analytical chemistry

pubs.acs.org/ac

Perspective

Analytical Challenges in Development of Chemoresistance Predictors for Precision Oncology

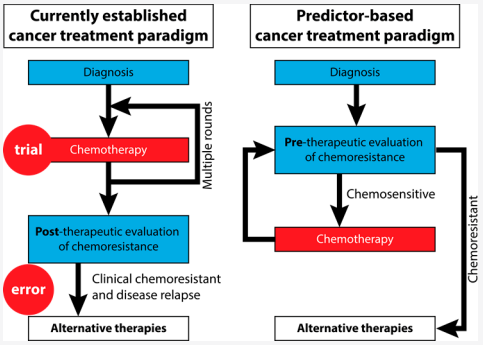
Mariana Bleker de Oliveira, Vasilij Koshkin, Geoffrey Liu, and Sergey N. Krylov*

Cite This: *Anal. Chem.* 2020, 92, 12101−12110

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Chemoresistance, i.e., tumor insensitivity to chemotherapy, shortens life expectancy of cancer patients. Despite the availability of new treatment options, initial systemic regimens for solid tumors are dominated by a set of standard chemotherapy drugs, and alternative therapies are used only when a patient has demonstrated chemoresistance clinically. Chemoresistance predictors use laboratory parameters measured on tissue samples to predict the patient's response to chemotherapy and help to avoid application of chemotherapy to chemoresistant patients. Despite thousands of publications on putative chemoresistance predictors, there are only about a dozen predictors that are sufficiently accurate for precision oncology. One of the major reasons for inaccuracy of predictors is inaccuracy of analytical methods utilized to measure their laboratory parameters: an inaccurate method leads to an inaccurate predictor. The goal of this study was to identify analytical challenges in chemoresistance-predictor development and suggest ways to overcome them. Here we describe principles of chemoresistance predictor development via correlating a clinical parameter, which manifests disease state, with a laboratory parameter. We further classify predictors based on the nature of laboratory parameters and analyze advantages and limitations of different predictors using the reliability of analytical methods utilized for measuring laboratory parameters as a criterion. Our eventual focus is on predictors with known mechanisms of reactions involved in drug resistance (drug extrusion, drug degradation, and DNA damage repair) and using rate constants of these reactions to establish accurate and robust laboratory parameters. Many aspects and conclusions of our analysis are applicable to all types of disease biomarkers built upon the correlation of clinical and laboratory parameters.



Chemotherapy, i.e., administration of cytotoxic or cytostatic agents targeting rapidly dividing cells, is the oldest and most-widely used modality of systemic cancer treatment.[1,2] The effectiveness of chemotherapy is, however, limited by chemoresistance, tumor insensitivity to cytotoxic drugs, which cause the death of the dividing cells. Chemoresistance can be pre-existing, when the tumor does not respond to the very first application of chemotherapy;[3] it can also be acquired during tumor exposure to chemotherapeutic agents.[4] Chemoresistance shortens the life expectancy of cancer patients,[5] and, eventually, may be responsible for up to 90% of cancer related deaths in the Western world.[6] This situation had been unavoidable for many decades when chemotherapy was a key component of cancer treatment, alongside radiation and surgery. However, recently, other treatments have been developed, such as immunotherapy, hormone therapy, molecularly targeted and signal transduction inhibitors, heat ablation, and cryotherapy.[7,8] Hence, selection of the most appropriate therapy matched to the right tumor, in the right patient, at the right time, known as precision oncology, has become a major focus of cancer research.

Despite all the new treatment options, initial systemic treatment regimens for solid tumors (which make up most cancers) are dominated by a set of standard chemotherapy drugs. Examples of such solid-tumor cancers are nonsmall cell lung cancer (NSCLC), which is usually treated with a combination of two drugs often including cisplatin or carboplatin, and breast cancer, which is treated with chemotherapeutics such as anthracyclines and taxanes.[9,10] Moreover, some blood cancers, such as leukemia and lymphomas, are also primarily treated with chemotherapy regimens, which include a combination of different chemotherapeutics.[11,12] Alternative chemotherapeutics, systemic immuno-targeting or novel local therapies are used when a patient has demonstrated clinical resistance to their initial chemotherapy option (Figure 1).[13,14] Confirming clinical chemoresistance in solid tumors requires multiple rounds of chemotherapy, which inevitably exposes patients to detrimental side effects of cytotoxic agents and causes a potentially critical delay of several months in the use of an alternative therapy. While standard first-line chemo-

Received: June 20, 2020
Accepted: August 13, 2020
Published: August 13, 2020

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

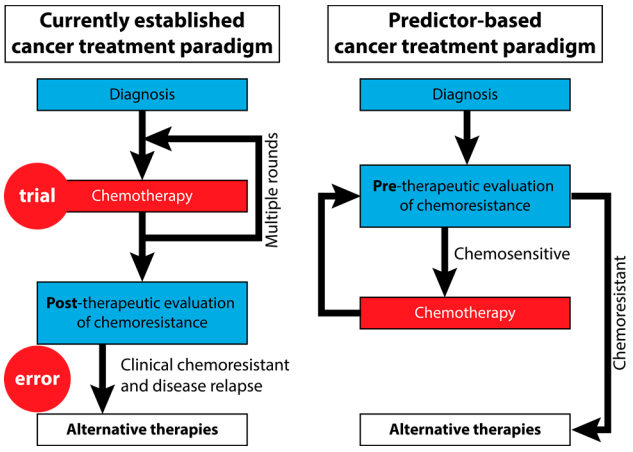


Figure 1. Flowcharts for current (trial and error) and prospective (predictor-based) cancer treatment paradigms. Blue boxes describe diagnostic and chemoresistance-evaluation steps; red boxes describe the chemotherapy steps. In the current paradigm, evaluation is done after multiple rounds of chemotherapy. In the predictor-based paradigm, evaluation is done prior to chemotherapy, preventing useless chemotherapeutic treatment of chemoresistant patients.

therapy regimens may remain the primary option for many cancers for a long time, precision oncology calls for improved methods to personalize treatments in order to improve clinical outcomes. As one treatment no longer fits all patients, every patient should be individually tested for chemoresistance before the beginning of treatment to select the optimal therapy.[15] Predictive biomarkers of cancer are being developed to guide the choice of therapies.[16] Chemoresistance predictors are predictive biomarkers which specifically predict resistance to chemotherapy;[17] other types of predictive biomarkers predict the effectiveness of other types of cancer treatments.[18]

Predictive biomarkers for guiding cancer therapy have been a research subject for decades.[19] Many tentative predictors have been suggested;[20−23] however, only a few proved to be clinically useful.[18,24−27] In total, 17 predictive biomarkers have been approved by FDA for guiding cancer therapy so far; among them 11 are chemoresistance predictors.[28] Evidently, the effectiveness of efforts aiming at the development of predictive biomarkers is alarmingly low, and there must be fundamental reasons for this.

Some important issues in the development of predictive biomarkers have been previously reviewed. Among them are the small size of study designs,[29] the lack of standardized protocols across institutions,[30] and inadequate state of technologies necessary for predictor discovery and validation.[25] Further, many predictors are reported in the form of odds or hazard ratios, without providing performance characteristics such as diagnostic sensitivity and specificity. There is, however, only limited systematic analysis of fundamental challenges in development of chemoresistance predictors and changes in approaches and practices that are needed to improve the effectiveness. This work is our attempt to provoke critical discussion on the fundamental issues of chemoresistance-predictor development. Many of these issues are common for all predictive biomarkers as well as for diagnostic and prognostic biomarkers.

Here, we describe the basic principles of chemoresistance predictor development, classify predictors based on the nature of a laboratory (lab) parameter utilized in them, and analyze advantages and limitations of different predictors. We focus on catalyst-based predictors (our term) with known mechanisms of reactions involved in drug resistance and based on kinetic lab parameters that have been proven to be accurate and robust. We would like to emphasize that developing a catalyst-based predictor requires the identification of a molecular mechanism of the catalyzed reaction.

FUNDAMENTALS OF CHEMORESISTANCE PREDICTORS

Concept of Chemoresistance Prediction. A chemoresistance predictor links a quantifiable clinical end point, serving as an indicator of clinically manifested chemoresistance, with a lab parameter measured before the treatment (see a schematic example in Figure 2). If there is a threshold

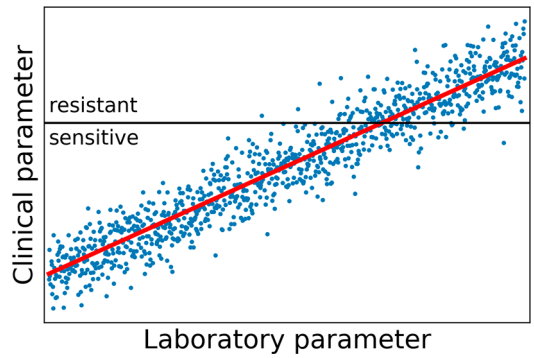


Figure 2. Schematic graphical representation of a chemoresistance predictor. A clinical parameter is a clinical end point such as time to tumor progression, while a laboratory parameter is a parameter measured on a biological sample obtained from a patient before chemotherapy is applied.

value of the clinical parameter that divides chemoresistance from chemosensitivity, then measuring the lab parameter before the treatment for individual patients can be used to predict their chemoresistance.

To establish a clinically useful predictor, two basic requirements should be satisfied: The first is biological: there must be a sufficiently strong biological association between the lab parameter and the clinical end point. The second is technical: methods used for measuring the lab parameter and quantifying the clinical end point must be reliable: precise, accurate, robust, and rugged (see Note S1 for current definitions by regulatory agencies). Importantly, a failure to satisfy the technical requirement automatically makes it impossible to know if the biological requirement is satisfied. Establishing reliable approaches for finding the two correlated parameters in Figure 2 must be step one in building a chemoresistance predictor. It is also important to keep in mind that the required performance parameters of chemoresistance predictors depend on the effectiveness of alternative therapy (see Note S2). In other words, biomarker development should only proceed if there is a very clear and unmet clinical need to guide therapy.

