



Research article

Utilizing temporal variations in chemotherapeutic response to improve breast cancer treatment efficacy

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Abstract: Though survival rates for women with stage I breast cancer have radically improved, treatment options remain poor for the 40% of women diagnosed with later-stage disease. For these patients, improved chemotherapeutic treatment strategies are critical to eradicate any disseminated tumor cells. Despite many promising new drugs *in vitro*, most ultimately fail in the clinic. One aspect often lost during testing is *in vivo* circulation half-lives rarely exceed 24 hours, whereas *in vitro* studies involve drug exposure for 2–3 days. Here, we show how mimicking these exposure times alters efficacy. Next, using this model we show how drug response is highly time-dependent by extending analysis of cell viability out to two weeks. Variations in response both with feeding and time were dependent on drug mechanism of action. Finally, we show that by implementing this temporal knowledge of drug effects to optimize scheduling of drug administration we are able to regain chemosensitivity in a Carboplatin-resistant cell line.

Keywords: high-throughput screening; drug discovery; pharmacodynamics; pharmacokinetics; breast cancer; chemotherapy

1. Introduction

Approximately one out of every twenty women in America will be diagnosed with breast cancer at stage II or later when the disease is no longer localized to the breast [1]. At this stage, even in patients with operable breast cancer given advanced drug cocktails survival to five years does not often exceed 60% [2]. In order to improve survival, it is critical to generate better chemotherapeutic treatments in order to eradicate any disseminated cancer cells. A main tool for discovery of new targets is high-throughput drug screening which accounts for approximately a quarter of successful new drugs [3] including successful cancer therapeutics such as erlotinib and sorafenib [4]. Moreover, with the advent of microfluidic technology high-throughput screening may be easily adapted to be performed on a per-patient basis enabling a personalized approach to cancer treatment [5]. As screening continues to expand over the coming years, it is critical to evaluate how key functional end points such as cytotoxicity are determined.

Despite the promise of modern screening technology, these systems do not fully mimic *in vivo* complexities and may have limited predictive value. Improved culture systems, such as transitioning to three-dimensional culture models and inclusion of stromal cells may offer improvements, but at the cost of increased experimental complexity [6]. Other improvements may come from selection of appropriate assays for cell death analysis [7]. In addition to these environmental changes, another key aspect not currently accounted for in high-throughput drug screens is the *in vitro* pharmacokinetic elements as well as the pharmacodynamics of drug action. Most screening protocols continually expose cancer cells to drugs for 2–3 days before analyzing viability. Not only are these exposure times significantly longer than typical circulation times, the time scale for evaluation is much shorter than the weeks to months typically analyzed *in vivo*. Moreover, the single time point for analysis relies on the assumption that all molecules will affect cells on the same time scale.

Here, we sought to account for these differences to improve information produced from *in vitro* drug screenings. First, based on analysis of *in vivo* half-lives showing circulation times of ~24 hours, we demonstrate how mimicking this exposure time *in vitro* alters the potency of multiple drugs. These alterations varied based on the drug's mechanism of action. Next, we demonstrate how typical 48–72 hour end points do not fully capture the activity of all compounds. This time-dependent variation in potency also varied based on drug mechanism of action, with DNA-targeting drugs like Carboplatin taking significantly longer to produce effects than microtubule-targeting drugs like Taxol or Vinblastine. Finally, since response rates for any single-agent therapy rarely exceed 50% [8] we implement this knowledge of drug effect times to optimize scheduling of drug combinations. By optimally timing administration of slower-acting carboplatin before faster-acting Taxol are able to regain chemosensitivity in a Carboplatin-resistant cell line. Taken together, this work demonstrates potential ways to increase the accuracy of high-throughput screens for cancer drug discovery and methods to optimally schedule drug administration for improved efficacy.

2. Materials and Methods

2.1. Cell Culture

Human breast carcinoma MDA-MB-231 (ATCC, Manassas, VA) were cultured in RPMI 1640 (Mediatech, Herndon, VA) containing 10% FBS (Atlanta Biologicals, Norcross, GA). Carboplatin-resistant MDA-MB-231 (231C) were isolated by cyclic drug administration followed by recovery period for approximately 8 months until resistance emerged (Figure S1) [9].

2.2. Chemotherapeutics

Cells were treated with the following drugs: Carboplatin (Enzo), Doxorubicin (Enzo), Paclitaxel (Enzo), Vinblastine (Enzo), and 5-Fluorouracil (Acros Organics). All were solubilized in DMSO except for Carboplatin, which was solubilized in water to maintain activity [10].

