Differential Determinants of Cancer Cell Insensitivity to Antimitotic Drugs Discriminated by a One-Step Cell Imaging Assay

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Abstract
Cancer cells can be drug resistant due to genetic variation at multiple steps in the drug response pathway, including drug efflux pumping, target mutation, and blunted apoptotic response. These are not discriminated by conventional cell survival assays. Here, we report a rapid and convenient high-content cell-imaging assay that measures multiple physiological changes in cells responding to antimitotic small-molecule drugs. Our one-step, no-wash assay uses three dyes to stain living cells and is much more accurate for scoring weakly adherent mitotic and apoptotic cells than conventional antibody-based assays. We profiled responses of 33 cell lines to 8 antimitotic drugs at multiple concentrations and time points using this assay and deposited our data and assay protocols into a public database (http://lincs.hms.harvard.edu/). Our data discriminated between alternative mechanisms that compromise drug sensitivity to paclitaxel and revealed an unexpected bell-shaped dose-response curve for BI2536, a highly selective inhibitor of Polo-like kinases. Our approach can be generalized, is scalable, and should therefore facilitate identification of molecular biomarkers for mechanisms of drug insensitivity in high-throughput screens and other assays.

Keywords
high-content screening, live cell imaging assay, image analysis, cancer cells, drug sensitivity, antimitotic drugs

Introduction
Understanding and combating variation in drug response is a central problem in cancer pharmacology. Acquired drug resistance is common, but large variation in response is also seen in drug-naive patients. Conceptually, variation in drug sensitivity and selection for resistance can occur at any step in the drug response pathway (Fig. 1). Common approaches to elucidating the genomic and mechanistic basis of response variation compare response between isogenic lines, for example, using RNA interference (RNAi)—mediated changes in gene expression or across a panel of cancer-derived cell lines. Typically, in these screens, response is quantified as the fraction of cells surviving at a fixed time point (often 3 days) following treatment with a dilution series of drug. These data are typically parameterized as a single EC50 value (drug concentration causing half-maximal killing). Less commonly, Emax (efficacy, the maximum response achievable from a drug) and a slope parameter are also extracted. This approach is simple and inexpensive, and the EC50 (also called GI50 for the drug concentration causing half-maximal growth inhibition) values it generates have been widely used to compare drugs and cell lines, notably in the NCI60 COMPARE analysis.1 This approach has been quite successful for predicting patient responses to kinase inhibitors as a function of their cancer genotype,2–4 but it has been less successful for other drug classes. A limitation of this approach is that it tells us little

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about the step or steps in the drug response pathway where a given cell line varies in response (Fig. 1). An approach that makes it possible to begin to understand the different mechanisms leading to variation in sensitivity would be very valuable when trying to determine the genotypic basis of drug resistance or insensitivity and response-predictive genetic biomarkers.

Discriminating different mechanisms that compromise drug sensitivity in cells in culture requires multiplexed readout of response. Typical multiplexed readouts include messenger RNA (mRNA) profiles, multiplexed gene expression reporters, and high-content imaging assays. These assays can be highly informative, but they are typically much more costly and complex than simple GI₅₀ measurements, which limits their application across large cell line panels at multiple drug concentrations. Furthermore, it can be difficult to infer alternative mechanistic effects on drug response pathways from gene expression and other multiplex readouts, in which the relationship between readout and the drug response pathway is complex. It would be useful to develop multiplexed assays that report directly on changes in cell physiology relevant to drug responses that are cheap enough to run across many cell lines and drug concentrations but informative enough to discriminate different mechanisms of drug sensitivity. Here, we developed such an approach using high-content screening (HCS; fluorescence microscopy with multiple markers followed by automated image analysis) as a multiplex readout of cell physiology.

Several considerations went into design of this HCS assay. Antibodies have been preferred as HCS markers due to their broad applicability, high specificity, and strong signal. However, fixation followed by antibody staining requires multiple wash steps that are time-consuming and bear the strong risk of selectively detaching cells that are loosely attached to the substrate. Cell detachment is problematic for accurate quantification of mitotic arrest and apoptosis, both of which weaken cell adhesion. Therefore, an imaging assay was developed in which living cells were labeled with three fluorescent dyes, followed by a fixation step to make the assay less time sensitive, but with no washes or medium changes. This lack of wash steps greatly increased the accuracy of scoring mitotic arrest and apoptosis. We demonstrate its utility by producing accurate dose-response curves at multiple time points for several aspects of cell physiology relevant to drug action. Together, these data comprise a pharmacological response signature that discriminates alternative mechanisms of compromised drug sensitivity to paclitaxel and reveal an unexpected dose-response profile for a Polo-like kinase inhibitor.

