$) of cells were substantially less sensitive (by up to $r = 16$ -fold) to treatment relative to the majority of the cell population (Figure 2A). We then defined and calculated “resistance enrichment ratio” for each of the f_a metrics (described based on viability, GR and DIP) or for phenotype rate constants (k_{death} and k_{stasis}) by normalizing each metric measured for the heterogeneous population to that in a homogeneous population (i.e. $\omega = 0$ or $r = 0$) at different times of treatment. Smaller resistance enrichment ratios represent greater ability to distinguish heterogeneous drug-tolerant subpopulations.

We first compared the ability of each metric to distinguish the presence of small subpopulations of drug-tolerant cells by analyzing how resistance enrichment ratio varies with ω and time (Figure 2B). Simulation results show that f_a metrics, defined based on either normalized cell viability or growth rate

inhibition (GR and DIP), are significantly less sensitive than k_{death} and k_{stasis} to the presence of drug-tolerant cells (Figure 2B). Furthermore, for any given initial fraction of drug-tolerant cells (ω), phenotype rate constants distinguished the emergence of drug resistance at earlier timepoints. Using similar simulations, we also tested how the relative level of drug resistance (r) in a fixed initial fraction of drug-tolerant cells would influence each of the drug response metrics. Simulation results show that for a given ω , phenotype rate constants detect subpopulations with weaker levels of resistance (i.e. smaller r values) and at earlier timepoints (Figure 2C).

Most conventional drug screening assays are performed following exposure of cells to drug for 3 to 5 days. While variations in drug potency are distinguishable in such assays, it is often suggested that longer periods of treatment are essential to distinguish the presence of drug-tolerant persisters that diminish the efficacy. However, our results show that phenotype rate constants can capture heterogeneities that would otherwise require significantly longer experiments when using population-level f_a metrics. The benefit of using phenotype rate constants would be especially significant in the case of potent drugs that induce substantial cell death, while sparing a small fraction of drug-tolerant cells. In particular, as the efficiency of drug-induced cell killing increases, the sensitivity of f_a metrics to detect drug tolerance in the surviving fraction of cells decreases.

Estimating probabilistic rate constants using time-lapse live cell microscopy

To experimentally capture stochastic processes of induction of cell death and inhibition of division in drug-treated tumor cell populations, we used time-lapse live cell microscopy and cells engineered to express two fluorescent reporters. The reporters included: (i) an H2B-Venus reporter which labels chromatin, allowing identification of nuclei and scoring cell death based on changes in nucleus morphology, and (ii) an mCherry-Geminin reporter for cell cycle progression [39] which allows tracking

of cell division events. Using a high-throughput, automated image analysis workflow (see Materials and Methods), the occurrence of individual phenotypic events (death and division) in single cells was tracked in time across a variety of drug treatment conditions (Supplementary Figure S1). To estimate time-dependent changes in probabilistic phenotype rate constants, the number of cell death and division events (N_{event}) were quantified over a series of uniform time intervals of length Δt . Phenotype rate constants were then estimated via normalizing N_{event} in each time interval to the length (Δt) and the average number of live cells over that time interval $[N_{live}(t \rightarrow t + \Delta t)]_{avg}$ as detailed below:

$$k_{death}(t) = \frac{N_{death}(t \rightarrow t + \Delta t)}{[N_{live}(t \rightarrow t + \Delta t)]_{avg} \Delta t} \quad (\text{Equation 5})$$

$$k_{division}(t) = \frac{N_{division}(t \rightarrow t + \Delta t)}{[N_{live}(t \rightarrow t + \Delta t)]_{avg} \Delta t} \quad (\text{Equation 6})$$

$$k_{stasis}(t) = k_{division}(no\ drug) - k_{division}(t) \quad (\text{Equation 7})$$

As a proof of concept, we monitored responses of two BRAF-mutated melanoma cell lines (COLO858 and MMACSF) following exposure to a BRAF inhibitor Vemurafenib at 6 doses for a period of ~120 h. Heterogeneity in drug response was then visualized through the estimation and analysis of phenotype rate constants, k_{death} and k_{stasis} , as a function of drug dose and time in each cell line (Figure 3A, B). In COLO858 cells, which have been shown to be initially sensitive but rapidly develop adaptive resistance to Vemurafenib [21,22], increasing drug concentration enhanced both the amplitude and the rate of increase in k_{death} and k_{stasis} within the first 36 h. After that, these responses were attenuated concurrent with the activation of drug-induced adaptive responses (Figure 3A). Responses of MMACSF cells involved a relatively monotonic and dose-dependent decrease in the number of live cells. At the highest drug concentration (3.2 μM), however, we observed two peaks of apoptotic response, one similar to COLO858 cells at $t \approx 36$ h and a higher peak later at $t \approx 108$ h (Figure 3B). These data are consistent with previous data reporting high sensitivity of MMACSF cells to 5 days of

exposure to Vemurafenib [21,22], but also highlight the impact of cell-to-cell heterogeneity and the presence of subpopulations of cells with different levels of drug tolerance.

In addition to interrogating dynamic aspects of heterogeneous drug response, we also tested the performance of our automated image analysis workflow by comparing the estimated phenotype rate constants with those measured from data generated by manual single-cell tracking using a MATLAB-based software [40]. The software allowed accurate tracking and cell fate annotation of individual cells across time-lapse images taken over a period of multiple days. Single-cell profiles from manual tracking confirmed heterogeneity in the number and timing of death and division events in cells exposed to drug. In COLO858, for example, cell-to-cell variability ranged from cells that died rapidly (as early as ~24 h) in response to high concentrations of Vemurafenib, to cells that survived but did not divide, to cells that slowly divided following a temporary delay in their cell cycle, the proportion and dynamics of which changed with drug dose (Figure 3C). By comparing rate constants between two image analysis methods across a variety of conditions in two cell lines, we identified quantitatively similar patterns (Supplemental Figure S2). This consistency confirms that the automated workflow would be suitable for high-throughput analysis of drug response.

We also used single-cell phenotype data to empirically evaluate the assumption of non-stationary *Poisson* process to model drug-induced death and division events. We compared the distribution of phenotypic events measured from time-lapse microscopy experiments with those simulated based on *Poisson* processes using estimates of phenotype rate constants. We observed similarity across patterns of response at the single-cell level and between distributions of events at the population level (Figures 3C-3E), suggesting that a simplified model of non-stationary *Poisson* process for drug-induced death and division events is a reasonable one.

Taken together, high-throughput estimation and analysis of phenotype rate constants and their

changes with time and dose provide an efficient tool to capture critical dynamic aspects of probabilistic and heterogeneous drug response that would be overlooked in bulk population assays.

Evaluating statistical independence of drug combination efficacies using probabilistic phenotype metrics

Among the most widely used reference models in evaluation of synergistic efficacy for cancer drug combinations is Bliss independence [16]. The Bliss model assumes that drug effects are consequences of probabilistic processes, and that two drugs act independently if their combined effect confers probabilistic or statistical independence:

$$P_{A+B}^I = P_A + P_B - P_A P_B \quad (\text{Equation 8})$$

where P_{A+B}^I describes the expected probability of the combinatorial effect of drugs A and B when they act independently. $0 \leq P_A \leq 1$ and $0 \leq P_B \leq 1$ represent probabilities of effect mediated by drugs A and B when tested individually. The Bliss combination index (CI) for drugs A and B may be calculated as:

$$CI_{A+B}^{Bliss} = \frac{P_{A+B}^I}{P_{A+B}} \quad (\text{Equation 9})$$

, where P_{A+B} describes the actual probability of effect induced by drugs A and B when used in combination. Synergistic combination efficacy is concluded if $CI < 1$, i.e. when the observed combinatorial effect exceeds the expected effect from the Bliss independence model. Despite its probabilistic definition, however, the Bliss model is broadly applied to a variety of f_a metrics (such as normalized viability or net growth rate inhibition), thereby leading to unreliable conclusions which are largely due to the following limitations. First, although f_a measurements satisfy the mathematical requirement of $0 \leq f_a \leq 1$, they do not have a probabilistic nature and thus do not necessarily follow the rules of probability theory. Second, f_a quantities are the result of two distinct probabilistic processes, induction of cell death and inhibition of cell division. These processes, even when induced by drugs with

the same probabilities, do not necessarily have the same impact on f_a . Third, for drugs A and B with $f_a < 1$, the Bliss model (when applied to f_a) is unable to account for the difference between being affected by drug A, drug B, or both. For example, consider the combined effect of two truly independent and purely cytostatic drugs A and B, whose phenotypic effects individually could be described by $P_{stasis} = 1$ (and $P_{death} = 0$). By Bliss independence when applied to f_a metrics such as viability or normalized growth rate inhibition, drugs A and B are expected to have a substantial cytotoxic effect and thus their combination would be scored incorrectly as antagonistic ($CI > 1$) (Figures 4A, B). To overcome such limitations and to avoid erroneous conclusions about drug combination efficacies, we propose to use probabilities of phenotypic events or their associated rate constants in evaluation of Bliss independence according to its definition.

