

# Dissecting Variability in Responses to Cancer Chemotherapy Through Systems Pharmacology

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Variability in patient responses to even the most potent and targeted therapeutics is now the primary challenge facing drug discovery and patient care, particularly in oncology and immune therapy. Variability with respect to mechanisms of induced resistance is observed both in drug-naïve patients and among those who are initially responsive. Genomics has developed powerful tools for systematic interrogation of disease genotype and transcriptional states (particularly in cancer) and for correlation of these measures with parameters of disease such as histological diagnosis and outcome. In contrast, mechanistic preclinical studies remain relatively narrowly focused, leading to many apparent contradictions and poor understanding of the determinants of response. We describe the emergence of a systems pharmacology approach that is mechanistic, quantitative, probabilistic, and postgenomic and promises to do for mechanistic pharmacology what genomics is doing for correlative studies. We focus on studies in cell lines (which currently dominate mechanism-oriented analysis), but our arguments are equally valid for real tumors studied in short-term culture as xenografts and, perhaps some time in the future, in humans.

## INTRODUCTION

Systems biology has emerged in the past decade as a means to analyze complex biological networks through a combination of high-throughput experimentation and computational analysis. In biomedical research, the dominant empirical approach of molecular biology has long coexisted with a much smaller effort in theoretical biology. In contrast, sustained attempts to combine experimental data with explanatory or predictive mathematical models—the essence of systems biology—are relatively new. The spirit of the effort would nonetheless have been familiar to the founders of modern pharmacology. Despite the marked immaturity of the tools and concepts available for effective systems-level interrogation of cells and tissues, this review argues for the development of a systems pharmacology that fuses systematic data collection with careful mathematical modeling of pharmacological problems. We restrict the discussion to the study and treatment of cancer, but most of our observations are relevant to other disease areas.

Classic pharmacology concerns itself with drug action at the cell and tissue levels, with particular emphasis on identifying targets and understanding drug–target interaction. The effect of variation in chemical structure on drug activity, namely, structure–activity relationships, lies at the heart of medicinal chemistry and the practical science of developing new medicines. However, interindividual variations in drug responses

have largely been the concern of pharmacogenomics, a branch of “personalized medicine” that has flourished in the genomic era. Pharmacogenomics has sought to explain, for example, patient-to-patient variation in drug metabolism based on polymorphisms in genes encoding cytochrome p450. In many diseases, variation in drug response at the level of the individual patient is a matter for consideration but not necessarily the dominant concern. In cancer pharmacology, however, with large interpatient variability being observed for virtually all targeted and cytotoxic agents, even with drug-naïve tumors, such variability attains great importance. This presumably reflects the combined effects of the genetic heterogeneity of tumors and normal human polymorphism. Given that the origins of variation are largely unknown and appear to be patient specific, they cannot easily be overcome through combination therapy or differences in dosing—at least not without patient-specific rationales, which do not yet exist for most drugs or diseases.

## CORRELATIVE AND MECHANISTIC APPROACHES

Approaches to understanding variations in drug response can be classified broadly as either correlative or mechanistic. The basic idea of correlative approaches is to match patients with drugs empirically, and to correlate responses with measurable parameters of disease such as histological diagnosis and with clinical factors such as family history, tumor size, and lymph node

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metastasis. Recently, and increasingly successfully, genomic profiling of tumors has been used to generate expression signatures (reviewed in refs. 1,2) and cancer genotypes (refs. 3,4) that are predictive of disease progression and drug response. In some cases, genomic data provide important mechanistic insight. Famously, epidermal growth factor receptors carrying mutant kinase domains (e.g., ErbB1-L858R) have been shown to have higher affinity for kinase inhibitors such as gefitinib,<sup>5</sup> thereby explaining the remarkable sensitivity of some lung cancers to treatment. Despite this, the application of genomics to pharmacology remains largely correlative, often involving the use of expression signatures.