### Materials and Methods

#### Cell Culture

Cell lines were from the Center for Molecular Therapeutics collection (Massachusetts General Hospital, Boston). Growth conditions for 33 cell lines are listed in Supplemental Table S1. All growth media were supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin.

#### Reagents and Antibodies

Paclitaxel and staurosporine were from Sigma (St. Louis, MO; cat. T7191 and S3939). BI-2536 was from Haoyuan Chemexpress (Shanghai, China; cat. HY-506980). Lyso Tracker-Red was from Invitrogen (Carlsbad, CA; cat. No L-7528). Hoechst 33342 was from Sigma (cat. No. B2261). DEVD-NucView488 caspase-3 substrate was from Biotium (Hayward, CA; cat. 10402). Rabbit anti–phospho-Histone H3 (Ser10) antibody was from Millipore (Billerica, MA; cat. 06-570); mouse anti–cleaved PARP (Asp214) antibody was from BD Pharmingen (San Jose, CA; cat. 552597). Alexa Fluor 488 goat anti–mouse IgG and Alexa Fluor 568 goat anti–rabbit IgG were from Invitrogen (cat. A-11001 and A-11011). For additional kinase inhibitors screened: VX-680 (cat. HY-10161), GSK1070916 (cat. HY-70044), KIN001-220 (cat. HY-70061), and MLN8054 (cat. HY-10180) were from Haoyuan Chemexpress; AZD1152-HQPA (cat. 1580) was from Axon Medchem (Groningen, the Netherlands); and MPS-1-IN-1 was provided by the laboratory of Nathanael Gray (Dana Farber Cancer Institute and Harvard Medical School, Boston, MA).

#### Cell Staining and Immunofluorescence

For both the dye-based and the antibody-based assays, cells are first seeded into clear-bottom black 384-well imaging plates (Corning 3712; Corning, Corning, NY) at 2000 to 3000 cells/30 µL medium/well. After allowing cells to settle down in the plates for 24 h, compounds are added either by multichannel pipettor or by robotic pin transfer. Either the dye-based or the antibody-based staining is performed at 24-, 48-, and 72-h time points after compound addition.

For the dye-based assay, 10 µL of a cocktail of reagents (4 µg/mL Hoechst 33342, 2 µM NucView488, and 4 µM
LysoTracker-Red in phosphate-buffered saline (PBS) is dispensed into each well (so that the final concentration of Hoechst 33342 is 1 µg/mL, NucView488 is 500 nM, and LysoTracker-Red is 1 µM) using a Matrix WellMate plate filler (Thermo Fisher Scientific, Hudson, NH). The plates are incubated in a tissue culture incubator (37 °C, 5% CO₂) for 1.5 h. Then, 40 µL of warm 2% formaldehyde in PBS is added to each well (final concentration 1%), using a Matrix WellMate. Plates are spun briefly in a table-top centrifuge at 1000 rpm while cells are being fixed for a total of 20 min at room temperature. After this, plates are sealed using aluminum plate seals (Corning 6570) and are imaged (best if imaged within the same day) using an ImageXpress Micro (Molecular Devices, Sunnyvale, CA) with 10× Plan Fluor objective lens and suitable filters (DAPI, FITC, and Texas Red). Four sites are imaged in each well.