When applied to probabilistic events of drug-induced cell death, Bliss independence for the combined cytotoxic effect of drugs A and B may be described as follows:

$$P_{death(A+B)}^I = k_{death(A+B)}^I dt = P_{death(A)} + P_{death(B)} - P_{death(A)}P_{death(B)} \approx (k_{death(A)} + k_{death(B)})dt \quad (\text{Equation 10})$$

, where $P_{death(A)}$ and $P_{death(B)}$ represent the probabilities with which drugs A and B induce cell death within a short time interval of dt , respectively. $P_{death(A+B)}^I$ represents the probability of death induced by the combination of drugs A and B when they act independently. $k_{death(A)}$, $k_{death(B)}$ and $k_{death(A+B)}^I$ represent rate constants associated with these probabilistic events, respectively. When applied to the conditional event of inhibition of cell division given that cells divide at a rate of $k_{division(no\ drug)}$ in the absence of drug, the Bliss independence may be described as follows:

$$P_{stasis(A+B)}^I = \frac{k_{stasis(A+B)}^I}{k_{division(no\ drug)}} = P_{stasis(A)} + P_{stasis(B)} - P_{stasis(A)}P_{stasis(B)} \quad (\text{Equation 11})$$

, where $P_{stasis(A)}$ and $P_{stasis(B)}$ represent the probabilities with which drugs A and B inhibit cell division given that cells would divide with a probability of $P_{division(no\ drug)} = k_{division(no\ drug)}dt$ within a short time

interval of dt . $P_{stasis}^I(A+B)$ represents the cytostatic effect for the combination of drugs A and B when they act independently. $k_{stasis}(A)$, $k_{stasis}(B)$ and $k_{stasis}^I(A+B)$ represent rate constants associated with these probabilistic events. Based on these definitions, the Bliss combination index for each individual drug-induced effect could be described as follows:

$$CI_{death}^{Bliss}(A+B) = \frac{P_{death}^I(A+B)}{P_{death}(A+B)} = \frac{k_{death}^I(A+B)}{k_{death}(A+B)} \quad (\text{Equation 12})$$

$$CI_{stasis}^{Bliss}(A+B) = \frac{P_{stasis}^I(A+B)}{P_{stasis}(A+B)} = \frac{k_{stasis}^I(A+B)}{k_{stasis}(A+B)} \quad (\text{Equation 13})$$

Systematic simulation results show that evaluating probabilistic independence based on drug-induced phenotypic events can distinguish a variety of possible drug interactions that would be otherwise overlooked when assessed on the basis of f_a quantities (Figure 4C). The discrepancy is particularly substantial under conditions where drug combinations have uneven cytotoxic and cytostatic interactions, e.g. when two compounds act synergistically with respect to inhibition of division but act independently or antagonistically with respect to induction of cell death, and vice versa.

Probabilistic phenotype metrics uncover target-specific differences in drug combination efficacies

We applied the probabilistic definition of Bliss independence to evaluate time-dependent changes in the efficacies of a group of 12 compounds in sequential combination with BRAF kinase inhibitor, Vemurafenib, plus MEK kinase inhibitor, Trametinib, in two BRAF-mutated melanoma cell lines COLO858 and MMACSF over the course of five days (see Methods for details). Single-cell drug responses were monitored using time-lapse fluorescence microscopy, and changes in probabilistic rate constants k_{stasis} and k_{death} were tracked for the entire period of experiment for each drug condition individually or in combinations (Supplementary Figures S3, S4). The list of compounds based on their nominal targets included two HDAC inhibitors (Fimepinostat and Givinostat), two BET bromodomain inhibitors (Birabresib and I-BET762), two KDM1A inhibitors (SP2509 and ORY-1001), a pan Jmj-

KDM inhibitor (JIB-04), a KDM5 inhibitor (CPI-455), two Tankyrase inhibitors (AZ6102 and NVP-TNKS656), and two CDK4/6 inhibitors (Palbociclib and Abemaciclib). These compounds were selected from two broad classes of anti-cancer drugs, referred to as epigenetic-modifying compounds and cell cycle inhibitors, which have been proposed to be used in combination with standard of care BRAF and MEK inhibitors to overcome drug-adapted subpopulations of cells in BRAF-mutant melanomas [10,21,41–48].

The analysis of variations in Bliss combination index, defined based on probabilistic cytotoxic and cytostatic actions, with drug and time (following unsupervised clustering) led to two major conclusions (Figure 5A). First, effective drugs with comparable mechanisms of action (e.g. BET inhibitors, HDAC inhibitors or CDK4/6 inhibitors) exhibited similar dynamic patterns of interaction with BRAF and MEK kinase inhibitors, suggesting that differences in probabilistic drug action and interactions are target-specific. Second, cytostatic and cytotoxic drug interactions among efficacious drug combinations often varied in time and did not necessarily correlate with one another. BET inhibitors, for example, exhibited a strong synergistic cytotoxic interaction ($CI_{death} < 1$) with the combination of BRAF and MEK inhibitors within 48-72 h of treatment in both COLO858 and MMACSF cell lines, whereas their interaction was scored as independent ($CI_{stasis} \approx 1$) with respect to inhibition of cell division (Figure 5A, B). Furthermore, the benefit of BET inhibitors combined with Vemurafenib and Trametinib diminished following 96 h, concomitant with the emergence of a small proliferating subpopulation ($k_{division} > 0$) (Figure 5B). CDK4/6 inhibitors acted independently with BRAF and MEK inhibitor combination to inhibit cell division within 72-96 h in both cell lines (Figure 5A and Supplementary Figures S3 and S4). Surprisingly, however, their effects on cell death appeared to be antagonistic especially in MMACSF cells (Figure 5C). This might be due the possibility that upon G0/G1 arrest, BRAF-mutant cells become less responsive to the effect of BRAF and MEK inhibitors, an

interesting observation which requires further investigation across more cell lines.

Altogether, experimental results and simulation outcomes suggest that dynamic measurements of the phenotype metrics and probabilistic evaluation of combination index reveal distinctive responses of cells to drug combinations that might be indistinguishable when assessed based on conventional assays. Phenotype metrics deconvolve differential (and sometimes opposing) degrees of drug effect on tumor cell killing versus inhibition of cell division, a potentially important consideration in choosing appropriate drug combinations. Furthermore, the probabilistic nature of these metrics makes them sensitive to cell-to-cell heterogeneities which are typically overlooked in conventional bulk drug response assays. They are therefore appropriate choices to assess synergistic efficacy in drug combinations aimed at blocking heterogeneous subpopulations of drug-tolerant cells.

Discussion

Synergistic interactions in cancer drug efficacy are typically assessed using Bliss independence or other models (e.g. Highest Single Agent approach [2]). These models are commonly applied to drug response measurements, whose outcomes are normalized to those measured in untreated controls to identify the fraction of cells affected (f_a) by drugs. Examples of these metrics include drug-induced changes in viability (normalized live cell count) or net growth rate inhibition, which are analyzed across drug doses and combinations. Synergistic efficacy is then concluded when the observed combinatorial effect on f_a metrics exceeds the expected effect from the null model. In this paper, we use basic probability theory and computer simulations to demonstrate that using f_a metrics may bias our estimation of drug combination effectiveness and synergistic efficacy, especially when the ultimate goal is to block heterogeneous drug-tolerant subpopulations. Instead, we propose to use direct measures of time-dependent probabilities of key drug-induced phenotypic events, i.e. induction of cell death and inhibition

of cell division, and their associated rate constants (k_{death} and k_{stasis}) to evaluate synergistic efficacy using probabilistic models such as Bliss.

Probabilistic phenotype metrics improve our ability to quantify drug efficacy and characterize drug combination interactions in the following three ways. First, in contrast to the commonly used f_a metrics, phenotype metrics are directly related to the probabilities of drug action in a cell population within any given time interval following drug exposure. Furthermore, they deconvolve differential degrees of drug effect on tumor cell killing versus inhibition of cell division, which may not be correlated in many cases. Second, k_{death} and k_{stasis} dramatically increase the sensitivity of short-term drug response assays to dynamic cell-to-cell heterogeneities and the presence (or emergence) of drug-resistant sub-clones, which are typically overlooked in conventional f_a based drug response analyses. This is a critical issue especially when heterogeneous tumor cell populations consist of cells that are differentially sensitive to drugs and that their sensitivity changes with time. Third, the probabilistic nature of phenotype metrics allows us to use them directly in unbiased evaluation of independence, synergistic or antagonistic efficacy in drug combinations using probabilistic models such as Bliss independence.

While we focused on Bliss independence as a probabilistic framework to study synergistic efficacy, phenotype metrics and their dose- and time-dependent variations could be used in other platforms for broad evaluation of synergy. A recently developed multi-dimensional framework (MuSyC) uses a two-dimensional extension of Hill equation to distinguish synergistic efficacy versus synergistic potency, thereby allowing for a comprehensive understanding of drug interactions. Such understanding not only helps with improving therapeutic efficacy via enhancing effect, but also reducing off-target toxicities via dose reduction [10]. Probabilistic phenotype rate constants follow dose-response patterns suitable to be fit by Hill equation and therefore could be used as input to platforms such as MuSyC.

Estimating probabilistic phenotype metrics requires continuous time-lapse experiments along

periods of multiple days, followed by computational single-cell analysis. Such integrative methods may not be cost-effective and not recommended for large-scale drug screening projects, in which many drugs are filtered out because of lack of potency. The benefit of these methods is significant, however, when there is a need for increased ability to distinguish cell-to-cell heterogeneities or detecting small subpopulations of drug-tolerant cells that influence drug efficacy rather than potency. Therefore, we envision that probabilistic analysis of single-cell phenotypes will complement currently available methods via improving our understanding of heterogeneity in drug response, thereby facilitating the discovery of more efficacious combination therapies to block drug-tolerant tumor cells.

Materials and Methods

Cell culture

BRAF-mutated melanoma cell lines used in this study were obtained from the Massachusetts General Hospital Cancer Center with the following primary sources: COLO858 (ECACC) and MMACSF (Riken Bioresource Center). Each cell line was independently authenticated by Short Tandem Repeats (STR) profiling by ATCC. COLO858 cells were grown in RMPI 1640 (Corning cellgro, Cat. 10-040 CV), and MMACSF cells were grown in DMEM/F-12 (Thermo Fisher Scientific, Cat. 11330-032). For both cell lines, growth media were supplemented with 5% fetal bovine serum (Thermo Fisher Scientific, Cat. 26140-079) and 1% sodium pyruvate (Thermo Fisher Scientific, Cat. 11360-070). We added penicillin and streptomycin at 100 U/ml (Thermo Fisher Scientific, Cat. 15140-122) and plasmocin at 0.5 μ g/ml (InvivoGen, Cat. ant-mpp) to all growth media. Cells were engineered to stably express H2B-Venus and mCherry-Geminin fluorescent reporters as described previously [21]. Engineered and parental cell lines were confirmed to grow at comparable rates in the absence of any treatment or in the presence of different concentrations of BRAF inhibitor Vemurafenib over 72 hours of treatment.