2.3. Sulforhodamine B Assay

In order to analyze cell viability, we quantified total protein with the sulforhodamine B (SRB) assay used for the NCI60 drug screening program [11] as described [12]. Following incubation, cells were fixed with 5% (wt/vol) trichloroacetic acid for one hour at 4 °C and then stained with 0.4% (wt/vol) sulforhodamine B (Acros Organics) in 1% (vol/vol) acetic acid. Plates were destained by washing in 1% (vol/vol) acetic acid. The remaining protein-bound dye was solubilized in 10 mM Tris base for OD determination using a Beckman Coulter DTX-800 Multimode Detector.

2.4. Drug Screening

Before screening, cells were plated at 10% of confluence and allowed to adhere overnight. The following day, cells were treated with serial dilutions of drugs spanning 4 decades and a Day 0 time point was collected. Unless otherwise indicated, the drugs were removed the following day and replaced with fresh growth media. Cells were then analyzed by the sulforhodamine B at indicated days post-treatment. For longer time points, cells were fed every 3 days.

2.5. Data Fitting

Values were fit to a conventional sigmoidal function:

$$y = E_{max} + \left(\frac{E_{min} - E_{max}}{1 + \left(\frac{C}{EC_{50}} \right)^b} \right)$$

where $y(C) = (OD(C) - \text{blank}) / (OD_{ctrl} - \text{blank})$ is viability, C is the given concentration, E_{max} is the maximum effect, E_{min} is the minimum effect (typically 100% viability), EC_{50} is the concentration

to see 50% effect, and b is the Hill Slope [13]. The concentration to inhibit growth by 50%, or IC_{50} , was determined as where $y = 50\%$. These parameters are shown graphically in Figure 1.

2.6. Statistics

All studies were performed in triplicate or more. The data are reported as mean \pm standard error of the mean. Statistical analysis was carried out using a student's t-test for comparison considering $p < 0.05$ to be significant ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$).

3. Results

3.1. Recapitulating *in vivo* pharmacokinetics

Following *in vivo* administration, drug metabolism and secretion will rapidly decrease the plasma concentration. We first selected a panel of commonly used chemotherapeutic drugs with various targets: doxorubicin which targets multiple pathways including DNA intercalation, cross-linking, and generation of free radicals [14], the antimetabolite 5-fluorouracil, DNA cross-linkers mitomycin C, carboplatin, and cisplatin, as well as microtubule-disrupting agents Taxol and vinblastine which promote microtubule stabilization and collapse, respectively. As shown in Table 1, the *in vivo* half-lives of these drugs rarely exceed one day, with most being on the order of hours [15–21]. We sought to mimic this by reducing *in vitro* exposure times to 24 hours before returning cells to growth media (Figure 2A) until processing for analysis. The triple-negative breast cancer (TNBC) cell line MDA-MB-231 was used as a model system due to the lack of therapeutic targets for TNBC.

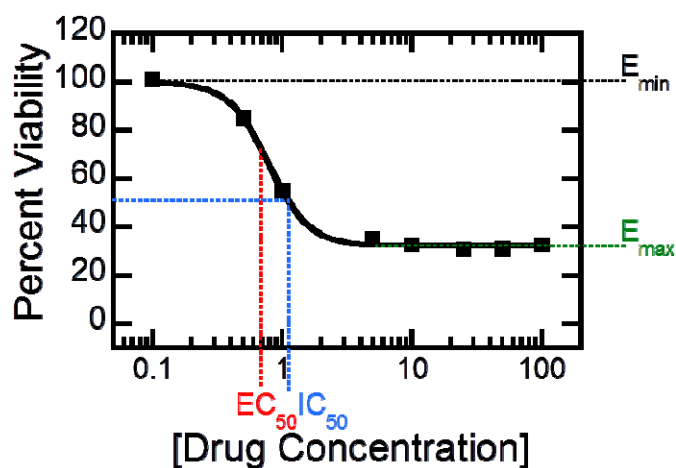


Figure 1. Graphical view of parameters used for fitting sigmoidal viability function.

Table 1. Clinical half-life values ($\tau_{1/2}$) for drugs used in this study and mechanisms of action.