Mechanistic studies of drug response seek a detailed understanding of interactions between drugs and their targets, the consequences of binding for downstream proteins, and, ultimately, the impact on cell fate. Such studies are heavily dependent on analysis of cell lines grown *in vitro*, but advances in intravital imaging<sup>6</sup> and *in situ* analysis of tumors<sup>7</sup> suggest that mechanistic studies will soon be possible in more sophisticated settings. It has long been known as exemplified by the NCI60 anticancer drug screen project, that cell lines exhibit up to 1,000-fold differences in the concentration of a drug required for 50% target inhibition (IC<sub>50</sub>; see data at NCI website).<sup>8</sup> Such differences have been studied extensively in the expectation that the origins of these clinical variations would become clear. Unfortunately, this expectation remains largely unfulfilled.

Given these failures, we should recall that some sources of variation in drug responses—such as the aforementioned epidermal growth factor receptor mutations—do translate faithfully from cell culture to the clinic.<sup>9</sup> Even in this case, however, the precise biochemical mechanisms that determine drug sensitivity remain obscure. Recent studies have demonstrated that epidermal growth factor receptor mutation is not the only factor in making cells highly responsive to kinase inhibitors; changes in the levels of myriad interacting proteins, including other cell surface receptors, are also involved.<sup>10,11</sup> Indeed, from the perspective of a cell biologist, it is remarkable how poor our understanding is of the consequences of drug–target interaction, even in cell culture.

### INTEGRATIVE ANALYSIS OF DRUG RESPONSES

In our view, one obstacle to identifying the causes of variability in drug response is the disjointed nature of measurements typically made during preclinical studies; a second is the informed, nonquantitative nature of most biochemical modeling. In contrast, genomicists routinely employ large-scale multidimensional analyses to identify informative correlations.<sup>12</sup> It is now time to undertake mechanistic studies that are quantitative, systematic, and computation-savvy in equal measure. The net result of such a systems pharmacology approach would be an integrated landscape of genotype–phenotype relationships in which banks of tumor cell lines would be studied for insight into the diversity of response mechanisms. Such a landscape would be constructed using single-cell methods (multifactorial imaging and flow cytometry, for example) and would therefore contain information on cell-to-cell variability and on differences in the

responsiveness of bulk populations as compared with specific subpopulations (e.g., tumor stem cells). In this view, we would not expect a one-to-one correspondence between real tumors and tumor cell lines, but across a landscape of responses we should nonetheless uncover many determinants of response (there is evidence for this from expression profiling and sequencing<sup>13,14</sup>). Translation of these findings from cell culture to humans would then involve working out (using biomarkers or *in vivo* assays) which of the response mechanisms are relevant to which patients or tumors.

### Getting drugs into cells

Drug companies perform careful pharmacokinetic and pharmacodynamic experiments to determine and optimize the time-dependent concentrations of compounds in circulation. However, remarkably few cell culture studies actually attempt to reproduce these time-dependent changes or even apply drugs at concentrations at or below peak plasma drug concentration. In part, this is because of the poor quality of the data detailing the actual levels of drugs achieved clinically in tumors and tumor cells. It is generally assumed that drugs get into cells simply by diffusion across the plasma membrane, but recent studies show that transmembrane transporters and carrier proteins probably play a dominant role.<sup>15</sup> To date, most of the research on the relationship between drug concentrations outside and inside cells has focused on a small subset of the 48 ATP-binding cassette transporters that can cause drug resistance *in vitro* when overexpressed,<sup>16</sup> while the roles of other transporters in efflux have been little studied.<sup>17</sup>

One approach is to develop fluorescent probes as drug surrogates, given that fluorescence measurements are readily adapted to high-throughput assays.<sup>18</sup> By combining fluorescent drugs with RNA interference in multiple cell lines, it should be possible to advance our understanding of factors that determine drug concentrations in cells. However, significant technology development is required to get around the problem that pharmacological properties of drugs are often dramatically altered by linking them to fluors.<sup>19</sup> Intravital imaging of drug responses in mice or rats is now becoming feasible and, coupled with suitably labeled compounds, should reveal how tumor vasculature, geometry, and genotype affect drug delivery to tumor cells. By combing through these data, it will be possible to construct data-driven, multiscale mathematical models that relate drug dosing in patients to drug concentrations in the cytosol of tumor cells (of varying genotypes). Modern microfluidic devices can then recreate relevant temporal and spatial gradients across tumors for careful mechanistic analysis *in vitro*.<sup>20</sup>