For the antibody-based assay, 30 µL of warm fixative/permeabilizing reagent (7.4% formaldehyde and 0.4% Triton X-100 in PBS) is dispensed into each well using a Matrix WellMate (final concentration of formaldehyde is 3.7% and Triton X-100 is 0.2%). Plates are spun briefly in a table-top centrifuge at 1000 rpm while cells are being fixed and permeabilized for a total of 20 min at room temperature. After fixation/permeabilization, wells are washed three times in PBS. Then, 20 µL of the mixture of primary antibodies (phospho-Histone H3, 1:500; cleaved PARP, 1:250) in blocking buffer (8% bovine serum albumin [BSA], 0.4% Triton X-100 in PBS) is added to each well with 20 µL of residual volume of PBS. Plates are incubated either at room temperature for 1 h or in the cold room overnight. After primary antibody incubation, the wells are washed three times in PBS. Then, 20 µL of the mixture of secondary antibodies (Alexa Fluor 488 goat anti–mouse IgG, 1:250; Alexa Fluor 568 goat anti–rabbit IgG, 1:250) in blocking buffer is added to each well with 20 µL of residual volume of PBS. The plates are incubated at room temperature for 1 to 2 h. After this, the wells are washed three times in PBS. Then, 20 µL of 2 µg/mL Hoechst 33342 in PBS is added to each well with 20 µL of residual volume of PBS (so that the final Hoechst concentration is 1 µg/mL). Plates are incubated at room temperature for 0.5 h. Wells are washed three times in PBS, and then the plates are sealed. Plates are imaged using ImageXpress Micro with the same settings as the dye-based assay.

**Live-Cell Imaging**

Cell lines HLF, HEC-1, 5637, and T24 were incubated with 11.11 µM, 1.2 µM, 137 nM, 45 nM, and 15 nM BI-2536 on a clear-bottom 96-well plate (655090; Greiner Bio-One, Monroe, NC) and imaged for 72 h on a Nikon Ti (Nikon, Tokyo, Japan) motorized inverted microscope with Perfect Focus System at 37 °C and 5% CO₂. Phase-contrast images were acquired using a 20×, 0.75 NA Plan Apochromat Nikon objective.

**Image and Data Analysis**

The large-scale data analysis used our in-house image analysis algorithm and was carried out on the Orchestra high-performance computation cluster at Harvard Medical School. The image analysis algorithms for both the dye-based and the antibody-based assays are developed in MATLAB, and detailed step-by-step analysis for the dye-based assay is illustrated in Supplemental Figure S1. (The image analysis algorithm for the antibody-based assay is similar to the dye-based assay but much simpler.) The image analysis methods are described in detail in the supplemental methods section.

Both algorithms share the same basic structure, which comprises two steps: nuclear segmentation and phenotypic classification. The nuclear segmentation step is used to identify individual nuclei and is identical for both assays since they rely on the same marker, Hoechst 33342, which is shown in panels a1 to a3 of Supplemental Figure S1. In the dye-based assay, we use LysoTracker-Red to help identify mitotic cells. Unlike traditional antibody-based mitotic markers, LysoTracker-Red labels all the cells but highlights mitotic cells as bright round objects due to their rounded-up morphology, as shown in panel b1 of Supplemental Figure S1. Due to this, traditional image analysis approaches to score cells based on the presence or absence of a marker signal would not work for this assay. Instead, a morphology-based algorithm was developed to extract bright rounded-up cells from LysoTracker-Red images, as illustrated in panels b1 to b3 in Supplemental Figure S1. To score apoptotic cells, bright spots are detected in the NucView488 channel, as shown in panels c1 to c2. In the dye-based assay, we also observed some objects with a large, round Hoechst stain and very faint NucView488 signal, but they lacked LysoTracker-Red signal around them, most likely due to the permeabilization or disassembly of the cell membrane. A couple of such objects are pointed out by blue arrows in Supplemental Figure S1. Judging from the morphology, we think they are NucView488-negative late-stage apoptosis cells, which was further confirmed by the time-lapse imaging experiment we performed.

For the antibody-based assay, the algorithm performs phenotypic classification by matching the spots detected from phospho-Histone H3 and cleaved PARP channels to the nuclear mask detected from the Hoechst channel. For both phospho-Histone H3 and cleaved PARP channels, the spot detection is carried out in the same way as the NucView488 channel in the dye-based assay. All phospho-Histone H3 positive cells get assigned as mitotic cells, and then all phospho-Histone H3 negative but cleaved PARP
positive cells are classified as apoptotic cells. The rest of the population then gets assigned as interphase cells.