Drug	$\tau_{1/2}$ (hours)	Mechanism of Action	Ref.
Doxorubicin	25	Multiple targets	[12]
5-Fluorouracil	0.2	Antimetabolite	[13]
Mitomycin C	0.8	DNA cross-linker	[14]
Carboplatin	3.0	DNA cross-linker	[15]
Cisplatin	0.8	DNA cross-linker	[16]
Taxol	6.4	Microtubule Stabilizer	[17]
Vinblastine	25	Microtubule De-stabilizer	[18]

For the DNA cross-linker carboplatin replacing the drug with growth media significantly decreased the IC_{50} value indicating an increase in potency (Figure 2B). We hypothesize that this cell death was induced by exposure to fresh growth factors, leading to subsequent apoptosis as cells attempted to amplify cross-linked DNA. The multiple-targeting doxorubicin was also significantly more potent when the drug was removed after 24 hours, possibly due to its ability to cross-link DNA (Figure 2C). In contrast, the antimetabolite 5-fluorouracil which acts as an irreversible suicide inhibitor of thymidylate synthase (TS) showed the exact opposite trend with a significantly increased IC_{50} value in the condition continually exposed to drug (Figure 2D). This could be attributed to recovery of TS function as new TS is synthesized in absence of the inhibitor. Microtubule targeting Taxol (Figure 2E) and vinblastine (Figure 2F) were not affected by exposure time. This finding is consistent with previous work across an large panel of cell lines treated with Taxol [22]. This may partially be because Taxol concentrates in cells to several orders of magnitude higher than in the media [23], and significant intracellular stores remained even after it was removed from the media. Presumably vinblastine could act by a similar mechanism.

3.2. Pharmacodynamics vary based on chemotherapeutic mechanism of action

After 3 days we found IC_{50} values of carboplatin to be on the order of 1000 μM , this value greatly exceeds the approximate max plasma concentration in vivo of ~ 50 μM [18,24]. We hypothesized this seeming *in vitro* failure with *in vivo* success may be in part to the hugely different time scales analyzed. Where tumor growth is tracked over weeks to months the vast majority of drug screening takes place over 2–3 days. To test if this difference may be partially attributed to these different time-scales, we sought to establish a detailed time line of drug potency for carboplatin, doxorubicin, Taxol, and vinblastine. For these studies, we moved forward with replacing the drug with growth media after 24 hours to best match *in vivo* exposure times and then feeding every 3 days thereafter for up to 2 weeks (Figure 3A–D). We found that by day 5 the IC_{50} for carboplatin had dropped two orders of magnitude to ~ 50 μM , closely mimicking clinically relevant concentrations (Figure 3A, E). This increase in potency continued through day 14 where the IC_{50} had dropped another order of magnitude to 8.6 ± 1.3 μM . To additionally quantify efficacy of the drug, we also