Analytical Performance of Methods. The requirement for precision and accuracy of methods used to determine clinical and lab parameters is self-evident. The key importance of robustness and ruggedness becomes clear when statistics is taken into consideration. The number of patients that should be used in the training and validation sets to establish a reliable

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

and accurate correlation between the lab parameter and the clinical end point in Figure 2 depends on the strength of this correlation and the quality of the analytical method. Very strong correlation accompanied by a very accurate method could allow a sample size of as few as tens of patients. However, the problem of chemoresistance predictors is that the methods are inaccurate, which precludes the assessment of the goodness of correlation. Accordingly, the required sample size may be as many as a thousand in each of the training and validation sets.[31] Even for common cancers, the required number of patients often exceeds those available in a single comprehensive cancer center. Developing reliable predictors for less common cancers will certainly require pooling together data across multiple institutions. Any systematic deviation in the lab parameter and/or the clinical end point due to changes in conditions, variability in commercial reagents and supplies, or variations in practices will result in the inability to use the data obtained in different facilities as a single set of parameters to build the correlation in Figure 2.[32]

Methods used for quantifying clinical end points have some limitations imposed by clinical practices and human nature. For example, accurately quantifying time to tumor progression requires precise timing of chemotherapy applications and clinical assessments, which is impossible due to issues associated with appointment scheduling and patient's physical fitness for treatments and assessments. To further complicate the matter, short survival may not even provide sufficient time for after-treatment assessments. Despite such limitations, these methods are considered reliable at least to develop existing clinically useful chemoresistance predictors. Based on this criterion, there are reliable methods for quantifying at least some clinical end points, e.g., progression free survival and overall survival.[20] For the purpose of this study, we assume that these methods provide a sufficient toolset for quantifying clinical end points; accordingly, we will focus solely on methods used for measuring lab parameters. Analytical methods used for measuring lab parameters are diverse, and their majority is deemed not to satisfy at least one of the necessary performance parameters (precision, accuracy, robustness, ruggedness). Therefore, our critical analysis concentrates on analytical performance of these methods. Moreover, here we use the nature of the lab parameters as a basis for predictor classification (Figure 3). The first level in this classification distinguishes between (i) predictors based on whole-tumor properties measured with *in situ* imaging and (ii) predictors based on lab parameters obtained with biochemical analyses of tissue samples or body-fluid specimens. Table S1 shows all FDA-approved predictive biomarkers of cancer, including chemoresistance predictors, and classifies them using the principles described below.

Whole-Tumor Properties. In principle, any quantifiable characteristic of a whole tumor, e.g., tumor size, levels of contrast uptake, vascularization, and oxygenation, can be used as a lab parameter to be correlated with a clinical end point in Figure 2. Such tumor characterization can be obtained with *in situ* imaging methods: ultrasound, X-ray, computed tomography, magnetic resonance imaging, and positron emission tomography.[33] Chemoresistance predictors based on *in situ* imaging have been studied in glioma,[33] and breast,[34] ovarian,[35] and rectal cancers.[36] While they can have clinical utility potentially, none of them have been proven to be clinically useful, and we do not consider them. Instead, we focus on predictors developed with biochemical analyses of tissue samples or body-fluid specimens. Such chemoresistance predictors are versatile; they constitute a larger area of research efforts,[30] and all known clinically used predictors belong to this category.

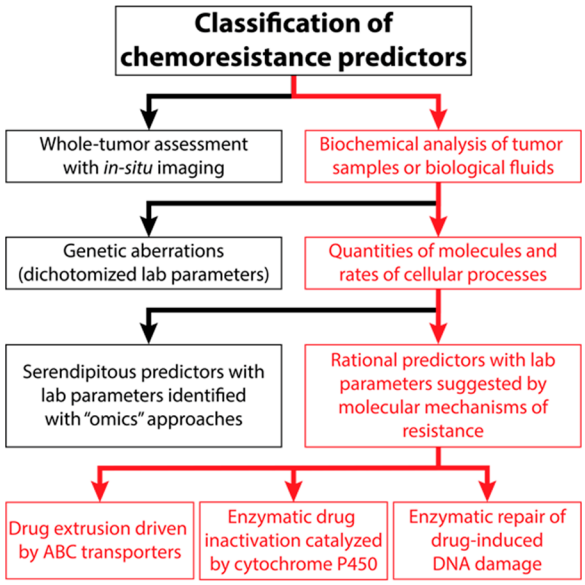


Figure 3. Classification of chemoresistance predictors emphasizing the branch of predictors based on lab parameters suggested by molecular mechanisms of chemoresistance.

Genomic Aberrations. Biochemical predictors of chemoresistance can be divided into two major categories. The first includes predictors based on genomic aberrations, e.g., mutations, single-nucleotide polymorphisms (SNPs), and chromosome deletion or translocation. Although many of these aberrations are quantifiable (e.g., the percentage of tumor cells carrying a specific mutation is measurable), such aberrations are typically listed as being either present or absent. When dichotomized in this fashion, the aberrations can be detected ruggedly with high precision, accuracy, and robustness via DNA sequencing or fluorescence *in situ* hybridization (FISH) for SNPs and chromosome abnormalities, for instance.[37,38] Dichotomization makes these predictors appear to have low uncertainty, which suggests them as methodologically ideal for the development of chemoresistance predictors. However, each of these tests has a threshold; for instance, FISH tests that indirectly measure the presence of an ALK fusion protein (which is the product of an ALK gene rearrangement) in the clinical setting utilizes a standard cutoff of 15% of cells demonstrating the ALK-fusion to be labeled ALK-fusion "present", based on initial clinical trial data, and in most cases, there is no doubt whether patients are positive or negative according to this cutoff.[39] However, there is a small proportion of cases where the cutoff is 15% ± 5% where there remains controversy over interpretation of positivity.[40] Dichotomization is also complicated by other factors such as the heterogeneity of cancerous tissues and errors in DNA sequencing associated with this heterogeneity.[41] Nonetheless, if a reasonably accurate and precise clinical end point is used along with a reasonably accurate, precise, robust, and rugged discrete lab parameter, then the presence or absence of sufficiently good correlation in Figure 2 can be interpreted unambiguously as the presence or absence of a solid biological link between the genomic aberration(s) and the clinical end point, respectively.

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

Despite the analytical advantage of discrete lab parameters, there are only a few reliable chemoresistance predictors based on genomic aberrations.[42] There are two reasons for relatively modest progress with this apparently rugged approach. The first reason is economical: the cost associated with accurate whole-genome sequencing limits the amount of available data for predictor development.[43] It is, thus, reasonable to expect that further advances in DNA sequencing will lead to more chemoresistance predictors based on genomic aberrations. The second reason is more fundamental: while the DNA sequence represents a key element in control of cellular processes, there are many other levels of cellular regulation, which can be associated with chemoresistance, e.g., epigenetic, transcriptional, post-translational, etc.[44] Therefore, it is widely accepted that predictors based on genomic aberrations are useful in a small minority of cancer patients who often then receive alternative therapies such as targeted agents or immunotherapy. The remaining majority of cancer patients still have to contend with standard chemotherapy agents and the potential for chemoresistance.[25]

Quantities of Molecules and Rates of Cellular Processes. The second large category of biochemical predictors of chemoresistance (Figure 3) includes predictors that measure quantities of molecules and rates of cellular processes as lab parameters.[25] Such lab parameters are continuous in nature and are inevitably prone to random and systematic errors. The extents of these errors depend on the performance of the analytical method used to measure the lab parameter.

Chemoresistance predictors in this large and diverse category can be further divided into two groups: serendipitous and rational. Serendipitous predictors are built with screening technologies, which can analyze thousands of mRNAs, proteins, post-translational modifications of proteins, miRNAs, metabolites, etc. simultaneously.[45] This approach can be termed a nondiscrete omics approach as it utilizes analytical tools of genomics, transcriptomics, proteomics, and metabolomics.[46] Subsets of molecules whose quantities correlate with a clinical end point are then identified, and these quantities are considered as a lab parameter for a tentative chemoresistance predictor. Most tentative chemoresistance predictors belong to this category,[30] but none of the FDA-approved predictors, for example, have been discovered using the nondiscrete omics approach.

Methods utilized for measuring lab parameters for serendipitous predictors include wide-panel hybridization assays (e.g., microarrays and Nanostring), nucleic acid sequencing methods (e.g., RNA-Seq), 2D gel, and mass spectrometry (MS).[30] All of them suffer from poor performance in one or more of the four critical parameters. For example, microarrays are semiquantitative and, thus, imprecise, inaccurate, nonrobust, and nonrugged.[47] Accordingly, they are suitable for wide screening and initial identification of tentative sets of molecules but not for populating Figure 2. Nanostring can identify up to 800 gene transcripts in a single run by detecting mRNA molecules with target-specific probe pairs;[48] however, it is inherently irreproducible.[49] Thus, it is also suitable only for the identification of preliminary relatively large sets of molecules. More accurate methods, such as RT-qPCR and ELISA are typically used for quantitative measurements of small sets of molecules identified from the wide screens.[50,51] However, RT-qPCR and ELISA have their own sources of inaccuracy associated, in particular, with the need to extract the molecules of interest from complex biological samples, and the error-prone enzymatic amplification-based mechanisms of detection.[52] The errors in enzymatic amplification-based detection are caused by multiple factors including exponential error accumulation in PCR, enzyme sensitivity to contaminants, and DNA thermal damage. RNA-Seq can determine the differential expression of genes and transcripts with very low amounts of RNA.[53] However, it suffers from issues associated with sample preparation (quantity and quality of recovered RNA), resulting in high variation of technical replicates. Additionally, analysis of genes with low expression levels in pooled samples can produce a false result of differential expression, when, in fact, this is due to the already existing high expression variance of those genes.[54] A pipeline approach with gradual exclusion of poorly performing serendipitous biomarker candidates and stronger ones moving to the next stages, along with the development of analytical methods for the most promising ones, was suggested as a way of improving the output of the omics-based efforts.[55] Overall, the current methods for measuring lab parameters in the category of serendipitous predictors can be considered as insufficiently precise, accurate, robust, and rugged for the reliable development of predictors and, thus, examples of robust and rugged markers using these methods are still to come.