**Dose-Response Curve Fitting**

All dose-response curves were fitted in Prism 6 (GraphPad Software, La Jolla, CA), using the following three-parameter nonlinear regression model:

\[
Y = E_{\text{min}} + \left( E_{\text{max}} - E_{\text{min}} \right) \left( 1 + 10^{\left( \log EC_{50} - X \right)} \right),
\]

where \( Y \) is the drug response; mitotic index, or apoptotic index in this case; \( X \) is the log of the drug concentration (in mM); \( E_{\text{min}} \) is the baseline response in the absence of drug; \( E_{\text{max}} \) is the maximum achievable response; and \( EC_{50} \) is the concentration that produces the half-maximal effect.

**Results**

**Comparison of Two HCS Assays for Profiling Antimitotic Drug Responses**

We first evaluated the performance of two HCS assays. One relied on antibody staining of fixed cells as has been typical in the HCS literature, the other on fluorescent dyes that stain living cells. The antibody assay used anti-phospho(Ser10) Histone H3 to mark mitosis and anti-cleaved PARP1 to mark apoptosis. The dye assay used LysoTracker-Red to visualize cell morphology and DEVD-NucView488 caspase-3 substrate to mark apoptosis.\(^\text{12}\) Both assays used the cell-permeable DNA dye Hoechst 33342 to mark nuclei. To make plate reading less time sensitive, cells were fixed after staining in the dye-based assay, but they were not washed before imaging. LysoTracker-Red is a fluorescent, lipophilic amine that accumulates in acidic compartments. But at high concentration (∼1 mM), it stained the whole cell, including acidic compartments and the cytosol. This enabled the identification of rounded-up mitotic cells by their brighter intensity and round morphology compared with interphase cells. Apoptotic cells were also round, but they stained less brightly with LysoTracker, presumably due to compromised plasma membrane integrity or lower adenosine triphosphate (ATP) levels. Major differences between the two assays are listed in Table 1. In addition to offering the washing-free feature, the dye-based assay is faster and cheaper.

Assay performances were compared using the human bladder tumor cell line 5637 treated with paclitaxel, a representative antimitotic drug. Cells were seeded at \( t = -24 \) h (∼3000 cells in 30 µL of growth medium/well, 48 replicate wells/treatment). Then, 200 nM paclitaxel or DMSO vehicle (<0.1% final) was added at \( t = 0 \). Two HCS assays were run in parallel at 24 and 48 h. Representative images from the two assays are presented in Figure 2A. In the no-drug control arm (left two columns of Fig. 2A), the two assays gave very similar results in terms of cell density, nucleus morphology, and % mitotic and % apoptotic cells. After 24 h of paclitaxel treatment (right two columns of Fig. 2A), both assays revealed a strongly elevated percentage of mitotic cells as expected. Mitotic index values were similar between both treatments, reflecting optimized washing conditions (a spin-down after each wash) designed to retain the weakly adherent mitotic cells in the antibody assay (Fig. 2B). However, we observed significantly fewer apoptotic cells in the antibody assay compared with the dye-based assay, judged from a lower percentage of cells in the cleaved PARP channel compared with the NucView488 channel (Fig. 2B). These data suggested that our wash protocol, even though optimized to retain mitotic cells, was selectively removing apoptotic cells. To confirm this, we tested the effect of washing on the dye-based assay of 5637 cells treated with paclitaxel for 48 h. Cells were imaged first without washing, and the same plate was imaged after three washes with PBS to mimic the wash procedure of the antibody assay. Representative images of the same field of view pre- and postwashing are shown in Figure 2C. The postwashing image has a much higher number of NucView488-positive cells compared with the postwashing image. In addition, the postwashing image contains many objects with round, puffy, blurred Hoechst staining and very weak LysoTracker-Red and NucView488 staining, which are completely missing from the postwashing image (three such cells are indicated by white arrows in Fig. 2C). Judging from their morphology and accumulation late in the time course, we believe these are late-stage apoptotic cells.