Reagents

Chemical inhibitors used in this study were obtained from Selleck Chemicals with the following catalog numbers: Vemurafenib (Cat. S1267), Trametinib (Cat. S2673), SP2509 (Cat. S7680), ORY-1001 (Cat. S7795), Palbociclib (Cat. S1116), Abemaciclib (Cat. S7158), AZ6102 (Cat. S7767), NVP-TNKS656 (Cat. S7238), Givinostat (Cat. S2170), Fimepinostat (CUDC-907; Cat. S2759), JIB-04 (Cat. S7281), CPI-455 (Cat. S8287), I-BET762 (Cat. S7189) and Birabresib (OTX-015; Cat. S7360). All chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solution, except Palbociclib of which the stock concentration was 5 mM.

Cell seeding and drug treatment

COLO858 and MMACSF cells expressing fluorescent reporters were seeded into Costar 96-well black clear-bottom tissue culture plates (Corning 2603) in 220 μ L full growth medium without phenol red at a density of 2000 and 3000 cells/well, respectively. Cells were counted using a TC20™ Automated Cell Counter (Bio Rad). In the case of Vemurafenib dose-response experiments, cells were treated ~24 h after seeding with either DMSO or five different concentrations of Vemurafenib (0.0316, 0.1, 0.316, 1 and 3.16 μ M) for a period of ~5 days. In the case of drug combination experiments, cells were treated (also 24 h after seeding) with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors at the following concentrations: Fimepinostat (0.02 μ M), Givinostat (0.2 μ M), Birabresib (0.5 μ M), I-BET762 (1 μ M), SP2509 (1 μ M), ORY-1001 (1 μ M), JIB-04 (0.2 μ M), CPI-455 (5 μ M), AZ6102 (1 μ M), NVP-TNKS656 (1 μ M), Palbociclib (1 μ M), and Abemaciclib (1 μ M); drug concentrations were chosen based on previous reports exhibiting maximal target inhibition in cells. After 24 h, Vemurafenib at 0.3 μ M plus Trametinib at 0.03 μ M, or DMSO control were added to each

treatment condition. All drug treatments were performed in at least 4 replicates using a Hewlett-Packard (HP) D300 Digital Dispenser.

High-throughput time-lapse live cell microscopy

Within 50-60 min after each treatment, cells were imaged every 10 min (for COLO858) and every 15 min (for MMACSF) using a Nikon Ti2-E inverted microscope with motorized stage, Perfect Focus System, 20× objective, and a Photometrics Prime 95B camera followed by 2×2 binning. The process of image acquisition was controlled using NIS element software. Illumination was powered by the Lumencor Spectra X light engine. H2B-Venus fluorescence was captured using 510 nm excitation and 535 nm emission at 25 ms exposure for MMACSF cells and 20 ms exposure for COLO858 cells. mCherry-Geminin fluorescence was captured using 575 nm excitation and a 629.5 nm emission at 80 ms exposure for MMACSF cells and 100 ms exposure for COLO858 cells. Throughout the entire period of image acquisition, environmental conditions were maintained at 37°C, 5% CO₂, and 93% humidity using an OkoLab Enclosure.

Image analysis and automated cell tracking workflow

Images were first processed using Fiji [49] for rolling ball background subtraction with radius of 20 pixels. Background-subtracted images were then analyzed using CellProfiler (3.1.8) for segmentation and classification of cellular phenotypic states, including cells that express high and low levels of Geminin (referred to as Geminin^{high} and Geminin^{low} cells, respectively), or live versus dead cells. Briefly, CellProfiler analysis (Supplementary Figure S1) involved: (1) edge enhancement and dark hole feature enhancement of the background-subtracted H2B images to facilitate segmentation; (2) segmenting individual cell nuclei using the Otsu thresholding method; (3) using nuclei segmentations as

masks to measure object intensities for all channels as well as object sizes and shapes; (4) classification of phenotypic states of each cell object using the classification model output from CellProfiler Analyst (2.2.1) [50] based on features measured from the previous step. Geminin^{high} versus Geminin^{low} cell classifiers and live versus dead cell classifiers were trained separately using fast gentle boosting algorithm in CellProfiler Analyst with eight and fifteen maximum rules, respectively. The training set used to develop each phenotype classifier was an annotated set, generated via manually sorting the cell object tiles into their corresponding phenotype classes in CellProfiler Analyst. The process of manual sorting followed by model training was iterated until approximately 80% of true positive accuracy was achieved.

Based on phenotype classifications of individual cells for each image output from CellProfiler, corresponding synthetic images were generated in MATLAB (2018b) for each phenotype of interest. Synthetic images contained synthetic pixels at locations of cells, indicating phenotypes of interest (i.e. Geminin^{high} or dead cells). Synthetic images for Geminin^{high} cells, dead cells, background-subtracted H2B and Geminin images acquired from the same site were merged into a single multi-channel image composite using Fiji. Image composites were then analyzed using Fiji plugin TrackMate (3.8.0) with TrackMate extras and Track Analysis extensions [51] for automated tracking. Synthetic pixels of a selected channel were detected by the Laplacian of Gaussian detector and spots were linked with Linear Assignment Problem (LAP) Tracker. Additional spots filtering (based on intensities from multiple channels) and track filtering (based on track duration, track median velocity, and velocity standard deviation) were implemented to optimize tracking results.

Estimating probabilistic phenotype rate constants from individual cell tracking data

Single-cell tracking data generated using TrackMate was analyzed using MATLAB. Transition of a live cell from Geminin^{high} to Geminin^{low} was recorded as a division event, whereas the beginning of a dead cell track was recorded as a death event (Supplementary Figure S1). To estimate time-dependent changes in probabilistic phenotype rate constants, k_{death} and $k_{division}$, the number of recorded cell death and division events (N_{death} and $N_{division}$) were quantified over a series of uniformly distributed time intervals ($t \rightarrow t + \Delta t$), where $\Delta t = 12$ h or 24 h. Normalizing N_{death} and $N_{division}$ to the length of each time interval (Δt) and the average number of live cells within the same interval $[N_{live}(t \rightarrow t + \Delta t)]_{avg}$, phenotype rate constant were estimated using Equations 5-7. As expected, we observed that the magnitude of noise in single-cell tracking data and consequently the relative error in the estimation of $k_{division}$ and k_{death} , increased under conditions where Geminin^{high} and dead cells were highly concentrated, respectively. To mitigate the effect of noise under such conditions, we imposed the following constraints in our estimation of $N_{division}$ (when $N_{death} < N_{division}$) and N_{death} (when $N_{death} > N_{division}$) during each time interval, respectively:

$$N_{division}(t \rightarrow t + \Delta t) = (N_{live}(t + \Delta t) - N_{live}(t)) - N_{death}(t \rightarrow t + \Delta t) \quad (\text{Equation 14})$$

$$N_{death}(t \rightarrow t + \Delta t) = N_{division}(t \rightarrow t + \Delta t) - (N_{live}(t + \Delta t) - N_{live}(t)) \quad (\text{Equation 15})$$

These constraints are consistent with the assumption that the overall change in the number of live cells during each time interval (Δt) must be equal to the number of division events minus the number of death events during the same time interval.

Verifying the accuracy of automated cell tracking workflow using manual single-cell tracking

To test the performance of our automated image analysis workflow, we compared the phenotype rate constants measured using data from the automated pipeline with those measured using data generated from manual single-cell tracking. This was accomplished using a MATLAB-based software, allowing

accurate single-cell tracking and cell fate annotation of individual cells across time-lapse images taken over a period of multiple days [40]. Briefly, the manual tracking method relies on identification of individual cells using intensity and shape information of the nuclear marker (H2B-Venus), track propagation using nearest neighbor criteria, and real-time user correction of tracking, and annotation of cell death and division events based on H2B and Geminin signal intensities. For each condition, about 150-250 cells pooled from four replicates were manually tracked and cell death and division events were recorded. Phenotype rate constants were then calculated using Equations 5-7.

Estimating fraction of cells affected (f_a) by drug

Currently, evaluation of Bliss independence (and other drug interaction frameworks) is based on fraction of cells affected (f_a), a normalized parameter between zero and one, that represents the fractional effect of drugs individually or in combination [52]. Conventionally, relative viability (or cell count normalized to an untreated control) measured at a fixed time-point (typically 72 or 96 h) following drug treatment has been used to calculate f_a :

$$f_a(\text{viability}) = 1 - \text{viability} \quad (\text{Equation 16})$$

Despite its wide-spread usage, however, the relative viability approach in assessing drug response suffers from a fundamental flaw, which is being confounded by variation in cell proliferation rates and assay duration. The reason is that cell count, which is used as a normalization factor in this approach, is non-linearly time-dependent. Therefore, new generation drug response metrics have recently been introduced to correct for this bias [5,6]. The nature of these metrics is based on modeling drug-induced changes in the net growth rate of the cancer cell population (instead of relative viability) as a function of drug dose. These metrics include drug-induced proliferation (DIP) rate [6] and growth rate (GR) inhibition [5], both of which consider and correct for the variability in growth rate that is irrelevant to

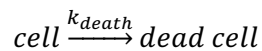
drug treatment via normalizing the net growth rate of the drug-treated cell population to that of the untreated control. Given the dynamic range of each metric, the definition of fraction of cells affected (f_a) for these new metrics, $f_a(DIP)$ and $f_a(GR)$, is modified as follows so that $0 \leq f_a \leq 1$:

$$f_a(DIP) = \frac{1-DIP}{1-\min(DIP)} \quad (\text{Equation 17})$$

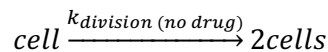
$$f_a(GR) = \frac{1-GR}{2} \quad (\text{Equation 18})$$