Despite the limitations of the analytical methods, promising putative serendipitous chemoresistance predictors have been proposed using omics strategies. In breast cancer, Smith et al. utilized an antibody microarray composed of 224 antibodies to identify differentially expressed proteins in sensitive and doxorubicin-resistant cell lines.[56] Decreased expression of cyclin B1, cyclin D2, cytokeratin 18, and p-ERK were correlated with doxorubicin resistance. Also in breast cancer, tandem MS was able to identify a difference in the expression of 15 proteins present in the basolateral plasma membrane of parental and mitoxantrone-resistant cell lines.[57] For NSCLC, Pasini et al. investigated a panel of tumor suppressor genes and genes related to stemness and drug resistance, and identified genes involved in DNA damage repair as possible predictors.[58] So far, the omics approach has been the most widely used and productive in the development of tentative biochemical predictors.[59] However, we emphasize that none of such predictors have proven to be clinically useful.

In contrast to the serendipitous predictors, which rely on wide screening for the identification of lab parameters, the rational predictors rely on molecular mechanisms which are known to drive chemoresistance at the cellular level (Figure 3). Some cytotoxic drugs induce DNA damage in rapidly dividing cells and consequently trigger apoptosis in these cells. The cytotoxic effect can be inhibited, i.e., tumor cells can exhibit chemoresistance, if (i) the drug is extruded from the cells before it reaches the nucleus, (ii) the drug is inactivated by xenobiotic-metabolizing enzymes before reaching DNA, and (iii) drug-induced DNA damage is rapidly repaired and does not induce apoptosis. These three molecular mechanisms of drug resistance are directly related to the chemotherapeutics and its target, unlike other resistance mechanisms, such as enhanced cell survival and decreased apoptotic signaling. Also, these three mechanisms are driven by specific catalysts (transporters or enzymes) that could be used to identify lab parameters. For example, it is possible to develop assays that measure the activities of molecular processes responsible for

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

the three resistance mechanisms and use these activities as lab parameters in Figure 2.

The rest of this perspective focuses on methodological approaches for development of chemoresistance predictors based on catalysts involved in drug extrusion, drug degradation, and DNA damage repair. The catalysts that participate in these three molecular mechanisms of chemoresistance are (i) multidrug-resistance (MDR) transporters belonging to the ATP-binding cassette (ABC) family of proteins and catalyzing drug extrusion;[60] (ii) metabolizing enzymes such as cytochrome P450 family (CYPs) catalyzing drug degradation,[61] and (iii) enzymes that participate in DNA damage repair pathways to detect the damage, slice the lesion, insert new bases to fill the gaps, and ligate the repaired DNA strands.[62]

EFFORTS TO DEVELOP CATALYST-BASED CHEMORESISTANCE PREDICTORS

Currently, there are no clinically reliable chemoresistance predictors based on drug extrusion, drug metabolism, or repair of drug-induced DNA damage. The reason for this absence may be biological: there may be no good association between the quantitative parameters characterizing these three processes and clinical end points. The reason may also be methodological: the current methods for measuring lab parameters may be insufficiently precise, accurate, robust, and rugged to facilitate collection of data required for establishing the correlation shown in Figure 2. In the next three sections, we categorize the methodological approaches currently used in the development of catalyst-based chemoresistance predictors (Figure 4).

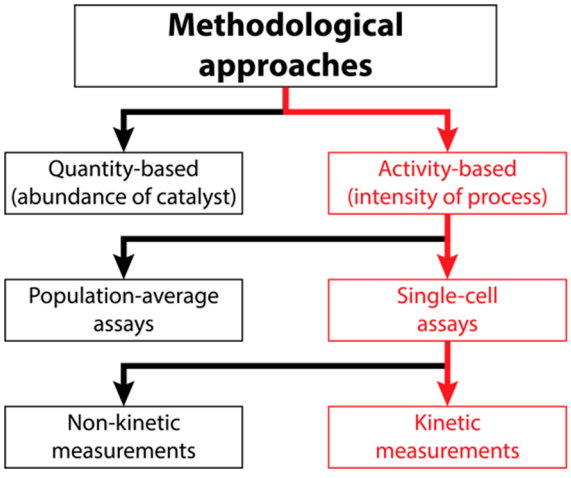


Figure 4. Methodological approaches utilized to measure a laboratory parameter while developing catalyst-based predictors; the focus is on the branch of catalyst-activity-based predictors.

Quantity of Catalysis vs their Activity. Lab parameters of catalytic processes of chemoresistance based on drug extrusion, drug degradation, and DNA damage repair can be categorized into abundance-based when the quantity (expression) of the catalyst is measured and activity-based when the intensity of the process is assessed through a quantifiable parameter (Figure 4). There are well-developed approaches for assessing levels of gene expression (amount of mRNA) or gene product (amount of the protein) with hybridization assays and immunoaffinity assays, respectively. However, in general, the amounts of mRNA and the protein are not enough to define the rate of reaction catalyzed by this protein. The reaction rate also depends on post-transcriptional and post-translational regulation, such as alternative splicing and editing of expressed transcripts and post-translational modifications to the protein.[63,64] Additional reasons for the functional insufficiency of expression assays are variation in concentrations of effectors and the dependence of reaction rate on the microenvironment, e.g., membrane microenvironment for transporters.[65,66] Therefore, expression levels of mRNA or catalyst proteins are not robust lab parameters. In contrast, the intensity of a catalytic process, or the activity of a catalyst, may be a base for a robust lab parameter assuming such a parameter is properly defined and can be measured. Thus, our following consideration focuses on catalytic activity of transporters and enzymes. Before delving into the issue of defining a suitable quantitative lab parameter based on catalyst activity, we will overview two types of approaches for studying such activities: a population-average approach and a single-cell approach.

Population-Average and Single-Cell Approaches. In general, the activity of transporters and enzymes can be studied with a population-average approach or with a single-cell approach (Figure 4). The population-average approach is inadequate for cancerous cells. Tumors are composed of cells that differ in their genome, transcriptome, proteome, metabolome, etc. resulting in heterogeneous cell populations. In population-average studies, an average characteristic of the entire cell population is measured and, thus, the heterogeneity of the cell population is completely disregarded (Figure 5).[67] As a result, population-average assays cannot detect minor subpopulations of cells, e.g., a subpopulation with an elevated level of drug resistance caused by higher activity of MDR transporters or enzymes involved in drug degradation and DNA damage repair. Accordingly, population-average assays cannot measure the size of a subpopulation of drug-resistant

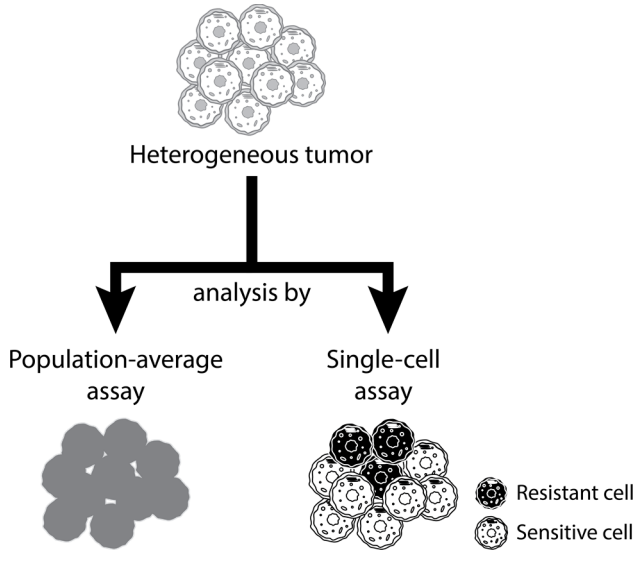


Figure 5. Conceptual representation of population-average vs single-cell assays. Population-average assays evaluate the average characteristic of a heterogeneous population, losing the information on heterogeneity, while single-cell assays can differentiate sensitive and resistant cell subpopulations present in the tumor. In this example, the heterogeneous population is composed of 30% of resistant cells and 70% of sensitive cells.

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

cells, while knowing this size is important because it correlates with clinical chemoresistance for an obvious reason: a greater number of drug-resistant cells in the tumor will result in a higher probability of some of such cells surviving chemotherapy and leading to disease relapse.[68]

Assessing population heterogeneity and measuring the size of a subpopulation of drug-resistant cells requires a single-cell approach. Single-cell assays analyze individual cells in the context of a large cell population and can, thus, identify and characterize small subpopulations of drug-resistant cells (Figure 5). If the size of the drug-resistant population can be measured, it can then be used as lab parameter to correlate with a clinical parameter in Figure 2. Collectively, techniques that can facilitate assessment of cell population heterogeneity are called cytometry.

Kinetic Approach for Identifying Population Heterogeneity with Regards to Catalyst Activity. There are two approaches for studying activity of catalysts at the single-cell level: nonkinetic and kinetic (Figure 4). A nonkinetic approach, such as measuring the amount of product formed at a certain time point, can be used to assess reaction rates and, therefore, characterize the subpopulation of drug-resistant cells. However, the nonkinetic approach is not robust, e.g., small variations in the substrate concentration may lead to significantly different results, i.e., different apparent reaction rates. Nonrobust measurement of reaction rates using a nonkinetic approach will, in turn, lead to nonrobust assessments of the size of drug-resistant subpopulation.[69] Flow cytometry is a nonkinetic single-cell approach that has been widely used to assess catalyst activity in order to determine the size of the drug-resistant subpopulation.[70] However, flow cytometry interrogates every cell at only one time point. Therefore, flow cytometry can only be used to determine pseudokinetic parameters which are inaccurate and nonrobust.[71]

To measure the size of a drug-resistant cell subpopulation and use it as a lab parameter, it is necessary to utilize a robust truly kinetic measure of reaction activity. A chemical reaction can be described through mechanisms that explain how reactants become products. Rate constants are quantitative parameters associated with reaction mechanisms, and they are the most accurate quantitative descriptors of the reaction. If a reaction is studied with a different instrument, a different substrate concentration or in a different time scale, rate constants will remain the same.