**Evaluation of Image Analysis Procedures**

Having chosen the dye-based HCS assay, we next evaluated the reliability of our automated phenotypic scoring algorithms. Figure 3A shows representative images of 5637 cells treated with DMSO alone, 200 nM paclitaxel for 24 h, and 200 nM paclitaxel for 48 h alongside the corresponding segmented images. Different phenotypes are highlighted with different-colored outlines in segmented images: interphase cells in white, mitotic cells in red, early apoptotic cells in green, and late-apoptotic cells in yellow. To evaluate the scoring accuracy of the automated image analysis, we

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**Table 1. Comparison between Two Imaging Assays That Count Nuclei, Mitotic Cells, and Apoptotic Cells.**

<table>
<thead>
<tr>
<th></th>
<th>Antibody-Based Assay</th>
<th>Dye-Based Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Involves washing?</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cell loss?</td>
<td>Yes</td>
<td>Minimal</td>
</tr>
<tr>
<td>Assay processing time</td>
<td>1–2 days</td>
<td>~2 h</td>
</tr>
<tr>
<td>Image quality</td>
<td>Great</td>
<td>Good</td>
</tr>
<tr>
<td>Assay reagent cost</td>
<td>~$116 per 384-well</td>
<td>~$27 per 384-well plate</td>
</tr>
</tbody>
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*Note: Figures and tables are placeholders and should be replaced with actual content.*
Figure 2. Comparison of antibody-based and dye-based assays using the human bladder tumor cell line 5637. (A) Comparison of individual channels and merged images from antibody-based assay and dye-based assay in the DMSO control (left) and 200-nM paclitaxel treatment for 24 h (right). (B) Bar graph comparison of the two assays in the DMSO and 200 nM paclitaxel for 24 h using parameters including total cell count, mitotic cell count, and apoptotic cell count. All cell counts were summed over four images acquired in the same well and then averaged over 48 replicates for each condition. (C) Comparison of individual channels and merged images of the same field of cells treated with 200 nM paclitaxel for 48 h before and after washing the dye-based assay plate.
randomly picked 12 images under each treatment condition and manually counted cells of different phenotypes to serve as benchmarks. Results from automated analysis are compared side-by-side with manual scoring in Figure 3B for all three treatment conditions. Under all three conditions, the automated analysis gave very similar results to those from the manual scoring. We noted slight overcounting of interphase cells and undercounting of mitotic and apoptotic cells in the automated analysis compared with the manual scoring. We were able to determine the source of these errors (see Suppl. Fig. S2) but did not attempt to modify the algorithms to correct for them because their magnitude was small compared with drug effects.

**Multidimensional Pharmacological Response Profiles of Multiple Cell Lines**

Thirty-three cell lines were chosen from the collection of the Center for Molecular Therapeutics at Massachusetts General Hospital\(^1\) on the basis of differential responses to...
antimitotic drugs in a conventional 3-day survival assay and similar proliferation rates. Proliferation rate is a known determinant of response to antimitotic drugs. We chose to minimize this source of variation in our cell line panel for simplicity. In principle, its contribution could be measured independently and corrected for, if desired, by extending the time course of the response assay for cells that grow slowly to time points longer than the 3-day time points used here. An important criterion in high-content analysis (HCA) is the ease of segmenting individual cells, which is more difficult in lines that tend to grow in clumps. Of the 33 lines initially selected, we were able to accurately score 20 (~60%) using our standard analysis protocol (Suppl. Table S1). Thus, our approach is broadly but not universally applicable to adherent cancer lines. Difficulty in scoring cell lines that tend to clump is a significant limitation of current HCS methodology.

Seven antimitotic drugs were selected to represent mechanisms of action currently under clinical trial, including inhibitors of Polo-like and Aurora kinases and of Kinesin 5 (also called KSP). Paclitaxel, which stabilizes microtubules, was included as a reference compound. Cells were treated over a wide range of drug concentrations (0.06 nM–11 µM, 3-fold dilution series) in duplicate and assayed at three time points (24, 48, and 72 h after drug addition). The median doubling time of the lines was 24 h (range, 18–36 h), so the assay duration covers at least two cell cycles. Data for eight drugs across 33 cell lines were deposited in the Library of Integrated Network-based Cellular Signatures (LINCS) database (http://lincs.hms.harvard.edu/) for public download and analysis. In Figures 4 and 5, we present a small snapshot of these data and discuss some of the interesting pharmacology they reveal.