Stochastic simulation of cytotoxic and cytostatic drug effects

We modeled phenotypic events in a heterogeneous tumor cell population as a series of independent stochastic reaction processes at a single-cell level. Drug-induced death events were described by the following reaction:



, where the rate constant of death k_{death} is defined such that a given cell dies with a probability of $k_{death}dt$ within a reasonably short time interval (dt). Cell division in the absence of drug could be described by the following reaction:



, where $k_{division\ (no\ drug)}$ is the inherent rate of division of a given cell. The cytostatic effect of a drug on a cell was described by a conditional probability ($P_{stasis} = P_{inhibition\ of\ division\ (with\ drug) | division\ (no\ drug)}$) with which it prevents a cell from dividing given that it would have divided in the absence of drug with a probability of $P_{division\ (no\ drug)}$. For cancer drugs that have inhibitory effect on cell division, the relationship between P_{stasis} and $P_{division}$ in the presence of drug could be derived as follows:

$$P_{division\ (no\ drug)} = P_{division\ (no\ drug) \cap division\ (with\ drug)} + P_{division\ (no\ drug) \cap inhibition\ of\ division\ (with\ drug)}$$

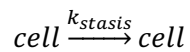
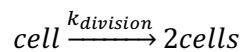
$$P_{division\ (no\ drug)} = P_{division\ (with\ drug)} + P_{inhibition\ of\ division\ (with\ drug) | division\ (no\ drug)} \cdot P_{division\ (no\ drug)}$$

$$P_{\text{inhibition of division (with drug) | division (no drug)}} = \frac{P_{\text{division (no drug)}} - P_{\text{division (with drug)}}}{P_{\text{division (no drug)}}$$

$$P_{\text{stasis}} = 1 - \frac{P_{\text{division}}}{P_{\text{division (no drug)}}$$

$$P_{\text{division}} = (1 - P_{\text{stasis}})P_{\text{division (no drug)}}$$

In the presence of drug, cell division and inhibition of cell division (stasis) could be described by the following reactions, respectively:



, where the rate constants are as follow:

$$k_{\text{stasis}} = P_{\text{stasis}}k_{\text{division (no drug)}}$$

$$k_{\text{division}} = (1 - P_{\text{stasis}})k_{\text{division (no drug)}}$$

The model assumes that the processes of drug-induced cell death and inhibition of cell division are independent of each other.

At the population level, *Poisson* processes of drug-induced phenotypic events in a tumor cell population were simulated using the Gillespie algorithm. Briefly, the Gillespie algorithm determines the time to the next reaction event in the cell population based on an exponential distribution that statistically characterizes the *Poisson* processes. The algorithm then stochastically determines whether the event is death or division based on probabilities that are proportional to the rates of these two processes (k_{death} and k_{division}). If the chosen event is division, then with probability P_{stasis} that division event is rejected.

Validation of non-stationary *Poisson* models for live cell microscopy data

To test whether a simplified model of non-stationary *Poisson* process may explain the distribution of

drug-induced death and division events in time-lapse microscopy data, we used maximum likelihood estimation to fit two non-stationary *Poisson* models, one to the single-cell death data and one to the single-cell division data. The rate function $k(t)$ of the non-stationary *Poisson* models used for data fitting was assumed to be a piece-wise function in time, where for each 12 h interval the rate was given by a single parameter. Hence, to capture 120 h of data, we set the rate function for each *Poisson* process with 10 parameters. The log-likelihood function for fitting a non-stationary *Poisson* model is given as follows:

$$l(\theta) = \sum_{j=1}^n \log\{k(t_j; \theta)\} - \int_0^T k(\tau; \theta) d\tau$$

, where θ is a vector of the 10 parameters to be estimated from the data, n is the number of data-points, t_i is the time of the i^{th} event and T is the end time of the experiment. The log-likelihood function was then maximized using the constrained optimization function ‘fmincon()’ in MATLAB. Using the fit parameters, we then simulated drug responses for 30 times and the normalized mean counts of phenotypic events were compared to that of the same data used for parameter estimation.

Simulations of combined drug responses with variable modes of drug interaction

For combined drug response simulations, we modified the Gillespie algorithm as follows. After determining the time of the next event, the algorithm stochastically determines whether that event is a death event induced by drug A, a death event induced by drug B, or a division event based on probabilities proportional to their rates of occurrence. In cases where the two drugs confer statistically independent cell killing, the probabilities of the next event being drug A-induced death and drug B-induced death are respectively proportional to their single drug-induced rates of death, i.e. $k_{death(A)}$ and $k_{death(B)}$, whereas the probability of the next event being a division event is proportional to the inherent division rate of the cell, $k_{division (no drug)}$. If the next event is division, then with a probability of $P_{stasis(A+B)}$

that division event is rejected. For independent cytostatic interactions, $P_{stasis(A+B)}$ is set to $P_{stasis(A+B)}^I$ as defined in Equation 11. In cases where drug combinations are not independent, $P_{stasis(A+B)}$ and $P_{death(A+B)}$ will be calculated as $P_{stasis(A+B)}^I$ and $P_{death(A+B)}^I$ divided by CI_{death} and CI_{stasis} to simulate different modes of drug interaction, respectively. For the purpose of comparison, we also evaluated Bliss combination index while replacing probabilistic metrics with f_a quantities measured for each drug condition individually and in combination.

Simulations of heterogeneous drug response in the presence of drug-tolerant subpopulations

We simulated drug treatment scenarios where the initial cell population consisted of heterogeneous subpopulations, in which a small fraction of cells was substantially less sensitive to treatment relative to the majority of the cell population. Stochastic arrival of death and inhibition of division events were modeled using Gillespie algorithm as described above, while considering two subpopulations: a larger sensitive (S) subpopulation and a small drug-tolerant or resistant (R) subpopulation. We initialized simulations with 300 cells, a small fraction of which (ω , varied from 0%-5%) had a more resistant phenotype, i.e. a lower death rate constant and a higher probability of stasis than that of the sensitive population, in the presence of drug. We modeled such resistant phenotype by defining the level of resistance ($r \geq 1$, varied from 1-16) as the fold-change in the rates of death and probability of stasis relative to the sensitive population. We used same fold-changes for death and cytostasis rates. In particular, parameters used for the sensitive population are: $k_{division}^S (no\ drug) = 0.035\ hr^{-1}$, $k_{death}^S (drug) = 0.03\ hr^{-1}$, $P_{stasis}^S (drug) = 0.8$. Parameters for the drug-tolerant subpopulation are $k_{division}^R (no\ drug) = 0.02\ hr^{-1}$, $k_{death}^R (drug) = k_{death}^S (drug) / r$, $P_{stasis}^R (drug) = P_{stasis}^S (drug) / r$. We assumed that phenotypic responses of both subpopulations are independent of each other and that daughter cells within the same subpopulation inherit the exact same probabilities of phenotypic events as their mother cells. The responses (i.e.

number of live cells, death and division events) of the two subpopulations were summed together to show the overall response of the entire cell population. To compare quantitatively the sensitivity of different metrics in capturing the differences in drug effect in the presence of phenotypic heterogeneity, we systematically varied the initial fraction of drug-tolerant subpopulation (ω) and its level of resistance (r) as input parameters in simulations. For each simulation, overall drug effect using different metrics (fraction affected or phenotype rate constants) were calculated. To evaluate the sensitivity of each metric to the presence of drug-tolerant subpopulations, we defined and calculated “resistance enrichment ratio” as the ratio of these metrics between two treatment scenarios, one in the presence of a heterogeneous population (varying $\omega > 0$ and $r > 1$) and one in the absence of heterogeneity ($\omega = 0$ and $r = 1$). The smaller the resistance enrichment ratio becomes, the more significant decrease in drug effect is captured by a given metric in the presence of drug-tolerant cells.

Hierarchical clustering

Unsupervised hierarchical clustering of combination index (CI) values estimated from the application of Bliss independence to probabilistic phenotype rate constants measured for 24 h time intervals of drug treatments was carried out using MATLAB 2018b and the Euclidean distance as the metric.

Statistical analysis

All data with error bars were presented as mean \pm standard error of the mean (SEM) using indicated numbers of replicates.

Acknowledgments

We thank members of the Fallahi-Sichani laboratory and Wendy Thomas for help and discussion. This

work was supported by awards from the Elsa Pardee Foundation and V Foundation for Cancer Research V2017-011, Department of Defense PRCRP Career Development Award W81XWH1810427, NIH grants R00-CA194163 and R35-GM133404 (to MFS), P30-CA046592 (University of Michigan Rogel Cancer Center), Rackham International Student Fellowship (to NCL), and NCI Training Grant award T32-CA009676 (to MK).

Author Contributions

NCL and MFS designed the project, performed the experiments, analyzed the results, and wrote the manuscript. NCL developed computational models and performed computational and statistical analysis. MK and MM assisted with experiments. DV assisted with data analysis. MFS supervised the research. All authors reviewed the manuscript.