Reaction rate constants measured for drug extrusion, drug degradation, and DNA damage repair can, thus, serve as a basis for accurate, precise, robust, and rugged characterization of the drug-resistant subpopulation. The size of the subpopulation of drug-resistant cells is viewed as a suitable lab parameter for correlation with the clinical parameter in Figure 2;[68] rate constants measured at the single-cell level can be used to determine this size in an accurate and robust way. Other parameters characterizing cell-population heterogeneity, such as peak skewness or peak width in a histogram of cell distribution, can be also assessed as lab parameters.[72]

A general approach in which the rate constant is used to characterize cell-population heterogeneity is termed cytometry of reaction rate constant (CRRC) (Figure S1).[69,71−77] This method is being developed using cancer cell lines with the final goal of analyzing tumor primary cells and circulating tumor cells. Unlike nonkinetic approaches, CRRC can provide a robust kinetic lab parameter. Recent work, in which CRRC was used to study cell population heterogeneity with respect to MDR transport, have proved that CRRC is accurate[78] and that it can detect subpopulations of cells with elevated rate constants.[79] Additionally, it was shown that many phenotypes do not change significantly in the first 96 h after cell dispersal from three-dimensional clusters,[80] suggesting that the kinetic constant measured on tissue samples disintegrated into single-cell suspension can be used to characterize MDR activity in solid tumors. In the most recent work, it was directly proven that cells obtained by disintegration of three-dimensional clusters maintain their MDR activity for 24 h.[81] These studies indicate that if the size of a subpopulation with greater rate constants of the three catalytic processes (drug-resistant subpopulation) is measured with CRRC, it can represent a reliable lab parameter for constructing correlation with the clinical parameter in Figure 2.[69]

Enzyme activity with kinetic approaches in single-cell assays has also been a subject of study by a few groups. Sunray et al. used kinetic parameters to characterize activity of cytosolic enzymes in peripheral blood mononuclear cells,[74] which served as a model for Blokh et al. in his study of cytosolic enzyme activity in breast cancer patients.[77] Blokh and colleagues used kinetic characteristics of esterase activity to construct a diagnostic model that provided the right diagnosis of breast cancer for 44 out of 50 patients. Afrimzon et al. also evaluated activity of esterases in lymphocytes from breast cancer patients utilizing kinetic parameters.[75] Their work reported a difference in enzyme activity in breast cancer patients when compared to healthy donors, and higher rate constant values were correlated with better prognostic status of breast cancer patients, indicating that this method could be an additional criterion for prognosis and monitoring of breast cancer. Furthermore, there are some studies evaluating enzymatic activity in single cells originated from tumor samples. Schwab et al. evaluated MDR activity in cells dispersed from colorectal tumors, providing evidence for future kinetic analysis of freshly isolated cells from solid tumors.[82] Ricci et al. analyzed ALDH activity and CD133 expression in cells from ovarian tumor samples; however, they were not able to find a predictive value of CD133 expression and ALDH activity since there was no correlation between those parameters and clinical features of the patients.[83] Another example of a sample that could be potentially evaluated by single-cell assays are circulating tumor cells, which are implied in tumor metastasis and progression; nevertheless, there are no reports on kinetic studies using this type of cell. We can anticipate that single-cell kinetic measurements of chemoresistance-driving processes by CRRC will significantly improve precision, accuracy, robustness, and ruggedness of predictors of clinical chemoresistance.

CRRC can facilitate the collection of measurements of the lab parameters for many patients across different care centers to populate the correlation graph in Figure 2 with a statistically significant number of points. Even more, if it is found that there is no correlation between the clinical and lab parameters, the reason for the lack of correlation would fall in the biological category. In addition, this method could show if individual mechanisms of chemoresistance could be used to build good predictors. If only one mechanism is being analyzed and there is failure to generate a strong correlation of the lab parameter with the clinical parameter, this will indicate that the predictor should be based on a combination of chemoresistance mechanisms.

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

FUTURE DIRECTIONS FOR CATALYST-BASED CHEMORESISTANCE PREDICTORS

As discussed above, the best way to identify drug-sensitive and drug-resistant subpopulations of cells in a heterogeneous population is to use a kinetic approach based on molecular mechanisms of chemoresistance: drug extrusion, drug degradation, and DNA damage repair (Figure S2). CRRC is a method that allows the identification of cells with differing catalyst (transporter of enzyme) activity and can be applied to these three cellular drug-resistance processes.

The first mechanism of chemoresistance analyzed by CRRC has been drug extrusion. MDR transporters are responsible for extrusion of chemotherapeutics, and they contribute to clinical chemoresistance in a variety of cancers, for instance, hematological malignancies and breast, ovarian, renal, and pulmonary tumors.[60] MDR transporters move a substrate against its natural flux caused by the concentration difference outside and inside the cell.[84] Three most related to chemoresistance transporters are P-glycoprotein (P-gp, also known as MDR1/ABCB1), MDR-associated protein 1 (MRP1, also known as ABCC1), and breast cancer resistance protein (BCRP, also known as ABCG2).[84] Feasibility of MDR activity as a predictor of therapy response has been suggested in acute myeloid leukemia,[85] and this activity can be assessed with fluorescent probes (fluorophores) with overlapping substrate specificities.[86] In studies of MDR with CRRC, a fluorophore (fluorescein) is added to the cells and allowed to enter and accumulate in the intracellular space. Further, the decrease in intracellular fluorescence is followed over time as an indicator of the transporters' activity. CRRC has been able to correctly identify subpopulations of cells with different MDR activity, i.e., different rate constants,[69] and the most recent work with MDR transporters have also shown that CRRC can be applied to cells from disintegrated multicellular spheroids, providing the basis for future CRRC use in cells from disintegrated tumor tissues.[81]

CRRC as a method to study MDR activity has been extensively evaluated and demonstrated to be suitable for attempts to develop chemoresistance predictors based on drug extrusion; however, the other two mechanisms of chemoresistance, drug degradation and DNA damage repair, have yet to be evaluated by CRRC. Cytotoxic drugs are metabolized by enzymes, namely, cytochrome P450 (CYP) (Figure S2) and transformed from lipophilic xenobiotics into water-soluble products that are more easily transported out of the cell.[61] CYPs have been linked with chemoresistance in various cancer types (ovarian, breast, colon, and liver cancers, for example),[87−90] since the greater deactivation of chemotherapeutics results in insufficient drug concentration in the interior of tumor cells. Additionally, there is evidence of the relevance of CYP activity in tumor cells when we consider the correlation between CYP expression and drug response.[91]

Evaluation of CYP activity differs from that of MDR in two aspects: localization of the catalyst and type of the substrate. While transporters are located in the cell membrane, CYPs are intracellular enzymes, located in the inner membrane of the endoplasmic reticulum or the mitochondria.[92] As for the substrate, a fluorophore is used for MDR transporters, because there is no chemical transformation of the substrate into a product. CYPs require a fluorogenic substrate, i.e., a nonfluorescent molecule that converts into a fluorescent product. There are many fluorogenic substrates for CYP assays: different O-alkyl derivatives of resorufin, fluorescein, 7-hydroxycoumarins, 6-hydroxyquinolines, and 4-methylsulfonylphenyl furanones. Some of them are substrates for many CYPs,[93,94] suggesting their use for assessing overall CYP activity required for kinetic measurements and identification of drug-sensitive and drug-resistant subpopulations. This fluorescence-based measurement of CYP activity has been vastly used in cell lines and, thus, provides a good basis of adapting this method for evaluation of CYP activity in tumor samples originated from patients.

The third chemoresistance mechanism, DNA damage repair, is utilized by cancer cells to repair the damage caused by cytotoxic chemotherapeutic agents and, hence, avoid cell apoptosis.[95] One relevant effector of the DNA damage repair machinery is ERCC1 (Figure S2). This endonuclease is involved in removal of DNA adducts caused by platinum-based chemotherapeutics. High ERCC1 expression levels have been shown to correlate with chemoresistance in NSCLC,[96] gastric,[97] and ovarian[98] cancers. Studies with colorectal,[99] melanoma,[100] and ovarian[101] cancer cell lines have also demonstrated a link between resistance to platinum-based drugs and ERCC1 expression levels. Furthermore, gene expression level of ERCC1 has been proposed as a predictor of poor clinical response for colorectal cancer patients after treatment with a fluoracil/oxaliplatin combination.[102] The level of ERCC1 protein has also been proposed as a predictor of tumor response and overall survival for patients with squamous cell carcinoma of the head and neck undergoing cisplatin-based concurrent chemoradiotherapy.[103] Moreover, it has been demonstrated that lung cancer patients whose tumors did not express ERCC1 presented better survival rates than patients with tumors expressing ERCC1, after treatment with cisplatin,[104] and that enhanced ERCC1 expression correlates with clinical resistance to platinum-based chemotherapeutic treatment in epithelial ovarian cancer patients.[105]

ERCC1 is a critical member of the DNA repair machinery, and its levels correlate with chemoresistance in several types of cancer, making this protein a good candidate for chemoresistance predictor to be evaluated by CRRC. Nonetheless, there are no fluorogenic substrates to evaluate ERCC1 activity with CRRC. This calls for biological chemists to develop fluorogenic substrates for this enzyme, so that ERCC1 can be a part of drug resistance predictor development together with MDR transporters and CYPs. One example of fluorogenic substrates used to assess DNA damage repair protein activity was developed by Beharry et al.[106] This substrate, employed in intact prostate immortalized cells, is specific for ALKBH3, a protein that oxidizes aberrant alkyl groups and restores guanine DNA bases after damage caused by alkylating agents.[107] Although there are reports associating ALKBH3 levels with increased tumor size in head and neck squamous cell carcinoma,[108] with an advanced tumor stage in pancreatic cancer,[109] and with drug resistance in prostate cancer,[110] this enzyme is not a pivotal effector of the DNA repair machinery regarding chemoresistance. So far, there have been few reports about ALKBH3 in studies related to chemoresistance, and therefore, this enzyme is not a well justified catalyst candidate for CRRC in predictor development. However, since there is a fluorogenic substrate for ALKBH3, this enzyme could still be a satisfactory testing system for CRRC in the DNA damage repair context.