### Consequences of target inhibition in the context of a cellular pathway

The majority of modern drug discovery focuses on identifying compounds that interact with targets in isolation. However, target proteins are invariably parts of complex pathways containing many other components, and there is rarely a direct correlation between the levels of the target and the consequences of its inhibition for downstream biochemistry.<sup>21</sup> In many cases,

proteins in parallel pathways take over or stress responses are induced, such that the cellular effects of target inhibition are mutated. Thus, potent monotherapies such as imatinib for chronic myeloid leukemia appear to be the exception rather than the rule.<sup>22</sup> However, understanding precisely how complex cellular networks respond to drugs at a mechanistic level is remarkably challenging, and most studies to date have been tightly focused. In fact, many arrive at seemingly contradictory conclusions—the controversy about whether epidermal growth factor receptor amplification is positively correlated with gefitinib sensitivity highlights this point.<sup>23,24</sup> This is an area in which systems biology clearly has a role because integrated, quantitative understanding of gene and protein networks is a fundamental goal of the field.<sup>25</sup> Some preclinical successes are already evident, including MM-121, a therapeutic antibody against the ErbB3 receptor whose development followed careful computational analysis of signaling pathways in tumor cells.<sup>26</sup> However, the experimental and computational tools required for dissecting the biochemistry of signaling networks (where most drug targets are found) are significantly less mature than those for gene regulatory networks (whose components have been less druggable). Therefore, significant effort will be required to advance network-oriented analysis of drug mechanisms.

**The role of the microenvironment**

Interactions between tumor and stromal cells are known to play major roles in the development of cancer,<sup>27</sup> and recent studies also demonstrate a role for the microenvironment in drug responses.<sup>28</sup> For example, the potency of trastuzumab changes dramatically when tumor cells are grown in 2D culture or 3D matrix<sup>29</sup> and when cells are plated on different extracellular matrices.<sup>30</sup> Even though many tumor cells can be induced to die *in vitro* when treated with chemotherapeutics, the relationship between these responses and tumor regression *in vivo* is unclear.<sup>31</sup> There is ample evidence that interaction with the immune system, specifically with natural killer cells, can lead to tumor regression.<sup>28</sup> It is now feasible to account for some of these effects through systematic experimentation *in vitro*: high-throughput 3D assays are available,<sup>32</sup> array-based methods make it possible to explore the effects of diverse extracellular matrices,<sup>33</sup> and cellular response to drugs can be assayed in the presence of diverse ligands.<sup>34</sup> Ultimately, high-throughput imaging methods will make it possible to explore interactions between tumor cells and immune cells, and the results from such studies should be superior to those of any mechanistic studies performed so far.

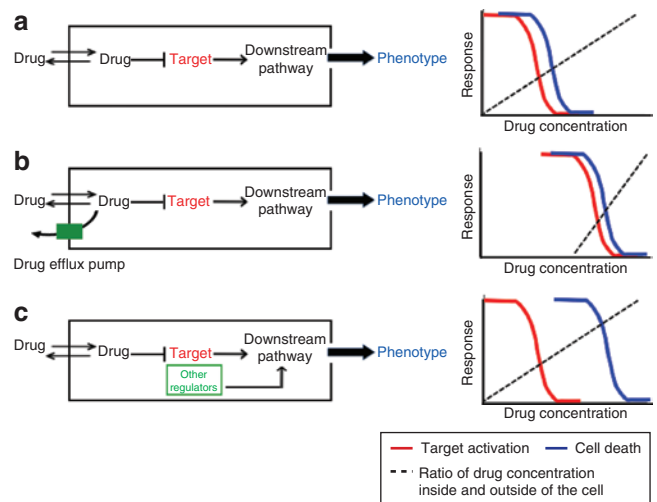
**The impact of cell-to-cell variability**