Alternative Mechanisms of Compromised Drug Sensitivity. Figure 4 shows dose-response data for the 24-h mitotic index and 48-h apoptotic index in three cell lines (CaSki, LNZTA3WT4, and Calu-1) responding to paclitaxel and BI2536, a potent and specific inhibitor of Plk family kinases. These three cell lines were chosen for their diverse responses. Data were fitted to conventional sigmoid functions (see Materials and Methods), and from these fits we estimated EC50 and Emax values (Suppl. Table S2). We note that even with paclitaxel, dose-response curves deviated significantly from true sigmoid shapes and were often somewhat bell shaped. This issue was examined further for BI2536, with which the bell-shaped dose response was more pronounced. With paclitaxel treatment, all three cell lines showed similar Emax values for mitotic arrest at 24 h (~20–40%) but with different EC50 values. Notably, there was a >10-fold shift to higher values in the EC50 for LNZTA3WT4 compared with CaSki and Calu-1. Emax for the apoptotic index at 48 h was high for both CaSki and Calu-1 but very low for Calu-1 (~5%). A similar right shift in EC50 (>10-fold) was also observed for the 48-h apoptotic index when comparing LNZTA3WT4 with CaSki. Comparison to BI2536, which targets Polo-like kinases, allows discrimination of mechanisms affecting drug sensitivity that are target specific versus more general differences in cell physiology.

Figure 4. The 24-h mitotic index and 48-h apoptotic index dose-response curves of three cell lines (CaSki, LNZTA3WT4, and Calu-1) against paclitaxel (top) and BI2536 (bottom).

Figure 5. Mitotic index, apoptotic index, and nuclear area dose-response curves of 5637 cells treated with BI2536 for 24, 48, and 72 h.
BI2536 tended to generate bell-shaped dose-response curves, which we discuss below. Neglecting this aspect for this discussion and using the upslope of the curve to generate EC$_{50}$ values, response variation for the three lines was strongly correlated between paclitaxel and BI2536; compared with the sensitive CaSki line, LNZTA3WT4 exhibited EC$_{50}$ values that were strongly shifted to higher concentrations, whereas Calu-1 exhibited low E$_{max}$ for apoptosis, but not for mitotic arrest, with less shift in EC$_{50}$. Since the two drugs target different proteins, target mutation is unlikely to account for the response variation. The strong right shift in EC$_{50}$ values for both drugs in LNZTA3WT4 (albeit to different extents) is consistent with observations caused by upregulation of drug efflux pumps, since this mechanism can act on drugs with diverse structures and can be overcome just by increasing drug concentration. Calu-1, in contrast, may upregulate an antiapoptotic protein such as Bcl-XL, which protects against apoptosis following mitotic arrest, since this mechanism cannot be reversed just by increasing drug concentration. Scanning across our whole data set, we noted many examples of both kinds of variation in drug sensitivity.

Off-target Physiology. Profiling BI2536, which primarily targets Plk1, Plk2, and Plk3, revealed an unexpectedly complex dose response. Plk1 is required for the assembly of normal mitotic spindles, and Plk1 inhibition leads to prolonged mitotic arrest followed by apoptosis. Figure 5 shows mitotic and apoptotic indices for this drug in 5637 cells at three time points (24, 48, and 72 h). Similar data were seen in other lines (see data deposited at http://lincs.hms.harvard.edu/). These curves look quite different from the conventional sigmoidal-shaped curves. The dose response for the mitotic index at 24 h is strongly bell shaped, with the highest mitotic index at ~50 nM. Apoptotic indices at all three time points also reach a peak at ~50 nM, then decrease as concentration increases, followed by a second rise at the highest two concentrations (3.7 µM and 11.1 µM). From the dose-response curves of mean nuclear areas of interphase cells in Figure 5, it appears that within the concentration range between 100 nM and 1 µM, where mitotic and apoptotic indices dip, the nuclear areas of interphase cells increase. To further investigate this unexpected dose-response behavior, we carried out phase-contrast time-lapse imaging on 5637 cells treated with different concentrations of BI2536 show that at 15 nM BI2536, almost all cells go through prolonged mitotic arrest and then die, whereas at 1.23 µM BI2536, cells sit throughout the movie without any obvious phenotypic changes except becoming slightly enlarged (Suppl. Videos S1 and S2). However, we cannot rule out off-target inhibition of other kinases, including other Plk family members. In any case, higher drug concentrations are causing an effect that is off-target with respect to cell physiology. Cell cycle arrest before mitotic entry protects cells from the cytotoxic effect of antimitotic drugs, which presumably accounts for the dip in the apoptosis curves around 1 µM. At even higher concentration (above 1 µM), BI2563 may start to exhibit further off-target cytotoxicity that contributes to the second rise of the apoptosis curve.