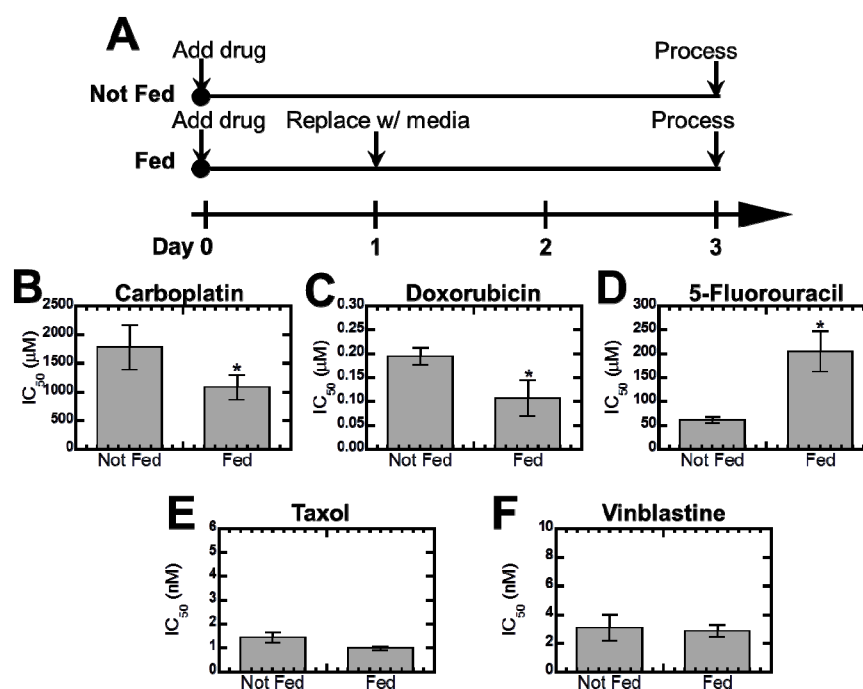


Figure 2. Effects of feeding on drug sensitivity. (A) Schematic of treatment time course for fed and not fed conditions. Invasive MDA-MB-231 breast cancer cells were allowed to adhere overnight before treating with serial dilutions spanning four decades. In the fed condition, drug was removed after 24 hours whereas in the unfed condition the drug was present for all 3 days. All plates were processed 3 days after treatment. (B) DNA cross-linking carboplatin. (C) Multiple-target doxorubicin. (D) Anti-metabolite 5-fluorouracil. (E) Microtubule-stabilizing Taxol. (F) Microtubule inhibitor vinblastine. Values are given \pm SEM, * $P < 0.05$.

analyzed changes in the maximum effect (E_{max} , or the percent viability at the bottom asymptote of the dose-response curve as shown in Figure 1) over the time course and found that while IC_{50} showed continual decreases throughout the 2 weeks E_{max} did not significantly decrease further after day 7 (Figure 3E). For doxorubicin (Figure 3B), E_{max} dropped to its minimum value by day 5 though the fold change was significantly less than for carboplatin while IC_{50} was time-independent (Figure 3F). Moreover, while carboplatin showed slight changes in the slope of the dose-response curve over time these changes were much more exaggerated with doxorubicin increasing nearly 8 fold over the course of two weeks (Figure 3I). In contrast to the DNA targeting carboplatin and multiple-targeting drug doxorubicin, the lowest IC_{50} value for both microtubule-targeting Taxol (Figure 3C, G) and vinblastine (Figure 3D, H) was at day 3, though the maximum effect followed similar trends as the previous drugs. This prevention of cell division by microtubule-targeting drugs may contribute to the increased potency at short time scales, while later time scales reflects more cytotoxicity. Since changes in drug potency and maximum effect were so much larger for carboplatin than any other drug, we further tested if this was specific to carboplatin or to DNA cross-linking drugs in general by repeating the studies with cisplatin and mitomycin C (Figure S2). Both drugs showed similar large drops in IC_{50} and E_{max} exceeding any drug except carboplatin, but cisplatin toxicity is observed much

earlier and undergoes a smaller decrease in IC_{50} with continued incubation. The smaller change in cisplatin also plateaus earlier than carboplatin and is consistent with the faster DNA cross-linking kinetics of cisplatin which induces more rapid DNA damage than carboplatin [25]. The delayed effect times observed in DNA crosslinking drugs could be due to the drug not inducing toxicity until DNA is amplified, DNA crosslinking kinetics being slower than those of simple binding for other drugs, or even delayed delivery as DNA targeting drugs would have to cross both the cell and nuclear membranes to exert effects.

3.3. Optimal drug scheduling based on pharmacodynamic information

Once we observed the altered pharmacodynamics between different drug classes, we next sought to use this information to improve therapeutic efficacy in a carboplatin-resistant MDA-MB-231 cell line (231C) derived from the parental MDA-MB-231 cells (231P) used thus far. We isolated this chemoresistant subpopulation by cyclic drug exposure and recovery until resistance was established, approximately 8 months (Figure S1). We hypothesized that we may be able to overcome this resistance by optimally timing the administration of a second chemotherapeutic such that the peak in the relative potency of both drugs overlap. For these purposes we chose Taxol which had its lowest IC_{50} (or highest potency) at day 3 (Figure 3G). In the Taxol control, we treated cells with 1 nM Taxol and 50 μ M carboplatin at day 0, producing dissynchronous peaks and thus no added benefit (Figure 4A). In the optimally scheduled condition, we treated with carboplatin at day 0 and Taxol at day 2 such that if the potencies were additive or synergistic the combination would induce more cell death (Figure 4B). While there was no significant difference in viability following treatment 1 nM Taxol or 50 μ M carboplatin individually, the combination treatment at day 0 did significantly improve response (Figure 4C). However, when optimally timed the viability of carboplatin-resistant cells was returned to that of parental cells by combination treatment. This result demonstrates that pharmacodynamic information can be implemented to improve drug efficacy, including recovering chemosensitivity.