Because of its reliance on population averages, traditional cancer pharmacology has addressed variance only between cells from genetically different cell populations. However, recent and potentially significant discoveries from careful single-cell studies *in vitro* have shown that even genetically identical cells can vary dramatically in phenotype as a consequence of stochastic biochemical processes that mediate protein expression.<sup>35–39</sup> Such cell-to-cell variation has the potential to impact responses to drugs in patients

and is an attractive explanation for the ubiquity of fractional killing by cancer chemotherapy (see ref. 40 for a recent review of this topic). In actual tumors, nongenetic sources of variation within clonal cell populations will converge with variations arising from genetic changes that occur over the course of tumor evolution, and with variations arising from differences in micro-environment. Recently, much of the variability in the responses of tumor cells to drugs has been ascribed to the presence of cancer stem cells (reviewed in refs. 41,42). We now need to apply single-cell assays and quantitative modeling to determine which of the observed phenomena arise from biochemical processes that are inevitably stochastic (the nongenetic variability discussed above), reflecting the rapid emergence of drug resistant subpopulations through epigenetic effects,<sup>43</sup> and which ones are explained by the presence of special subpopulations of primitive stem-like cells. Resolving this question is likely to have a fundamental impact on our choice of which drugs to explore therapeutically.

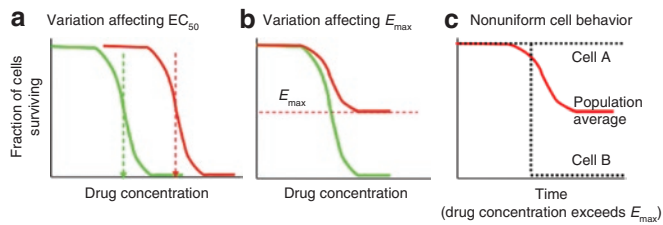
**MOVING BEYOND IC<sub>50</sub>: DEVELOPING MULTIFACTORIAL PHARMACOLOGICAL RESPONSE SIGNATURES**

For many years, the efficacy of cancer drugs in cell culture has been measured via inhibition of cell growth or induction of cell death (the ubiquitous GI50 assay). This assay is inexpensive and



**Figure 1** Different types of variation in drug response affect dose–response curves differently. The panels on the left illustrate a simple drug response pathway in cancer cells. The drug crosses the cell membrane via transporters and inhibits its oncogenic target, leading to alteration of the downstream pathways and ultimately affecting the cell phenotype. A real example of such a pathway is inhibition of the Bcl-Abl kinase by imatinib.<sup>22</sup> The panels on the right illustrate dose–response curves for three measured parameters: the ratio of drug concentrations inside and outside the cell (dotted lines), the fraction of activated targets (red lines), and cell fate response (blue lines). (a) Cancer cell in which the drug is effective. (b) Cancer cell that overexpresses a drug efflux pump for which the drug is a substrate, such as ABCG2. Dose–response for both target activation and phenotypic readouts are shifted to the right, and the cell is drug resistant. This cell could be sensitive to co-drugging with an ABCG2 inhibitor. (c) Cancer cell that overexpresses another oncogenic target, such as the *Bcl2* oncogene, acting downstream of the original target. The dose–response for target activation is unaffected, whereas the dose–response for phenotype is shifted to the right. This cell could be sensitive to co-drugging with a Bcl2 inhibitor.