Discussion

Cell-based HCS has gained popularity in drug discovery in recent years due to the rich information it reveals about cellular response and low per-well cost. It is obviously useful for antimitotic drugs, where mitotic arrest provides an activity biomarker upstream of cell death. However, similar upstream biomarkers (DNA damage markers, kinase substrate phosphorylation markers, etc.) could be substituted in protocols assaying cancer drugs with different mechanisms. We compared two marker combinations for profiling antimitotic drug responses, using antibodies versus cell-permeant dyes. The dye-based assay was clearly superior in its ability to quantify apoptosis, due to loss of weakly adherent dead cells during the washes needed for the antibody method. This advantage outweighed the potentially greater molecular specificity of the antibody method for identifying mitotic cells. Cell morphology and staining intensity in the LysoTracker channel were sufficient to accurately identify mitotic cells in all the lines we tested. In addition, LysoTracker-Red enabled scoring of late-stage apoptotic cells. These comprised >30% of the cell population under certain conditions (e.g., CaSki treated with paclitaxel at...
137 nM or higher) and would likely be underscored by other methods, particularly any that requires wash steps.

A common challenge to automated image analysis is the clumping or clustering behavior of certain cell lines, which normally results in poor image segmentation and therefore poor statistics. (Images from a typical well-behaved cell line and a clumpy cell line are shown in Suppl. Fig. S4.) Among all the cell lines we tested, ~40% exhibited some level of clumpiness either before or after drug treatment. The image analysis algorithm we employed was not able to yield accurate single-cell segmentations as expected for these clumpy lines. However, the robustness of our analysis algorithm still enabled us to generate reasonable and reproduceable dose-response curves for clumpy lines despite their nonideal growth patterns.

Our assay provided far more details on drug response than a conventional 3-day survival assay, as illustrated by the examples in Figures 4 and 5. Perhaps the most obvious benefit of this new assay is that it provides information that allows discrimination of different mechanisms of compromised drug sensitivity due to right shifts on the dose-response axis versus lack of apoptosis, despite good response with an upstream biomarker (in our case, mitotic arrest). The former might result from drug efflux pumping, the latter from changes in expression of Bcl2 family members, although many other molecular causes are possible. These are fundamentally different mechanisms, and the steps one might take to combat them in the clinic are quite different. It is possible, in principle, to discriminate these alternative mechanisms that affect drug sensitivity in conventional 3-day cell survival assays by careful measurement of E\text{max} and EC\text{50} as well as testing whether the cell count at 3 days goes below the initial seeding density. In practice, it is difficult to design experiments that robustly monitor these parameters. The key diagnostic for compromised drug sensitivity due to drug efflux or related mechanisms was that all aspects of the response increase in parallel by increasing drug concentration (e.g., LNZTA3WT4 in Fig. 4). The key diagnostic of compromised drug sensitivity due to lack of apoptosis is that an upstream biomarker, in this case mitotic arrest, exhibits typical EC\text{50} and E\text{max} values, whereas E\text{max} for apoptosis is drastically depressed (e.g., Calu-1 in Suppl. Table S2). Both types of insensitivity were common in our panel. Similar discriminations could be made for responses to any anticancer drug where the end point is killing the cancer cell and upstream biomarkers are available. We believe that attempts to relate drug sensitivity to genotypic markers would be much more successful if these alternative mechanisms were separately correlated to genotype. Addition of more biomarkers up and down the drug response pathway would further increase the power of our approach to discriminate alternative mechanisms of drug response/nonresponse. Methods that directly measure candidate proteins that might affect drug responses, at the RNA or protein level, will provide complementary data. By increasing our ability to discriminate causes of variation in drug sensitivity, we may be better able to fight this phenomenon in the clinic.

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