4. Discussion

Since the National Cancer Institute implemented its high-throughput screening approach based on a panel of 60 human cancer cell lines in 1990 tens of thousands of drugs have been tested [13]. Despite the number of successful cancer therapeutics originating from high-throughput screening and the immense amount of data gleaned from these studies, most new compounds fail during phase III clinical trials [26]. Undoubtedly this failure arises from several factors. The most obvious may be the drastic difference in growth in a 2D petri dish and the complexity of a 3D tumor with surrounding matrix and stromal cells. This may be further complicated by irreversible genetic changes caused by *in vitro* culture [27]. Indeed, moving to patient-derived xenografts does appear to be an improved screening tool for drug validation, but would simply be impossible to implement for initial drug screening [26]. Though it may not be possible to fully recapitulate these *in vivo* complexities of cancer for initial screens, improvements to current *in vitro* screening protocols may lead to higher success rates.

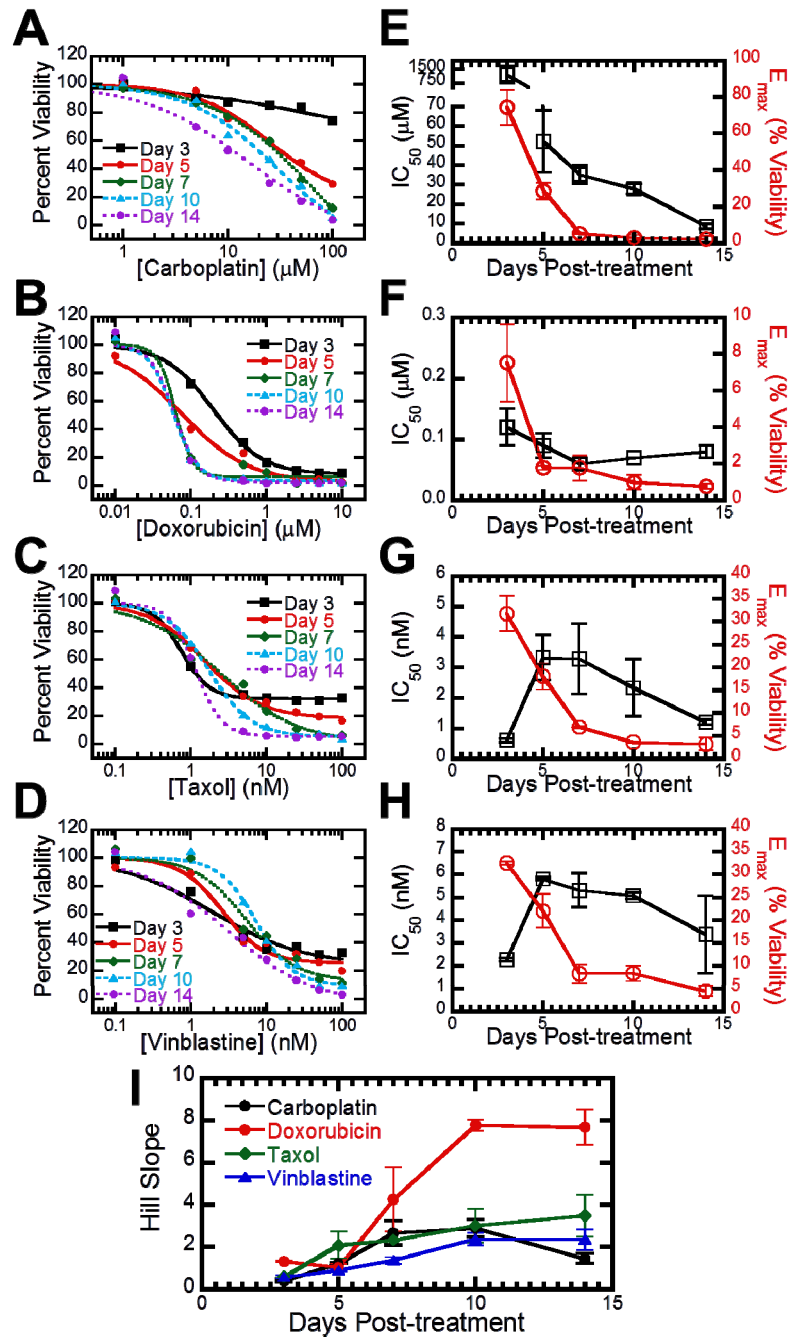


Figure 3. Time-dependent drug response profiles (A-D) Dose-response viability curves for drugs indicated on x-axis over two weeks of treatment. Invasive MDA-MB-231 breast cancer cells were all exposed to drug for 24 hours and then returned to growth media until indicated time point, feeding every 3 days as required. (E-H) Analysis of parameters fit from dose-response curves as described in methods. Black squares indicate concentration to induce cell viability by 50% (IC_{50}) and red circles indicate maximum effect (E_{max} , the bottom asymptote of viability curve) over two week evaluation period. (I) Slope of the dose-response curve over the 2 week evaluation period.