Altogether, MDR transporters, drug metabolizing enzymes, and DNA repair enzymes are well studied, and mechanisms of

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

their catalytic activity are understood. Thus, these three processes catalyzed by these types of catalysts form a group of chemoresistance mechanisms for which the catalyst-based approach in predictor development can be utilized.

CONCLUSIONS

In order to develop a reliable chemoresistance predictor, not only is good correlation of the clinical parameters and lab parameters needed but also a method that allows the measurement of both parameters in an accurate, precise, robust, and rugged way. If the method to measure the lab parameters follows these four requirements, then it can be used to test whether there is a strong correlation between the lab and clinical parameters. If the correlation is present and the correlation coefficient is found to be sufficiently high, this finding can be a starting point in the multistep development of a clinically useful chemoresistance predictor.

Although the nature of genomic aberration provides a theoretical analytical advantage, there are several post-transcriptional steps of regulation of gene expression that can impact the cells' response to chemotherapeutics. Therefore, genomic aberrations are not found to be the driver of chemoresistance in most cases. Instead, assessment of potential predictors that evaluate quantities of molecules (e.g., proteins) and rates of reactions (e.g., enzymatic ones) should be actively pursued. The most common approach for this evaluation is to identify possible molecular candidates with omics techniques and then quantify the candidate molecules using more robust techniques, such as immunoassays. The caveats of this approach are that (i) the quantity of molecules, e.g., proteins, may not correlate well with their activities and (ii) the omics approach is a brute-force one that does not rely on known mechanisms of cellular resistance to cytotoxic agents. The limitations of molecular quantity-based predictors emphasize the importance of predictors based on known cellular processes of resistance to cytotoxic agents, such as drug extrusion, drug degradation, and DNA damage repair. These mechanisms are driven by catalysts (transporters and enzymes) and, hence, are termed catalyst-based predictors.

For catalyst-based predictors, the commonly used methods that evaluate expression levels of genes and proteins, rather than protein activity, have been criticized for providing insufficiently high correlation with activity of drug-resistance processes. In addition, population-average approaches disregard characteristics of a drug-resistant cell subpopulation (e.g., its size) as potential lab parameters. Cytometry techniques, in contrast, provide single-cell resolution and allow the development of chemoresistance predictors based on the characteristics of the minor drug-resistance cell subpopulation as such subpopulation can give rise to tumor relapse after chemotherapy. The extension of data analysis to determination of rate constants (instead of nonkinetic rates) will improve accuracy, precision, robustness, and ruggedness of the assay required for studying combined multicenter patient cohorts. Moreover, the development of CRRC-based predictors of chemoresistance, focused on drug extrusion, drug degradation, and DNA damage repair, that is, catalyst-based predictors, has the potential to improve the accuracy and reliability of drug resistance prediction and eventually allow rationalized decisions on the best treatment options for individual cancer patients.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c02644.

Note S1, current definitions of precision, accuracy, robustness, and ruggedness by regulatory agencies; Note S2, consideration of effectiveness of alternative therapy; Table S1, predictive biomarkers of cancer approved by FDA for clinical use; Figure S1, schematic representation of cytometry of reaction rate constant; Figure S2, three molecular mechanisms of chemoresistance; and supporting references (PDF)

AUTHOR INFORMATION

Corresponding Author

Sergey N. Krylov − *Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto M3J 1P3, Canada;* orcid.org/0000-0003-3270-2130; Email: skrylov@yorku.ca

Authors

Mariana Bleker de Oliveira − *Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto M3J 1P3, Canada*

Vasilij Koshkin − *Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto M3J 1P3, Canada*

Geoffrey Liu − *Department of Medicine, Medical Oncology, Princess Margaret Cancer Centre, Toronto M5G 2M9, Canada*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.0c02644

Author Contributions

The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript before submission.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Dr. Sven Kochmann for technical assistance. This work was supported by the NSERC Discovery Grant (Grant No. 238990) to S.N.K.

REFERENCES

(1) Chabner, B. A.; Roberts, T. G., Jr. *Nat. Rev. Cancer* **2005**, *5*, 65−72.

(2) Perez-Herrero, E.; Fernandez-Medarde, A. *Eur. J. Pharm. Biopharm.* **2015**, *93*, 52−79.

(3) Luqmani, Y. A. *Med. Princ. Pract.* **2005**, *14* (Suppl1), 35−48.

(4) Wu, Q.; Yang, Z.; Nie, Y.; Shi, Y.; Fan, D. *Cancer Lett.* **2014**, *347*, 159−166.

(5) Brunt, E. M.; Paradis, V.; Sempoux, C.; Theise, N. D. *Hepat. Oncol.* **2015**, *2*, 255−273.

(6) Rueff, J.; Rodrigues, A. S. *Methods Mol. Biol.* **2016**, *1395*, 1−18.

(7) Padma, V. V. *Biomedicine (Taipei)* **2015**, *5*, 19.

(8) Feinberg, B.; Kish, J.; Dokubo, I.; Wojtynek, J.; Lord, K. *Am. J. Manag. Care* **2019**, *25*, 270−272.

(9) Chang, A. *Lung Cancer* **2011**, *71*, 3−10.

(10) Waks, A. G.; Winer, E. P. *JAMA* **2019**, *321*, 288−300.

(11) Buchner, T.; Hiddemann, W.; Berdel, W.; Wormann, B.; Schoch, C.; Loffler, H.; Haferlach, T.; Schumacher, A.; Staib, P.; Balleisen, L.; Gruneisen, A.; Rasche, H.; Aul, C.; Heyll, A.; Lengfelder,