Figure Legends

Figure 1. Probabilistic description of drug-induced phenotypic events. (A) Schematic representation of phenotypic effects of drug action in a cell population. Drug effect is described as probabilistic events, involving induction of cell death and inhibition of cell division, at a single-cell level. Cytotoxic effect of a drug on a given cell is described by the probability with which it induces cell death per unit of time (P_{death}). The cytostatic effect of drug on a given cell is described by a conditional probability (P_{stasis}) with which it prevents the cell from dividing given that the same cell would have divided in the absence of drug with a probability of $P_{division (no drug)}$. **(B)** Dose-dependent changes in phenotype rate constants (k_{death} and k_{stasis}) in simulation of drug effect in a population of cells. **(C)** Model outputs showing variations in the fraction of cells affected (f_a) at $t = 96$ h corresponding to phenotype rate constant values shown in (B). f_a may be calculated in three different ways based on bulk response metrics such as

relative viability and net growth rate inhibition (GR and DIP) following normalization to an untreated control. **(D)** Simulation results comparing f_a quantities at $t = 96$ h with probabilistic measures of drug action, P_{death} quantified per unit of time (h) and conditional probability P_{stasis} , and the overall probability with which a drug induces cell death or inhibits cell division ($P_{death \cup stasis}$) across a variety of conditions, representing drugs with different levels of cytotoxic and cytostatic effect. Each data-point represents the mean of 30 stochastic simulations. Cells grow from an initial number of $N_{live}(t=0) = 1000$ and at a rate of $k_{division (no drug)} = 0.025 \text{ h}^{-1}$.

Figure 2. Probabilistic rate constants capture time-dependent heterogeneities in phenotypic responses. **(A)** Schematic representation of drug response in a heterogeneous cell population. Prior to drug-treatment, cells consist of a dominantly drug-sensitive population plus a small fraction ($\omega \leq 5\%$) of drug-tolerant subpopulation which is r times more drug-resistant than the majority of cells. Upon drug treatment, the drug-tolerant subpopulation is gradually enriched over time. **(B-C)** Analysis of our ability to distinguish the presence of drug-tolerant subpopulation based on variations in resistance enrichment ratio for different drug response metrics with time, ω (B) and r (C). We defined and calculated resistance enrichment ratio for each of the f_a metrics (described based on viability, GR and DIP) or for phenotype rate constants (k_{death} and k_{stasis}) by normalizing each metric measured for a heterogeneous population to that in a homogeneous population (i.e. $\omega = 0$ or $r = 0$) at different times of treatment. Smaller resistance enrichment ratios represent greater ability to distinguish heterogeneous drug-tolerant subpopulations. Data shown are mean values from 50 simulations.

Figure 3. Dynamic analysis of heterogeneous drug response using estimates of probabilistic phenotype rate constants from time-lapse live cell microscopy. **(A-B)** Dynamics of (A) COLO858

and (B) MMACSF cell responses to BRAF inhibitor Vemurafenib across 6 doses (0-3.2 μM). Time- and dose-dependent changes in live cell count and estimates of k_{death} , $k_{division}$ and k_{stasis} for time intervals of $\Delta t = 12$ h are shown. Experimental data for Vemurafenib concentrations of 0 and 0.032 μM are shown until 48 h, that is when cells reached confluency under these conditions. Data are shown as mean \pm SEM across four replicates. (C) Single-cell profiles of COLO858 response to Vemurafenib depicted based on manual tracking of individual cells exposed to different concentrations of Vemurafenib as described in (A). Each cell track is presented horizontally along time axis. Division events are marked as red circles. Transition from white to black represents a cell death event. Times at which cells spend out of field of view are shown in light green. (D) Single-cell profiles of COLO858 response to Vemurafenib simulated based on *Poisson* processes using rate parameters estimated from COLO858 experimental data along 12 h time intervals. (E) Comparison of normalized distributions of division and death events along 12 h time intervals between experiments performed in COLO858 cells and the simulated responses for the same conditions. Experimental data-points represent pooled data from all four replicates. Simulated data-points represent mean \pm SEM across 30 simulations.

Figure 4. Probabilistic phenotype metrics, but not f_a based metrics, reveal statistical independence

in drug combination efficacies. (A) Simulation results shown for the effect of two independent and purely cytostatic drugs, A and B, with identical dose-effect profiles used individually and in combination. Dose-effect profiles of drugs A and B are shown as P_{death} and P_{stasis} , quantified per unit of time (h). Normalized changes in relative viability and growth rate (GR) inhibition are reported for each condition at 48 h and the predicted combination effects are shown for scenarios where either the probabilistic metric P_{death} and P_{stasis} or f_a quantities (based on viability and GR) were used in the evaluation of Bliss independence. (B) Bliss combination index values calculated (at $t = 48$ h) using

different drug response metrics, f_a (viability), f_a (GR) and P_{stasis} , in simulations of combined effects of two independent and identical drugs with variable cytostatic effects represented by variations in P_{stasis} .

The rate of cell division in the absence of drug was simulated as $k_{division (no drug)} = 0.035 \text{ h}^{-1}$. (C)

Simulation results quantifying Bliss combination index values (at $t = 48 \text{ h}$) calculated using f_a response metrics, f_a (viability) and f_a (GR), in comparison with probabilistic combination index values (CI_{death} and CI_{stasis}). Each data-point represents the mean of 10 simulations for a drug combination condition with a given set of probabilistic drug interaction condition quantified as CI_{death} and CI_{stasis} . Conditions where $CI_{death} = CI_{stasis}$ are highlighted in yellow. Representative simulations were performed using an initial live cell number of $N_{live} (t = 0) = 2000$, $k_{division (no drug)} = 0.035 \text{ h}^{-1}$, $k_{death (drug A)} = k_{death (drug B)} = 0.01 \text{ h}^{-1}$, $P_{stasis (drug A)} = P_{stasis (drug B)} = 0.2$.

Figure 5. Probabilistic phenotype metrics uncover target-specific differences in drug combination efficacies and their interactions. (A) Unsupervised clustering of Bliss combination index values

(CI_{death} and CI_{stasis}) calculated using probabilistic metrics k_{death} and k_{stasis} in COLO858 and MMACSF cells between 48-120 h of exposure to various drugs in sequential combination with Vemurafenib and Trametinib. Cells were treated initially for 24 h with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors (3rd compound) at the following concentrations: Fimepinostat (0.02 μM), Givinostat (0.2 μM), Birabresib (0.5 μM), I-BET762 (1 μM), SP2509 (1 μM), ORY-1001 (1 μM), JIB-04 (0.2 μM), CPI-455 (5 μM), AZ6102 (1 μM), NVP-TNKS656 (1 μM), Palbociclib (1 μM), and Abemaciclib (1 μM). Nominal targets of compounds are highlighted. After 24 h, Vemurafenib at 0.3 μM plus Trametinib at 0.03 μM , or DMSO control were added to each treatment condition. Combination index data-points represent mean values across 2-3 replicates. NaN data-points represent conditions where the effect of drug combination or that of the independence model are within measurement error,

making the ratio (combination index) unreliable. **(B)** Estimated dynamics of changes in live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for COLO858 and MMACSF cell responses to the combination of Vemurafenib (0.32 μM) and Trametinib (0.032 μM), BET bromodomain inhibitor I-BET762 (1 μM), their triple combination, or vehicle (DMSO) control. **(C)** Estimated dynamics of changes in live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for MMACSF cell responses to the combination of Vemurafenib (0.32 μM) and Trametinib (0.032 μM), CDK4/6 inhibitors Palbociclib (1 μM) and Abemaciclib (1 μM), their triple combination, or vehicle (DMSO) control. $k_{division}$ (no drug) used for the estimation of k_{stasis} for each cell line was estimated using cell division data averaged for the first 24 h in cells treated with DMSO only. In conditions where confluency was achieved, data-points were replaced with the last available data-point (dotted line). Data-points represent mean \pm SEM across 2 or 3 replicates.

Supplementary Figure Legends

Figure S1. Overview of the time-lapse image analysis pipeline to quantify occurrence of single-cell phenotypic events from time-lapse live cell microscopy data. The automated image analysis pipeline involves four steps: (1) Each background (BG) subtracted H2B image was segmented in CellProfiler for nucleus identification. For each nucleus object, a variety of features (e.g. mean signal intensities across multiple channels, area and shape) were measured. (2) To classify the phenotypes of interest (i.e. live or dead cells, Geminin^{high} or Geminin^{low} cells) in each image, classification models were trained in CellProfiler Analyst based on feature measurements of the user-annotated training sets. (3) Based on phenotype classifications of individual cells for each image output from CellProfiler, corresponding synthetic images were generated in MATLAB for each phenotype of interest. Synthetic images contained synthetic pixels at locations of Geminin^{high} or dead cells. To facilitate tracking of individual

cells, relative intensities of the synthetic pixels for each phenotype were scaled with the mean intensity of the signal associated with that phenotype. For example, intensities of death synthetic pixels were scaled with the mean H2B signal intensities of individual cells, whereas intensities of the Geminin^{high} synthetic pixels were scaled with the mean Geminin signal intensities. (4) Synthetic pixels for each phenotype were tracked separately in TrackMate. Since Geminin level drops at the M phase, a division event is marked when the Geminin track ends. The beginning of a death track is also marked as a death event.

Figure S2. Probabilistic rate constants of phenotypic events measured using automated tracking is consistent with the rate constants acquired from manual single-cell tracking across different cell lines and drug conditions. (A-B) Probabilistic rate constants of death (k_{death}) and division events ($k_{division}$) measured in (A) COLO858 and (B) MMACSF cells treated with Vemurafenib at the indicated doses, using automated tracking analysis pipeline (top row) versus manual tracking (bottom row) on the same set of time-lapse images. For each condition, the automated tracking estimates at each timepoint are the mean values across four replicated wells. Error bars represent SEM. The rate constants calculated from manual tracking data are based on individually tracked cells pooled from four replicated wells, including about 150-220 cells per condition.