**Figure 2** Cell-to-cell variability in drug sensitivity. Individual cells in an isogenic population will always have slightly different biochemical properties because of stochastic fluctuation in protein levels. They may also have differences in biological state regulation, e.g., different cell cycle states. These variations at the single-cell as well as population levels will affect the perceived drug responses, measured as  $EC_{50}$ . **(a)** A standard view of drug sensitivity; two cell populations vary in  $EC_{50}$  value, as measured by population-level survival curves at a fixed time point. Green depicts a more drug-sensitive population; red depicts a more drug-resistant one. **(b)** A less commonly considered possibility, in which two cell populations differ in  $E_{max}$  for survival and not in  $EC_{50}$ . Variation in drug penetration, for example, is expected to alter only  $EC_{50}$  because increasing drug concentrations will eventually achieve maximal efficacy. The effects of variation after the drug reaches the target are less predictable. Depending on the kinetic nature of the drug response pathways, they could cause variations in  $EC_{50}$ ,  $E_{max}$ , or both. **(c)** Single-cell behavior as a function of time in an isogenic population for which  $E_{max}$  is  $\sim 50\%$  survival. In order for the whole population to show the smooth red curve illustrated in **a** and **b**, individual cells in the population must behave nonuniformly, choosing either death (cell B) or survival (cell A). In order to measure this nonuniform behavior directly, single-cell measurements such as high-content imaging and flow cytometry must be used.

can be applied to any cell line or drug, but a dose–response curve for cell survival interrogates the whole pathway and fails to capture the underlying mechanistic complexity. Variation has been documented in every step in a typical drug response pathway (Figure 1) for at least some drugs in some cancer cells. Consequently, this leads to a complex and compounding relationship between in-cell drug concentrations, target engagement, and existence of pathway redundancy. For example, mutations that affect drug uptake and efflux will presumably shift the GI50 value but not the slope of the response or the level of maximum response,  $E_{max}$  (Figure 2a). In contrast, the presence of tumor stem cells and nongenetic variability are expected to have an impact on  $E_{max}$  but not necessarily on the GI50 value (Figure 2b,c).

Real drug responses are complex, time-dependent, and probabilistic at the single-cell level. The systematic analysis we envision must therefore monitor responses at multiple time points using quantitative, single-cell assays. Integrating this data into pharmacological response signatures with the same contextual richness and systems-level information as genetic signatures will not be simple, and the development of better modeling and informatics emerges as an urgent priority. However, by linking response signatures collected *in vitro* with genomic signatures collected both *in vitro* and from patients, it should be possible to better translate preclinical insight into effective treatment.

## CONCLUSIONS

Modern drug discovery is effective at identifying small molecules and therapeutic antibodies that inhibit oncoproteins implicated in human disease. Although important cancer genes no doubt remain to be identified, and many new drugs remain to

be developed, the fundamental challenge in cancer therapy is no longer finding targets and drugging them effectively. Instead, it centers on solving three related problems: translation, variation, and resistance. Responses to specific therapies are less dramatic and specific in patients than had been hoped, often because the consequences of target inhibition for protein networks in tumor and normal cells are unknown; considerable patient-to-patient variation is observed, and methods for predicting response on the basis of pretreatment status are not particularly effective (with some dramatic exceptions<sup>44</sup>); and resistant tumors usually emerge over the course of treatment, and it is often unclear how these should be treated, or prevented in the first case.

We believe that these challenges can be addressed with a systems approach to pharmacology that is (i) mechanistic in explaining tumor cell phenotypes in terms of the detailed biology of disease genes, drug targets, and other biomolecules; (ii) quantitative in applying mass-action kinetics and other mathematical formalisms to predict the behaviors of ensembles of interacting proteins through knowledge of their individual biochemistries; (iii) probabilistic in accounting for the variability between cells with respect to drug response, with the attendant likelihood that only a fraction of tumor cells will arrest or die in response to treatment with a chemotherapeutic drug; (iv) postgenomic in analyzing diverse tumors in the light of knowledge of their genetic differences and with the possibility of applying powerful knockout/-in and RNA interference strategies to alter genotypes in preclinical studies; and (v) integrative in assuming that determinants of drug response are multifactorial, that physiological, morphological, and genetic features are important, and that multiple interacting pathways rather than single genes or proteins must be studied.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest.

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