E.; Ludwig, W.-D.; Maschmeyer, G.; Eimermacher, H.; Karow, J.; Frickhofen, N.; Hirschmann, W.-D.; Sauerland, M.-C. *Rev. Clin. Exp. Hematol.* **2002**, *6*, 46−59.
(12) Ansell, S. M. *Mayo Clin. Proc.* **2015**, *90*, 1152−1163.
(13) Brasseur, K.; Gevry, N.; Asselin, E. *Oncotarget* **2017**, *8*, 4008−4042.
(14) Luo, W.; Yang, G.; Qiu, J.; Luan, J.; Zhang, Y.; You, L.; Feng, M.; Zhao, F.; Liu, Y.; Cao, Z.; Zheng, L.; Zhang, T.; Zhao, Y. *Cancer Med.* **2019**, *8*, 6403−6413.
(15) Krzyszczyk, P.; Acevedo, A.; Davidoff, E. J.; Timmins, L. M.; Marrero-Berrios, I.; Patel, M.; White, C.; Lowe, C.; Sherba, J. J.; Hartmanshenn, C.; O'Neill, K. M.; Balter, M. L.; Fritz, Z. R.; Androulakis, I. P.; Schloss, R. S.; Yarmush, M. L. *Technology (Singap World Sci.)* **2018**, *06*, 79−100.
(16) Kalia, M. *Metab., Clin. Exp.* **2015**, *64*, S16−21.
(17) Taylor, C. R. Introduction to Predictive Biomarkers: Definitions and Characteristics. In *Predictive Biomarkers in Oncology*; Badve, S., Kumar, G. L., Eds.; Springer, 2019; pp 3−18.
(18) La Thangue, N. B.; Kerr, D. J. *Nat. Rev. Clin. Oncol.* **2011**, *8*, 587−596.
(19) Willemse, P. H.; Sleijfer, D. T.; Schraffordt Koops, H.; De Bruijn, H. W.; Oosterhuis, J. W.; Brouwers, T. M.; Ockhuizen, T.; Marrink, J. *Oncodev. Biol. Med.* **1981**, *2*, 129−134.
(20) Lloyd, K. L.; Cree, I. A.; Savage, R. S. *BMC Cancer* **2015**, *15*, 117.
(21) Volm, M.; Efferth, T. *Front. Oncol.* **2015**, *5*, 282.
(22) Nicolini, A.; Ferrari, P.; Duffy, M. J. *Semin. Cancer Biol.* **2018**, *52*, 56−73.
(23) Allen, K. E.; Weiss, G. J. *Mol. Cancer Ther.* **2010**, *9*, 3126−3136.
(24) El Bairi, K.; Atanasov, A. G.; Amrani, M.; Afqir, S. *Biomed. Pharmacother.* **2019**, *109*, 2492−2498.
(25) Ludwig, J. A.; Weinstein, J. N. *Nat. Rev. Cancer* **2005**, *5*, 845−856.
(26) Dracopoli, N. C.; Boguski, M. S. *Trends Pharmacol. Sci.* **2017**, *38*, 41−54.
(27) Duffy, M. J.; O'Donovan, N.; Crown, J. *Cancer Treat. Rev.* **2011**, *37*, 151−159.
(28) Twomey, J. D.; Brahme, N. N.; Zhang, B. *Drug Resist. Updates* **2017**, *30*, 48−62.
(29) Gosho, M.; Nagashima, K.; Sato, Y. *Sensors* **2012**, *12*, 8966−8986.
(30) Chatterjee, S. K.; Zetter, B. R. *Future Oncol.* **2005**, *1*, 37−50.
(31) Ioannidis, J. P.; Panagiotou, O. A. *JAMA* **2011**, *305*, 2200−2210.
(32) Goossens, N.; Nakagawa, S.; Sun, X.; Hoshida, Y. *Transl. Cancer Res.* **2015**, *4*, 256−269.
(33) O'Connor, J. P.; Aboagye, E. O.; Adams, J. E.; Aerts, H. J.; Barrington, S. F.; Beer, A. J.; Boellaard, R.; Bohndiek, S. E.; Brady, M.; Brown, G.; Buckley, D. L.; Chenevert, T. L.; Clarke, L. P.; Collette, S.; Cook, G. J.; deSouza, N. M.; Dickson, J. C.; Dive, C.; Evelhoch, J. L.; Faivre-Finn, C.; et al. *Nat. Rev. Clin. Oncol.* **2017**, *14*, 169−186.
(34) Panzeri, M. M.; Losio, C.; Della Corte, A.; Venturini, E.; Ambrosi, A.; Panizza, P.; De Cobelli, F. *Contrast Media Mol. Imaging* **2018**, *2018*, 8329041.
(35) Deen, S. S.; Priest, A. N.; McLean, M. A.; Gill, A. B.; Brodie, C.; Crawford, R.; Latimer, J.; Baldwin, P.; Earl, H. M.; Parkinson, C.; Smith, S.; Hodgkin, C.; Patterson, I.; Addley, H.; Freeman, S.; Moyle, P.; Jimenez-Linan, M.; Graves, M. J.; Sala, E.; Brenton, J. D.; Gallagher, F. A. *Sci. Rep.* **2019**, *9*, 10742.
(36) Musio, D.; De Felice, F.; Magnante, A. L.; Ciolina, M.; De Cecco, C. N.; Rengo, M.; Redler, A.; Laghi, A.; Raffetto, N.; Tombolini, V. *BioMed Res. Int.* **2013**, *2013*, 740195.
(37) Lih, C. J.; Harrington, R. D.; Sims, D. J.; Harper, K. N.; Bouk, C. H.; Datta, V.; Yau, J.; Singh, R. R.; Routbort, M. J.; Luthra, R.; Patel, K. P.; Mantha, G. S.; Krishnamurthy, S.; Ronski, K.; Walther, Z.; Finberg, K. E.; Canosa, S.; Robinson, H.; Raymond, A.; Le, L. P.; et al. *J. Mol. Diagn.* **2017**, *19*, 313−327.
(38) Ratan, Z. A.; Zaman, S. B.; Mehta, V.; Haidere, M. F.; Runa, N. J.; Akter, N. *Cureus* **2017**, *9*, No. e1325.
(39) Dacic, S.; Villaruz, L. C.; Abberbock, S.; Mahaffey, A.; Incharoen, P.; Nikiforova, M. N. *Oncotarget* **2016**, *7*, 82943−82952.
(40) Martin, V.; Bernasconi, B.; Merlo, E.; Balzarini, P.; Vermi, W.; Riva, A.; Chiaravalli, A. M.; Frattini, M.; Sahnane, N.; Facchetti, F.; Mazzucchelli, L.; Sessa, F.; Tibiletti, M. G. *J. Thorac. Oncol.* **2015**, *10*, 595−602.
(41) Schmitt, M. W.; Prindle, M. J.; Loeb, L. A. *Ann. N. Y. Acad. Sci.* **2012**, *1267*, 110−116.
(42) Oldenhuis, C. N.; Oosting, S. F.; Gietema, J. A.; de Vries, E. G. *Eur. J. Cancer* **2008**, *44*, 946−953.
(43) Sawyers, C. L. *Nature* **2008**, *452*, 548−552.
(44) Wilting, R. H.; Dannenberg, J. H. *Drug Resist. Updates* **2012**, *15*, 21−38.
(45) Tainsky, M. A. *Biochim. Biophys. Acta, Rev. Cancer* **2009**, *1796*, 176−193.
(46) Hu, Z. Z.; Huang, H.; Wu, C. H.; Jung, M.; Dritschilo, A.; Riegel, A. T.; Wellstein, A. *Methods Mol. Biol.* **2011**, *719*, 547−571.
(47) Draghici, S.; Khatri, P.; Eklund, A. C.; Szallasi, Z. *Trends Genet.* **2006**, *22*, 101−109.
(48) Kulkarni, M. M. *Curr. Protoc. Mol. Biol.* **2011**, 25B.10.1−25B.10.17.
(49) Godoy, P. M.; Barczak, A. J.; DeHoff, P.; Srinivasan, S.; Etheridge, A.; Galas, D.; Das, S.; Erle, D. J.; Laurent, L. C. *Cell Rep.* **2019**, *29*, 4212−4222.
(50) Morey, J. S.; Ryan, J. C.; Van Dolah, F. M. *Biol. Proced. Online* **2006**, *8*, 175−193.
(51) Zhu, Z. Q.; Tang, J. S.; Gang, D.; Wang, M. X.; Wang, J. Q.; Lei, Z.; Feng, Z.; Fang, M. L.; Yan, L. *Mol. Med. Rep.* **2015**, *12*, 1157−1162.
(52) Bustin, S.; Nolan, T. *Eur. J. Clin. Invest.* **2017**, *47*, 756−774.
(53) Wang, Z.; Gerstein, M.; Snyder, M. *Nat. Rev. Genet.* **2009**, *10*, 57−63.
(54) Williams, A. G.; Thomas, S.; Wyman, S. K.; Holloway, A. K. *Curr. Protoc. Hum. Genet.* **2014**, *83*, 11.13.1−11.13.20.
(55) Drabovich, A. P.; Martínez-Morillo, E.; Diamandis, E. P. *Biochim. Biophys. Acta, Proteins Proteomics* **2015**, *1854*, 677−686.
(56) Smith, L.; Watson, M. B.; O'Kane, S. L.; Drew, P. J.; Lind, M. J.; Cawkwell, L. *Mol. Cancer Ther.* **2006**, *5*, 2115−2120.
(57) Rahbar, A. M.; Fenselau, C. *J. Proteome Res.* **2005**, *4*, 2148−2153.
(58) Pasini, A.; Paganelli, G.; Tesei, A.; Zoli, W.; Giordano, E.; Calistri, D. *Transl. Oncol.* **2012**, *5*, 461−468.
(59) Quezada, H.; Guzmán-Ortiz, A. L.; Díaz-Sánchez, H.; Valle-Rios, R.; Aguirre-Hernández, J. *Bol. Med. Hosp. Infant. Mex.* **2017**, *74*, 219−226.
(60) Sharom, F. J. *Pharmacogenomics* **2008**, *9*, 105−127.
(61) Nebert, D. W.; Russell, D. W. *Lancet* **2002**, *360*, 1155−1162.
(62) Hakem, R. *EMBO J.* **2008**, *27*, 589−605.
(63) Kumar, D.; Bansal, G.; Narang, A.; Basak, T.; Abbas, T.; Dash, D. *Proteomics* **2016**, *16*, 2533−2544.
(64) Filipowicz, W.; Bhattacharyya, S. N.; Sonenberg, N. *Nat. Rev. Genet.* **2008**, *9*, 102−114.
(65) Cervera, J.; Meseguer, S.; Mafe, S. *Sci. Rep.* **2016**, *6*, 35201.
(66) Wu, L.; Wang, P.; Lü, J. *Sci. China: Technol. Sci.* **2018**, *61*, 1175−1183.
(67) Strzelecka, P. M.; Ranzoni, A. M.; Cvejic, A. *Dis. Models & Mech.* **2018**, *11*, No. dmm036525.
(68) Mabert, K.; Cojoc, M.; Peitzsch, C.; Kurth, I.; Souchelnytskyi, S.; Dubrovska, A. *Int. J. Radiat. Biol.* **2014**, *90*, 659−677.
(69) Koshkin, V.; Kochmann, S.; Sorupanathan, A.; Peng, C.; Ailles, L. E.; Liu, G.; Krylov, S. N. *Anal. Chem.* **2019**, *91*, 4186−4194.
(70) Lee, F. Y. F.; Siemann, D. W. *Int. J. Radiat. Oncol., Biol., Phys.* **1989**, *16*, 1315−1319.
(71) Walling, M. A.; Shepard, J. R. *Chem. Soc. Rev.* **2011**, *40*, 4049−4076.
(72) Gough, A.; Stern, A. M.; Maier, J.; Lezon, T.; Shun, T. Y.; Chennubhotla, C.; Schurdak, M. E.; Haney, S. A.; Taylor, D. L. *SLAS Discovery* **2017**, *22*, 213−237.