Figure S3. Dynamic responses of COLO858 cells to epigenetic-modifying compounds and cell cycle inhibitors in sequential combination with Vemurafenib plus Trametinib. Estimated dynamics of changes in live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for COLO858 cell responses to the combination of Vemurafenib (0.32 μ M) and Trametinib (0.032 μ M), a 3rd compound (including epigenetic-modifying compounds or cell cycle inhibitors), their triple

combination, or vehicle (DMSO) control. Cells were treated initially for 24 h with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors (3rd compound) at the following concentrations: Fimepinostat (0.02 μM), Givinostat (0.2 μM), Birabresib (0.5 μM), I-BET762 (1 μM), SP2509 (1 μM), ORY-1001 (1 μM), JIB-04 (0.2 μM), CPI-455 (5 μM), AZ6102 (1 μM), NVP-TNKS656 (1 μM), Palbociclib (1 μM), and Abemaciclib (1 μM). After 24 h, Vemurafenib at 0.3 μM plus Trametinib at 0.03 μM , or DMSO control were added to each treatment condition. $k_{division}$ (no drug) used for the estimation of k_{stasis} is estimated using cell division data averaged for the first 24 h in cells treated with DMSO only. In conditions where confluency was achieved, data-points were replaced with the last available data-point (dotted line). Data-points represent mean \pm SEM across 2 or 3 replicates.

Figure S4. Dynamic responses of MMACSF cells to epigenetic-modifying compounds and cell cycle inhibitors in sequential combination with Vemurafenib plus Trametinib. Estimated dynamics of changes in live cell count, k_{death} , k_{stasis} and $k_{division}$ measured from time-lapse live cell microscopy data for MMACSF cell responses to the combination of Vemurafenib (0.32 μM) and Trametinib (0.032 μM), a 3rd compound (including epigenetic-modifying compounds or cell cycle inhibitors), their triple combination, or vehicle (DMSO) control. Cells were treated initially for 24 h with DMSO control or one of the epigenetic-modifying compounds or cell cycle inhibitors (3rd compound) at the following concentrations: Fimepinostat (0.02 μM), Givinostat (0.2 μM), Birabresib (0.5 μM), I-BET762 (1 μM), SP2509 (1 μM), ORY-1001 (1 μM), JIB-04 (0.2 μM), CPI-455 (5 μM), AZ6102 (1 μM), NVP-TNKS656 (1 μM), Palbociclib (1 μM), and Abemaciclib (1 μM). After 24 h, Vemurafenib at 0.3 μM plus Trametinib at 0.03 μM , or DMSO control were added to each treatment condition. $k_{division}$ (no drug) used for the estimation of k_{stasis} is estimated using cell division data averaged for the first 24 h in cells treated with DMSO only. In conditions where confluency was achieved, data-points were replaced with

the last available data-point (dotted line). Data-points represent mean \pm SEM across 2 or 3 replicates.

References

1. Greco WR, Bravo G, Parsons JC. The search for synergy: a critical review from a response surface perspective. *Pharmacol Rev.* 1995;47: 331–385.
2. Berenbaum MC. What is synergy? *Pharmacol Rev.* 1989;41: 93–141.
3. Foucquier J, Guedj M. Analysis of drug combinations: current methodological landscape. *Pharmacol Res Perspect.* 2015;3. doi:10.1002/prp2.149
4. Geary N. Understanding synergy. *Am J Physiol Endocrinol Metab.* 2013;304: E237-253. doi:10.1152/ajpendo.00308.2012
5. Hafner M, Niepel M, Chung M, Sorger PK. Growth rate inhibition metrics correct for confounders in measuring sensitivity to cancer drugs. *Nat Methods.* 2016;13: 521–527. doi:10.1038/nmeth.3853
6. Harris LA, Frick PL, Garbett SP, Hardeman KN, Paudel BB, Lopez CF, et al. An unbiased metric of antiproliferative drug effect in vitro. *Nat Methods.* 2016;13: 497–500. doi:10.1038/nmeth.3852
7. Margue C, Philippidou D, Kozar I, Cesi G, Felten P, Kulms D, et al. Kinase inhibitor library screening identifies synergistic drug combinations effective in sensitive and resistant melanoma cells. *J Exp Clin Cancer Res.* 2019;38. doi:10.1186/s13046-019-1038-x
8. Crystal AS, Shaw AT, Sequist LV, Friboulet L, Niederst MJ, Lockerman EL, et al. Patient-derived models of acquired resistance can identify effective drug combinations for cancer. *Science.* 2014;346: 1480–1486. doi:10.1126/science.1254721
9. de Lange J, Ly LV, Lodder K, Verlaan-de Vries M, Teunisse AF a. S, Jager MJ, et al. Synergistic growth inhibition based on small-molecule p53 activation as treatment for intraocular melanoma. *Oncogene.* 2012;31: 1105–1116. doi:10.1038/onc.2011.309
10. Meyer CT, Wooten DJ, Paudel BB, Bauer J, Hardeman KN, Westover D, et al. Quantifying Drug Combination Synergy along Potency and Efficacy Axes. *Cell Syst.* 2019;8: 97-108.e16. doi:10.1016/j.cels.2019.01.003
11. Yadav B, Wennerberg K, Aittokallio T, Tang J. Searching for Drug Synergy in Complex Dose–Response Landscapes Using an Interaction Potency Model. *Comput Struct Biotechnol J.* 2015;13: 504–513. doi:10.1016/j.csbj.2015.09.001
12. Bliss CI. The calculation of microbial assays. *Bacteriol Rev.* 1956;20: 243–258.
13. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984;22: 27–55.

14. Loewe S, Muischnek H. Effect of combinations: Mathematical basis of problem. *Arch Exp Pathol Pharmacol.* 1926;114: 313–326.
15. Gaddum JH. *Pharmacology.* 1st ed. Oxford University Press; 1940.
16. Bliss CI. The toxicity of poisons applied jointly. *Ann Appl Biol.* 1939;26: 585–615.
doi:10.1111/j.1744-7348.1939.tb06990.x
17. Fallahi-Sichani M, Honarnejad S, Heiser LM, Gray JW, Sorger PK. Metrics other than potency reveal systematic variation in responses to cancer drugs. *Nat Chem Biol.* 2013;9: 708–714.
doi:10.1038/nchembio.1337
18. Yang R, Niepel M, Mitchison TK, Sorger PK. Dissecting variability in responses to cancer chemotherapy through systems pharmacology. *Clin Pharmacol Ther.* 2010;88: 34–38.
doi:10.1038/clpt.2010.96
19. Azad NS, Posadas EM, Kwitkowski VE, Steinberg SM, Jain L, Annunziata CM, et al. Combination Targeted Therapy With Sorafenib and Bevacizumab Results in Enhanced Toxicity and Antitumor Activity. *J Clin Oncol.* 2008;26: 3709–3714. doi:10.1200/JCO.2007.10.8332
20. Sosman JA, Flaherty KT, Atkins MB, McDermott DF, Rothenberg ML, Vermeulen WL, et al. Updated results of phase I trial of sorafenib (S) and bevacizumab (B) in patients with metastatic renal cell cancer (mRCC). *J Clin Oncol.* 2008;26: 5011–5011.
doi:10.1200/jco.2008.26.15_suppl.5011
21. Fallahi-Sichani M, Becker V, Izar B, Baker GJ, Lin J-R, Boswell SA, et al. Adaptive resistance of melanoma cells to RAF inhibition via reversible induction of a slowly dividing de-differentiated state. *Mol Syst Biol.* 2017;13: 905. doi:10.15252/msb.20166796
22. Fallahi-Sichani M, Moerke NJ, Niepel M, Zhang T, Gray NS, Sorger PK. Systematic analysis of BRAF(V600E) melanomas reveals a role for JNK/c-Jun pathway in adaptive resistance to drug-induced apoptosis. *Mol Syst Biol.* 2015;11: 797. doi:10.15252/msb.20145877
23. Jenkins JL. Drug discovery: Rethinking cellular drug response. *Nat Chem Biol.* 2013;9: 669–670.
doi:10.1038/nchembio.1365
24. Gascoigne KE, Taylor SS. Cancer cells display profound intra- and interline variation following prolonged exposure to antimetabolic drugs. *Cancer Cell.* 2008;14: 111–122.
doi:10.1016/j.ccr.2008.07.002
25. Cohen AA, Geva-Zatorsky N, Eden E, Frenkel-Morgenstern M, Issaeva I, Sigal A, et al. Dynamic proteomics of individual cancer cells in response to a drug. *Science.* 2008;322: 1511–1516.
doi:10.1126/science.1160165
26. Berenbaum MC. In vivo determination of the fractional kill of human tumor cells by chemotherapeutic agents. *Cancer Chemother Rep.* 1972;56: 563–571.

27. Emmons MF, Faião-Flores F, Smalley KSM. The role of phenotypic plasticity in the escape of cancer cells from targeted therapy. *Biochem Pharmacol.* 2016;122: 1–9. doi:10.1016/j.bcp.2016.06.014
28. Kochanowski K, Morinishi L, Altschuler S, Wu L. Drug persistence - from antibiotics to cancer therapies. *Curr Opin Syst Biol.* 2018;10: 1–8. doi:10.1016/j.coisb.2018.03.003
29. Hangauer MJ, Viswanathan VS, Ryan MJ, Bole D, Eaton JK, Matov A, et al. Drug-tolerant persister cancer cells are vulnerable to GPX4 inhibition. *Nature.* 2017;551: 247–250. doi:10.1038/nature24297
30. Shaffer SM, Dunagin MC, Torborg SR, Torre EA, Emert B, Krepler C, et al. Rare cell variability and drug-induced reprogramming as a mode of cancer drug resistance. *Nature.* 2017;546: 431–435. doi:10.1038/nature22794
31. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell.* 2010;141: 69–80. doi:10.1016/j.cell.2010.02.027
32. Bivona TG, Doebele RC. A framework for understanding and targeting residual disease in oncogene-driven solid cancers. *Nat Med.* 2016;22: 472–478. doi:10.1038/nm.4091
33. Wei G, Margolin AA, Haery L, Brown E, Cucolo L, Julian B, et al. Chemical Genomics Identifies Small-Molecule MCL1 Repressors and BCL-xL as a Predictor of MCL1 Dependency. *Cancer Cell.* 2012;21: 547–562. doi:10.1016/j.ccr.2012.02.028
34. Ramirez M, Rajaram S, Steininger RJ, Osipchuk D, Roth MA, Morinishi LS, et al. Diverse drug-resistance mechanisms can emerge from drug-tolerant cancer persister cells. *Nat Commun.* 2016;7: 10690. doi:10.1038/ncomms10690
35. Rambow F, Rogiers A, Marin-Bejar O, Aibar S, Femel J, Dewaele M, et al. Toward Minimal Residual Disease-Directed Therapy in Melanoma. *Cell.* 2018;174: 843-855.e19. doi:10.1016/j.cell.2018.06.025
36. Oxnard GR. The cellular origins of drug resistance in cancer. *Nat Med.* 2016;22: 232–234. doi:10.1038/nm.4058
37. Tyson DR, Garbett SP, Frick PL, Quaranta V. Fractional proliferation: a method to deconvolve cell population dynamics from single-cell data. *Nat Methods.* 2012;9: 923–928. doi:10.1038/nmeth.2138
38. Palmer AC, Sorger PK. Combination Cancer Therapy Can Confer Benefit via Patient-to-Patient Variability without Drug Additivity or Synergy. *Cell.* 2017;171: 1678-1691.e13. doi:10.1016/j.cell.2017.11.009
39. Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, et al. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell.* 2008;132: 487–498. doi:10.1016/j.cell.2007.12.033

40. Reyes J, Chen J-Y, Stewart-Ornstein J, Karhohs KW, Mock CS, Lahav G. Fluctuations in p53 Signaling Allow Escape from Cell-Cycle Arrest. *Mol Cell*. 2018;71: 581-591.e5. doi:10.1016/j.molcel.2018.06.031
41. Smalley KSM, Lioni M, Dalla Palma M, Xiao M, Desai B, Egyhazi S, et al. Increased cyclin D1 expression can mediate BRAF inhibitor resistance in BRAF V600E-mutated melanomas. *Mol Cancer Ther*. 2008;7: 2876–2883. doi:10.1158/1535-7163.MCT-08-0431
42. Roesch A, Vultur A, Bogeski I, Wang H, Zimmermann KM, Speicher D, et al. Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B(high) cells. *Cancer Cell*. 2013;23: 811–825. doi:10.1016/j.ccr.2013.05.003
43. Emmons MF, Faião-Flores F, Sharma R, Thapa R, Messina JL, Becker JC, et al. HDAC8 Regulates a Stress Response Pathway in Melanoma to Mediate Escape from BRAF Inhibitor Therapy. *Cancer Res*. 2019;79: 2947–2961. doi:10.1158/0008-5472.CAN-19-0040
44. Maertens O, Kuzmickas R, Manchester HE, Emerson CE, Gavin AG, Guild CJ, et al. MAPK Pathway Suppression Unmasks Latent DNA Repair Defects and Confers a Chemical Synthetic Vulnerability in BRAF-, NRAS-, and NF1-Mutant Melanomas. *Cancer Discov*. 2019;9: 526–545. doi:10.1158/2159-8290.CD-18-0879
45. Wang L, Leite de Oliveira R, Huijberts S, Bosdriesz E, Pencheva N, Brunen D, et al. An Acquired Vulnerability of Drug-Resistant Melanoma with Therapeutic Potential. *Cell*. 2018;173: 1413-1425.e14. doi:10.1016/j.cell.2018.04.012
46. Echevarría-Vargas IM, Reyes-Urbe PI, Guterres AN, Yin X, Kossenkov AV, Liu Q, et al. Co-targeting BET and MEK as salvage therapy for MAPK and checkpoint inhibitor-resistant melanoma. *EMBO Mol Med*. 2018;10. doi:10.15252/emmm.201708446
47. Yu Y, Schleich K, Yue B, Ji S, Lohneis P, Kemper K, et al. Targeting the Senescence-Overriding Cooperative Activity of Structurally Unrelated H3K9 Demethylases in Melanoma. *Cancer Cell*. 2018;33: 322-336.e8. doi:10.1016/j.ccell.2018.01.002
48. Su Y, Lu X, Li G, Liu C, Kong Y, Lee JW, et al. Kinetic Inference Resolves Epigenetic Mechanism of Drug Resistance in Melanoma [Internet]. *Cancer Biology*; 2019 Aug. doi:10.1101/724740
49. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9: 676–682. doi:10.1038/nmeth.2019
50. Jones TR, Carpenter AE, Lamprecht MR, Moffat J, Silver SJ, Grenier JK, et al. Scoring diverse cellular morphologies in image-based screens with iterative feedback and machine learning. *Proc Natl Acad Sci*. 2009;106: 1826–1831. doi:10.1073/pnas.0808843106
51. Tinevez JY. TrackMate: An open and extensible platform for single-particle tracking. [Internet]. 2016. Available: <http://www.sciencedirect.com/science/article/pii/S1046202316303346>

52. Roell KR, Reif DM, Motsinger-Reif AA. An Introduction to Terminology and Methodology of Chemical Synergy-Perspectives from Across Disciplines. *Front Pharmacol.* 2017;8: 158.
doi:10.3389/fphar.2017.00158