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

(73) Kubitscheck, U.; Pratsch, L.; Passow, H.; Peters, R. *Biophys. J.* **1995**, *69*, 30−41.
(74) Sunray, M.; Zurgil, N.; Shafran, Y.; Deutsch, M. *Cytometry* **2002**, *47*, 8−16.
(75) Afrimzon, E.; Zurgil, N.; Shafran, Y.; Sandbank, J.; Orda, R.; Lalchuk, S.; Deutsch, M. *Cancer Epidemiol., Biomarkers Prev.* **2004**, *13*, 235−241.
(76) Blokh, D.; Stambler, I.; Afrimzon, E.; Shafran, Y.; Korech, E.; Sandbank, J.; Orda, R.; Zurgil, N.; Deutsch, M. *Cancer Detect. Prev.* **2007**, *31*, 489−498.
(77) Blokh, D.; Afrimzon, E.; Stambler, I.; Korech, E.; Shafran, Y.; Zurgil, N.; Deutsch, M. *Comput. Methods Programs Biomed.* **2007**, *85*, 210−213.
(78) Koshkin, V.; Krylov, S. N. *Anal. Chem.* **2011**, *83*, 6132−6134.
(79) Koshkin, V.; Yang, B. B.; Krylov, S. N. *PLoS One* **2013**, *8*, No. e79222.
(80) Koshkin, V.; Ailles, L. E.; Liu, G.; Krylov, S. N. *J. Cell. Biochem.* **2017**, *118*, 154−162.
(81) Koshkin, V.; Bleker de Oliveira, M.; Peng, C.; Ailles, L.; Liu, G.; Covens, A.; Krylov, S. N. *Anal. Chem.* **2020**, *92*, 9348.
(82) Schwab, R.; Micsik, T.; Szokolóczi, O.; Schafer, E.; Tihanyi, B.; Tihanyi, T.; Kupcsulik, P.; Diófalvi, K.; Mersich, T.; Besznyak, I., Jr.; Zarand, A.; Mihalik, R.; Sarkadi, B.; Kéri, G.; Pap, A.; Jakab, F.; Kopper, L.; Petak, I. *Assay Drug Dev. Technol.* **2007**, *5*, 541−550.
(83) Ricci, F.; Bernasconi, S.; Porcu, L.; Erba, E.; Panini, N.; Fruscio, R.; Sina, F.; Torri, V.; Broggini, M.; Damia, G. *Am. J. Cancer Res.* **2013**, *3*, 221−229.
(84) Vasiliou, V.; Vasiliou, K.; Nebert, D. W. *Hum. Genomics* **2008**, *3*, 281−290.
(85) Kappelmayer, J.; Hevessy, Z.; Apjok, A.; Jakab, K. T. *EJIFCC* **2013**, *23*, 117−123.
(86) Lebedeva, I. V.; Pande, P.; Patton, W. F. *PLoS One* **2011**, *6*, No. e22429.
(87) Downie, D.; McFadyen, M. C.; Rooney, P. H.; Cruickshank, M. E.; Parkin, D. E.; Miller, I. D.; Telfer, C.; Melvin, W. T.; Murray, G. I. *Clin. Cancer Res.* **2005**, *11*, 7369−7375.
(88) Murray, G. I.; Foster, C. O.; Barnes, T. S.; Weaver, R. J.; Ewen, S. W.; Melvin, W. T.; Burke, M. D. *Br. J. Cancer* **1991**, *63*, 1021−1023.
(89) Stenstedt, K.; Hallstrom, M.; Johansson, I.; Ingelman-Sundberg, M.; Ragnhammar, P.; Edler, D. *Anticancer Res.* **2012**, *32*, 3869−3874.
(90) Zhang, K.; Jiang, L.; He, R.; Li, B. L.; Jia, Z.; Huang, R. H.; Mu, Y. *Tumor Biol.* **2014**, *35*, 7669−7673.
(91) Verma, H.; Singh Bahia, M.; Choudhary, S.; Kumar Singh, P.; Silakari, O. *Drug Metab. Rev.* **2019**, *51*, 196−223.
(92) Nelson, D. R. *Hum. Genomics* **2009**, *4*, 59−65.
(93) Cohen, L. H.; Remley, M. J.; Raunig, D.; Vaz, A. D. *Drug Metab. Dispos.* **2003**, *31*, 1005−1015.
(94) Donato, M. T.; Jiménez, N.; Castell, J. V.; Gómez-Lechón, M. J. *Drug Metab. Dispos.* **2004**, *32*, 699−706.
(95) Bouwman, P.; Jonkers, J. *Nat. Rev. Cancer* **2012**, *12*, 587−598.
(96) Lord, R. V.; Brabender, J.; Gandara, D.; Alberola, V.; Camps, C.; Domine, M.; Cardenal, F.; Sánchez, J. M.; Gumerlock, P. H.; Tarón, M.; Sánchez, J. J.; Danenberg, K. D.; Danenberg, P. V.; Rosell, R. *Clin. Cancer Res.* **2002**, *8*, 2286−2291.
(97) Kwon, H. C.; Roh, M. S.; Oh, S. Y.; Kim, S. H.; Kim, M. C.; Kim, J. S.; Kim, H. J. *Ann. Oncol.* **2007**, *18*, 504−509.
(98) Hassan, M.; Watari, H.; AbuAlmaaty, A.; Ohba, Y.; Sakuragi, N. *BioMed Res. Int.* **2014**, *2014*, 150845.
(99) Boyer, J.; McLean, E. G.; Aroori, S.; Wilson, P.; McCulla, A.; Carey, P. D.; Longley, D. B.; Johnston, P. G. *Clin. Cancer Res.* **2004**, *10*, 2158−2167.
(100) Li, W.; Melton, D. W. *Oncogene* **2012**, *31*, 2412−2422.
(101) Hector, S.; Bolanowska-Higdon, W.; Zdanowicz, J.; Hitt, S.; Pendyala, L. *Cancer Chemother. Pharmacol.* **2001**, *48*, 398−406.
(102) Shirota, Y.; Stoehlmacher, J.; Brabender, J.; Xiong, Y. P.; Uetake, H.; Danenberg, K. D.; Groshen, S.; Tsao-Wei, D. D.; Danenberg, P. V.; Lenz, H. J. *J. Clin. Oncol.* **2001**, *19*, 4298−4304.
(103) Jun, H. J.; Ahn, M. J.; Kim, H. S.; Yi, S. Y.; Han, J.; Lee, S. K.; Ahn, Y. C.; Jeong, H. S.; Son, Y. I.; Baek, J. H.; Park, K. *Br. J. Cancer* **2008**, *99*, 167−172.
(104) Olaussen, K. A.; Dunant, A.; Fouret, P.; Brambilla, E.; Andre, F.; Haddad, V.; Taranchon, E.; Filipits, M.; Pirker, R.; Popper, H. H.; Stahel, R.; Sabatier, L.; Pignon, J.-P.; Tursz, T.; Le Chevalier, T.; Soria, J.-C. *N. Engl. J. Med.* **2006**, *355*, 983−991.
(105) Du, P.; Wang, Y.; Chen, L.; Gan, Y.; Wu, Q. *Oncol. Lett.* **2016**, *12*, 857−862.
(106) Beharry, A. A.; Lacoste, S.; O'Connor, T. R.; Kool, E. T. *J. Am. Chem. Soc.* **2016**, *138*, 3647−3650.
(107) Sedgwick, B.; Bates, P. A.; Paik, J.; Jacobs, S. C.; Lindahl, T. *DNA Repair* **2007**, *6*, 429−442.
(108) Pilžys, T.; Marcinkowski, M.; Kukwa, W.; Garbicz, D.; Dylewska, M.; Ferenc, K.; Mieczkowski, A.; Kukwa, A.; Migacz, E.; Wołosz, D.; Mielecki, D.; Klungland, A.; Piwowarski, J.; Poznański, J.; Grzesiuk, E. *Sci. Rep.* **2019**, *9*, 13249.
(109) Yamato, I.; Sho, M.; Shimada, K.; Hotta, K.; Ueda, Y.; Yasuda, S.; Shigi, N.; Konishi, N.; Tsujikawa, K.; Nakajima, Y. *Cancer Res.* **2012**, *72*, 4829−4839.
(110) Shimada, K.; Nakamura, M.; Ishida, E.; Higuchi, T.; Yamamoto, H.; Tsujikawa, K.; Konishi, N. *Cancer Sci.* **2008**, *99*, 39−45.

https://dx.doi.org/10.1021/acs.analchem.0c02644
Anal. Chem. 2020, 92, 12101−12110

Supporting Information for:

Analytical Challenges in Development of Chemoresistance Predictors for Precision Oncology

Mariana Bleker de Oliveira,[1] Vasilij Koshkin,[1] Geoffrey Liu,[2] and Sergey N. Krylov[1]

[1]*Department of Chemistry and Centre for Research on Biomolecular Interactions, York University, Toronto M3J 1P3, Canada*

[2]*Department of Medicine, Medical Oncology, Princess Margaret Cancer Centre, Toronto M5G 2M9, Canada*

ABSTRACT

Chemoresistance, *i.e.*, tumor insensitivity to chemotherapy, shortens life expectancy of cancer patients. Despite the availability of new treatment options, initial systemic regimens for solid tumors are dominated by a set of standard chemotherapy drugs, and alternative therapies are used only when a patient has demonstrated chemoresistance clinically. Chemoresistance predictors use laboratory parameters measured on tissue samples to predict patient's response to chemotherapy and help to avoid application of chemotherapy to chemoresistant patients. Despite thousands of publications on putative chemoresistance predictors, there are only about a dozen predictors that are sufficiently accurate for precision oncology. One of the major reasons for inaccuracy of predictors is inaccuracy of analytical methods utilized to measure their laboratory parameters: an inaccurate method leads to an inaccurate predictor. The goal of this study was to identify analytical challenges in chemoresistance-predictor development and suggest ways to overcome them. Here we describe principles of chemoresistance predictor development *via* correlating a clinical parameter, which manifests disease state, with a laboratory parameter. We further classify predictors based on the nature of laboratory parameters and analyze advantages and limitations of different predictors using the reliability of analytical methods utilized for measuring laboratory parameters as a criterion. Our eventual focus is on predictors with known mechanisms of reactions involved in drug resistance (drug extrusion, drug degradation, and DNA damage repair) and using rate constants of these reactions to establish accurate and robust laboratory parameters. Many aspects and conclusions of our analysis are applicable to all types of disease biomarkers built upon the correlation of clinical and laboratory parameters.

TABLE OF CONTENTS

Note S1: Current definitions of precision, accuracy, robustness and ruggedness by regulatory agencies

Precision is defined as "the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions".[1] Accuracy is defined as "the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found".[1] The robustness of an analytical procedure is defined as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage".[1] Ruggedness of an analytical method is defined as "the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, *etc.*".[2]

Note S2: Consideration of effectiveness of alternative therapy

Deciding to replace an established frontline chemotherapy with an alternative therapy in an individual patient requires that chemoresistance be very reliably predicted. False-positive results in a chemoresistance test may lead to unjustified replacement of chemotherapy with a less effective alternative therapy for non-chemoresistant (*i.e.* chemosensitive) patients.[3] According to the definition of chemosensitivity, the effectiveness of frontline chemotherapy (E_{chemo}) is equal to unity for chemosensitive patients: $E_{chemo} = 1$. The effectiveness of the alternative therapy (E_{alt}) is lower than E_{chemo} for chemosensitive patients, *e.g.* $E_{alt} < 1$. On the other hand, E_{alt} can be presumed to be the same for chemoresistant and chemosensitive patients as the alternative therapy has a mechanism of action different from the cytotoxicity mechanism of the first line chemotherapeutic agent. The value of E_{alt} will define the required quality of a chemoresistance test. Two parameters of a chemoresistance-predictor test define its quality: sensitivity and specificity. Sensitivity of a chemoresistance predictor is defined as a fraction of patients who are correctly predicted to be chemoresistant among those who are truly chemoresistant, and thus should be treated with the alternative therapy. Specificity, in turn, is a fraction of patients who are correctly predicted to be chemosensitive among those who are truly chemosensitive, and who should be treated with chemotherapy.[4] The requirement for a predictor test is that for chemosensitive patients, E_{alt} should be higher than the negative likelihood ratio, which is a ratio between the probability of false-detection of chemoresistance (1 − assay sensitivity) and that of correctly ruling out chemoresistance (sensitivity),[5] which can be summarized as:

$$E_{alt} > (1 - \text{sensitivity})/\text{specificity}$$

Values for assay sensitivity and specificity can vary between 0 and 1. When applying real values of alternative therapy effectiveness (E_{alt}) to the above inequality, the need for high sensitivity and specificity becomes evident. For example, for metastatic colorectal cancer patients, the effectiveness of second-line therapy is 0.61;[6] therefore, assay sensitivity and specificity must satisfy inequality $(1 - \text{sensitivity})/\text{specificity} < 0.61$. When sensitivity is set at 1, the numerator is equal to zero, and any specificity value would make the statement true. In contrast, when specificity is set at 1, sensitivity needs to be higher than 0.39 to satisfy the inequality. In another example, for renal cell carcinoma patients, the effectiveness of second-line therapy is only 0.13;[7] therefore, assay sensitivity and specificity must satisfy inequality $(1 - \text{sensitivity})/\text{specificity} < 0.13$. Again, for sensitivity of 1, any specificity value would be sufficient; however, when specificity is set at 1, sensitivity should be higher than 0.87. The above examples are solely to illustrate how the effectiveness of an alternative therapy imposes requirements on the sensitivity and specificity of a chemoresistance-predictor test. Higher values of E_{alt} allow more tolerance for assay sensitivity while low values of E_{alt} impose very strict requirements on this characteristic. Assay specificity is a less restrictive parameter, but it is also important as low specificity must be compensated by the increase in sensitivity. In clinical practice, the actual threshold set for the negative likelihood ratio may also be influenced by factors other than treatment effectiveness: issues such as cost effectiveness, toxicity of chemotherapy versus alternative regimen, and patient preference may supersede or alter the specific thresholds; however, we will take the purist approach, assuming that all other non-effectiveness factors are equal between treatment options.