Figure 1

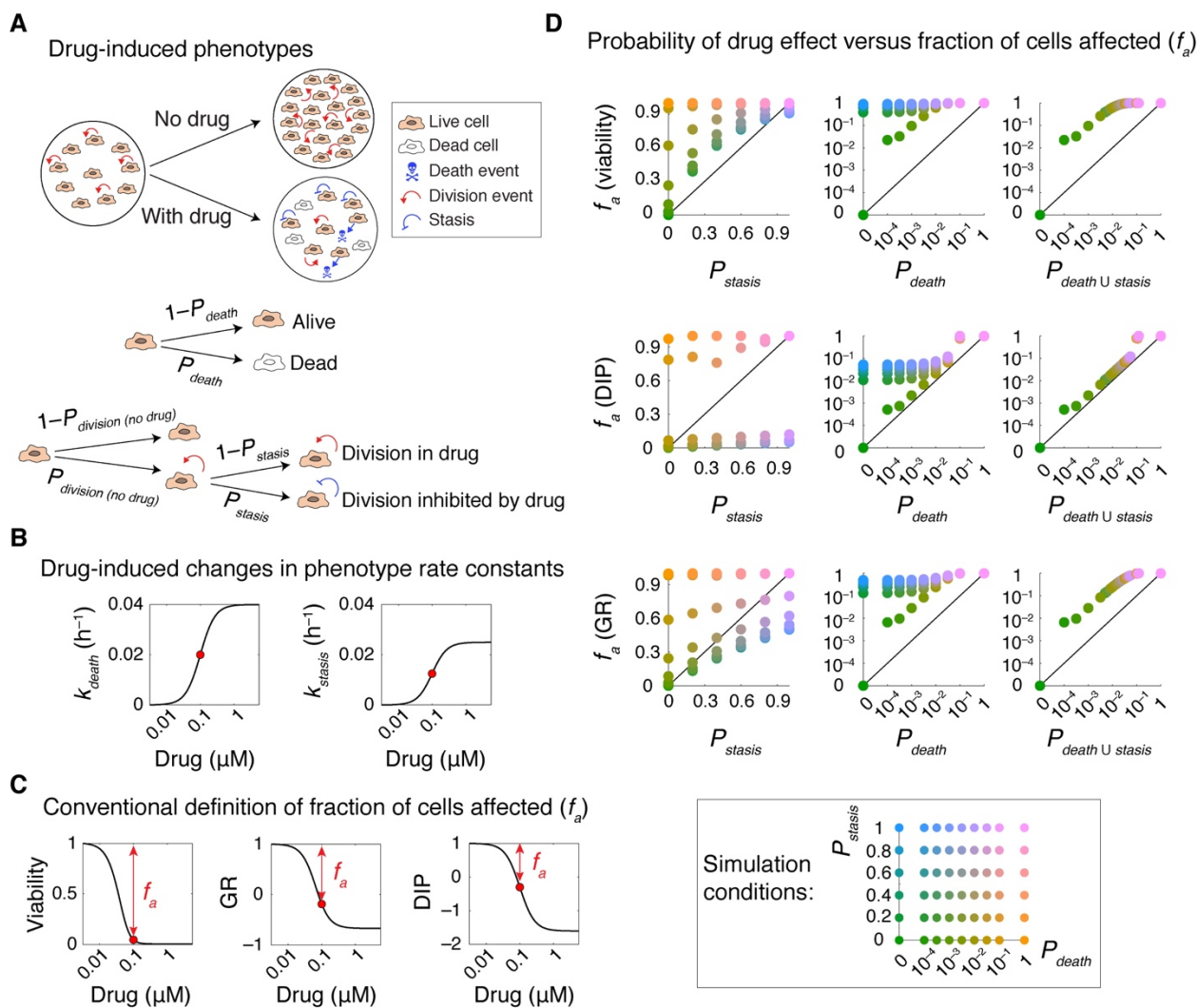


Figure 2

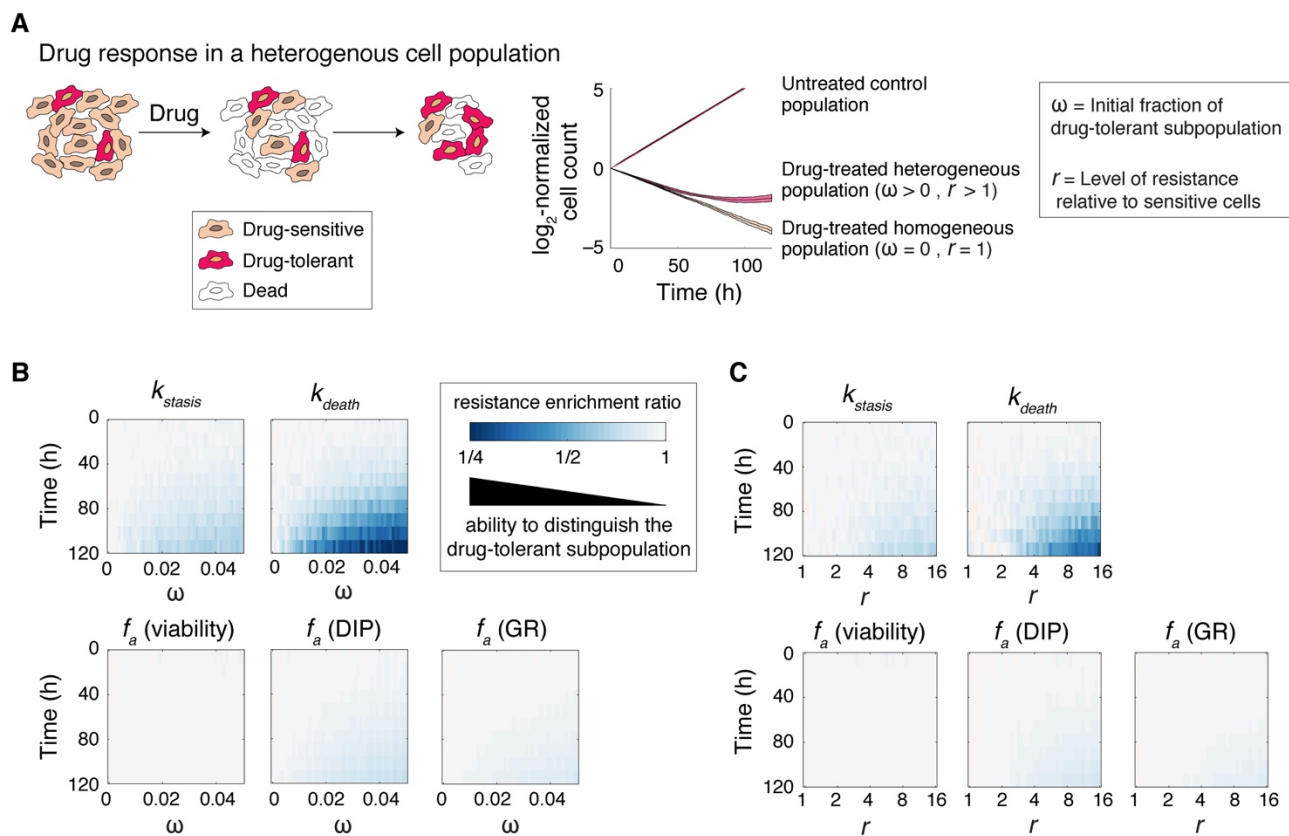


Figure 3

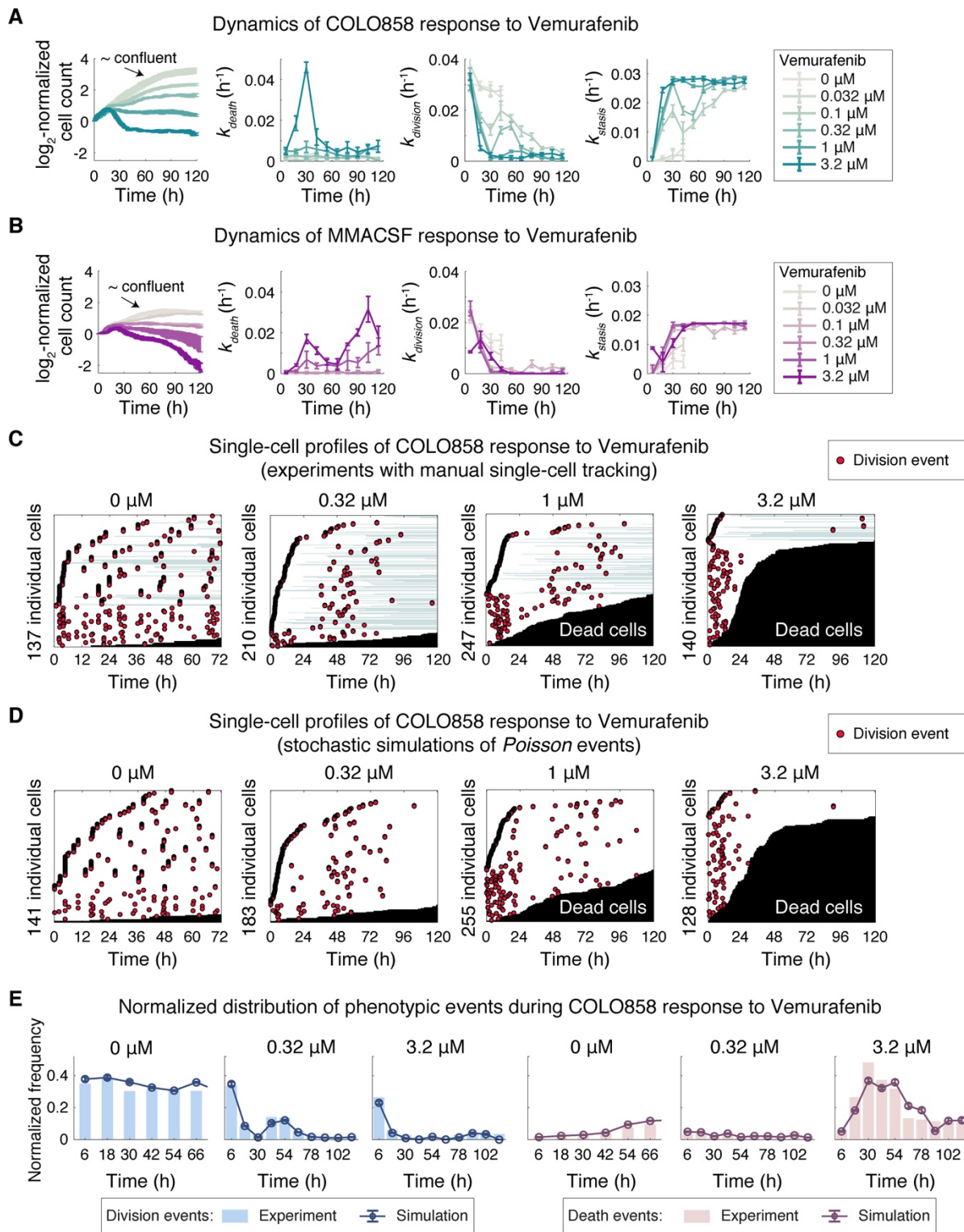


Figure 4

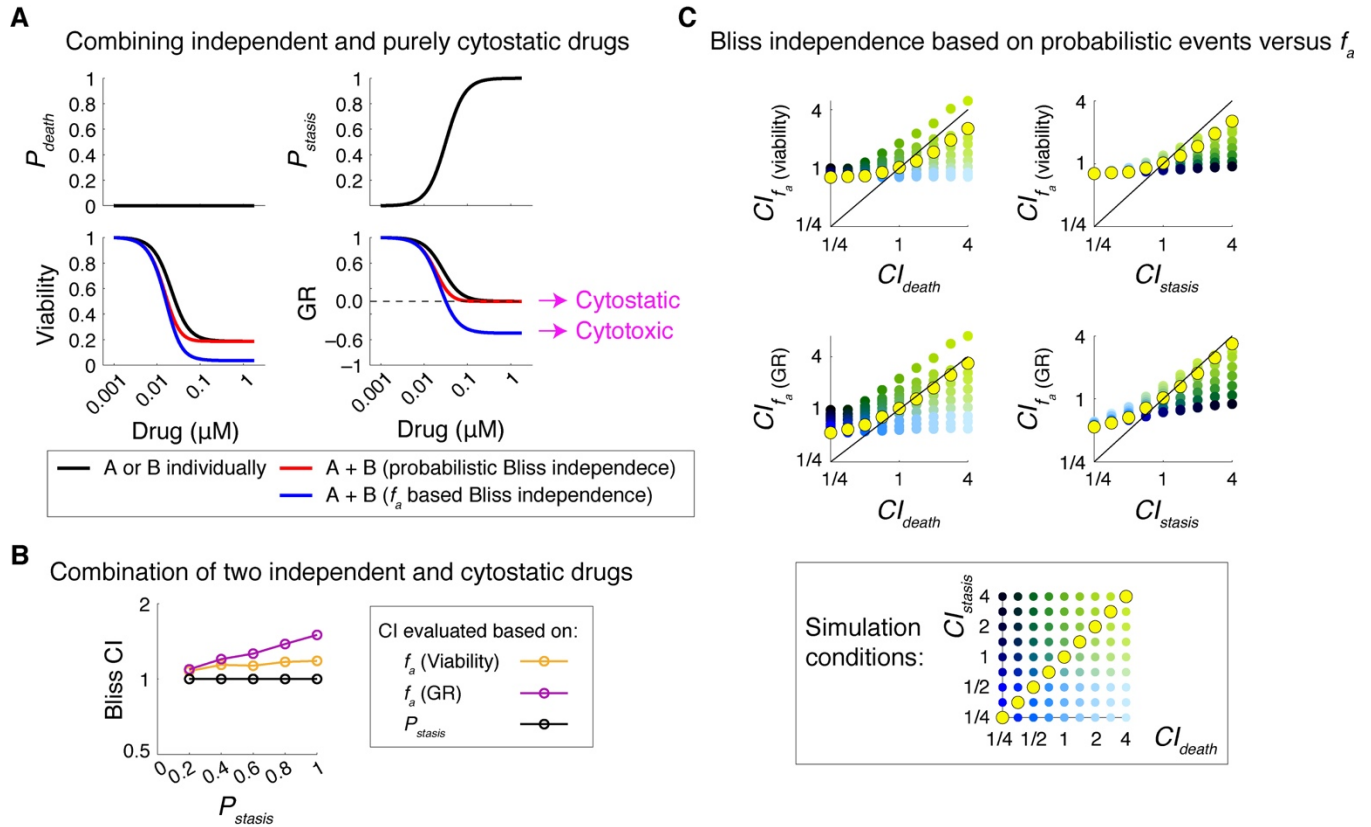


Figure 5