It is important to emphasize that diagnostic sensitivity and specificity of a chemoresistance predictor depend on the choice of the threshold which divides cases presumed to be chemoresistant and chemosensitive (horizonal line in Figure 2 in the main text). When the threshold is lowered, more true-chemoresistant cases are identified at the expense of a growing number of false-chemoresistant cases. Thus, lowering the threshold increases the sensitivity but decreases the specificity. A plot of sensitivity versus 1 – specificity is called receiving operating characteristic (ROC) curve, which can evaluate the diagnostic ability of a test to truly discriminate between the two different states of the patients,[8] *i.e.* chemoresistance or chemosensitivity. The area under the curve (AUC) is an important performance characteristic of the predictor, and it can be interpreted as the average value of sensitivity for all possible values of specificity.[8] AUC = 1.0 indicates perfect separability of chemoresistance from

chemosensitivity and corresponds to an ideal predictor. A predictor with AUC = 0.5 cannot separate chemoresistance from chemosensitivity, meaning that the predictor test yields chemoresistant or chemosensitive classifications at chance, without any correlation with the true status of the patient.[9]

If a reliable chemoresistance predictor is available, a test for chemoresistance should take place before the first round of treatment for every patient considered for chemotherapy. Such a test should also be used before the subsequent rounds of treatment for patients who did not show pre-existing chemoresistance but had eventual cancer progression (*i.e.* tumor growth or development of new metastasis) at any time point after the start of therapy.[10] Chemoresistance prediction, if reliable, is thus envisioned to benefit patients with both pre-existing and acquired resistance.

Table S1: Predictive biomarkers of cancer approved by FDA for clinical use.*

Predictor classification	Year***	Cancer type
Genetic aberrations (11)		
ALK (gene rearrangement)**	2010	NSCLC
BRAF (mutation)	2010	Melanoma
BRCA (mutation)**	2011	Breast; ovarian
BCR–ABL (chromosome translocation)	2001	CML
c-KIT (mutation)	2002	GIST
EGFR (mutation)**	2010	NSCLC
IDH (mutation)	2013	Glioma
KRAS (mutation)**	2008	NSCLC; CRC
PDGFR (mutation)	2002	CML; GIST
PML–RAR (chromosome translocation)	2001	APL
ROS1 (gene rearrangement)**	2017	NSCLC
Quantities of molecules (6)		
EGFR (protein expression)**	2004	CRC
ER (protein expression)**	1977	Breast
HER2 (protein expression)**	1998	Breast
HER2 (gene amplification)**	1998	Breast
PDL-1 (protein expression)**	2015	NSCLC; melanoma; bladder
MSI-H (alteration in the number of repeated DNA bases in microsatellites)**	2017	Adult and pediatric unresectable or metastatic solid tumors with the biomarker.

Abbreviations: ER, estrogen receptor; MSI-H, microsatellite instability high; NSCLC, non-small-cell lung cancer; CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumors; CRC, colorectal cancer; APL, acute promyelocytic leukemia.

* Prognostic biomarkers such as Mammaprint or Oncotype Dx that are not tied to the effectiveness of specific systemic therapies are not included in the table.

** Chemoresistance predictors.

*** Year in which the biomarker was either approved by FDA (with or without an accompanying drug) or was implemented for clinical use.

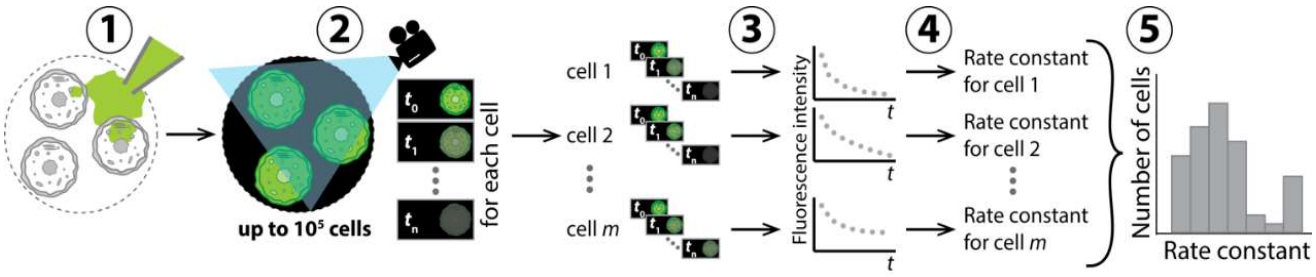


Figure S1: Schematic representation of Cytometry of Reaction Rate Constant. 1) A fluorescent substrate is added to the cells. 2) The reduction in cellular fluorescence intensity as a result of the catalytic reaction is monitored over time as sequential images are captured. 3 and 4) Individual reaction rate constants are determined for each single cell. 5) The values of rate constants are plotted on a "number of cells *vs*. rate constant" histogram to determine cell-population heterogeneity. Adapted from Koshkin *et al., Anal. Chem.* 2019, *91*, 4186–4194.

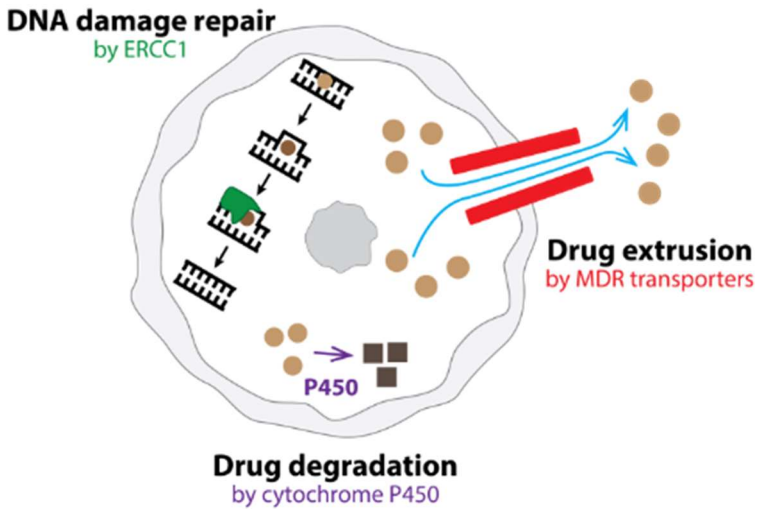


Figure S2. Three molecular mechanisms of chemoresistance. The three mechanisms directly related to the chemotherapeutic agent include: drug extrusion by MDR transporters, drug degradation by cytochrome P450, and repair of drug-induced DNA damage by ERCC1.

Supporting References

(1) Validation of analytical procedures: text and methodology Q2(R1). International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, 2005.

(2) USP 42–NF 37. In *Supplement 1: February 2019. Supplement 2: June 2019.*: United States Pharmacopeia Convention Inc., 2018.

(3) Pletcher, M. J.; Pignone, M. Evaluating the clinical utility of a biomarker: a review of methods for estimating health impact. *Circulation* **2011**, *123*, 1116–1124.

(4) Janes, H.; Pepe, M. S.; McShane, L. M.; Sargent, D. J.; Heagerty, P. J. The Fundamental Difficulty With Evaluating the Accuracy of Biomarkers for Guiding Treatment. *J. Natl. Cancer Inst.* **2015**, *107*, djv157.

(5) Attia, J. Diagnostic tests: Moving beyond sensitivity and specificity: Using likelihood ration to help interpret diagnostic tests. *Aust. Prescr.* **2003**, *26*, 111–113.

(6) Bazarbashi, S.; Hakoun, A. M.; Gad, A. M.; Elshenawy, M. A.; Aljubran, A.; Alzahrani, A. M.; Eldali, A. Efficacy of second-line chemotherapy after a first-line triplet in patients with metastatic colorectal cancer. *Curr. Oncol.* **2019**, *26*, e24–e29.

(7) Herbst, R. S.; Soria, J. C.; Kowanetz, M.; Fine, G. D.; Hamid, O.; Gordon, M. S.; Sosman, J. A.; McDermott, D. F.; Powderly, J. D.; Gettinger, S. N.; Kohrt, H. E.; Horn, L.; Lawrence, D. P.; Rost, S.; Leabman, M.; Xiao, Y.; Mokatrin, A.; Koeppen, H.; Hegde, P. S.; Mellman, I., *et al*. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* **2014**, *515*, 563–567.

(8) Hanley, J.A; McNeil, B.J. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* **1982**,143, 29–36.

(9) Hajian-Tilaki, K. Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation. *Caspian J. Intern. Med*. **2013**, 4, 627–635.

(10) Perrone, M. G.; Luisi, O.; De Grassi, A.; Ferorelli, S.; Cormio, G.; Scilimati, A. Translational Theragnostic of Ovarian Cancer: Where do we stand? *Curr. Med. Chem.* **2019** published online ahead of print.