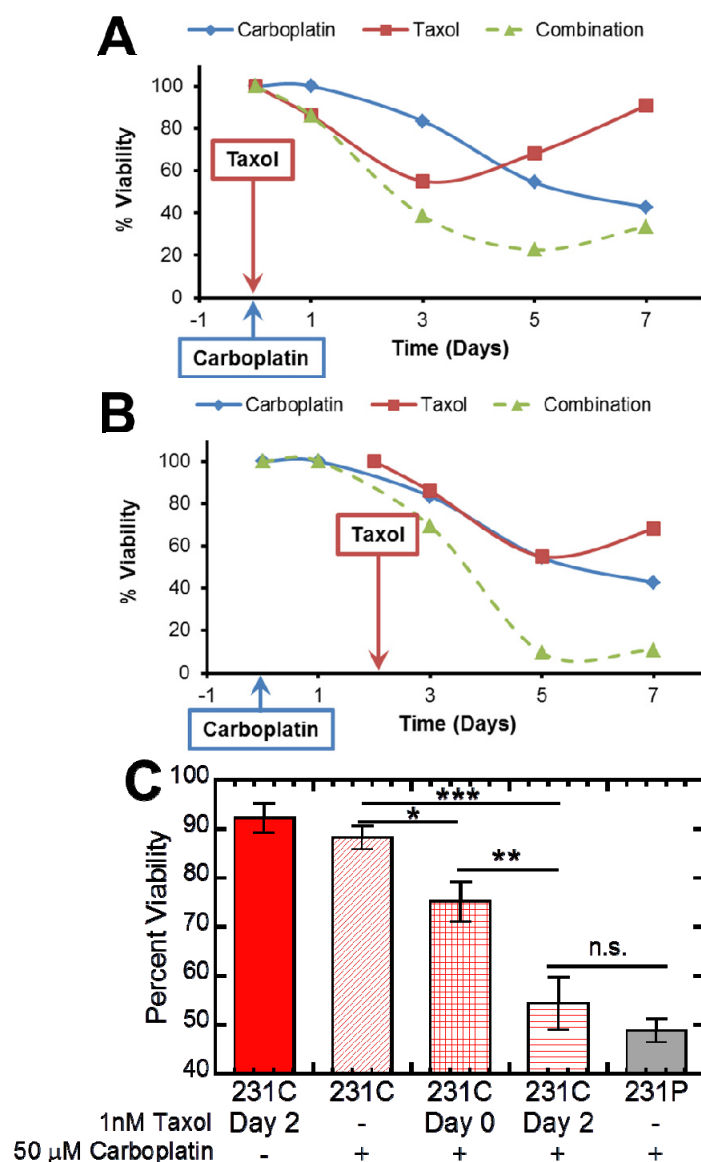


Figure 4. Optimal scheduling improves drug efficacy to overcome carboplatin resistance. (A-B) Treatment schedule overlaid with plots of parental cell viability over time treated with 1 nM Taxol and 50 μ M carboplatin. If given together, maximal potencies do not overlap resulting in less hypothetical combination effect, here plotted as the sum of both potencies (A). If optimally scheduled such that potency peaks overlap, combined theoretical potency could result in increased cell death (B). (C) Carboplatin-resistant MDA-MB-231 cells (231C, see Figure S1) or parental cells (231P) were treated with 50 μ M carboplatin on day 0 and 1nM Taxol as indicated for 24 hours. On day 3, all cells were moved to fresh media before processing on day 5. While treatment with both carboplatin and Taxol on day 0 did result in some increased cell death, when optimally scheduled Taxol on day 2 with carboplatin on day 0 further decreased viability and returned recovered carboplatin sensitivity to parental levels. Values are given \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The first step we took to mimic *in vitro* conditions is using drug exposure times based on *in vivo* half-lives. While previous work has shown that drugs used in this study including Taxol [28], 5-FU [29], carboplatin [28], and doxorubicin [30] are retained within tumors for much longer than their systemic half-life, this aspect should be recapitulated in our system where drugs are allowed to accumulate inside the cell for a limited time before removal. For instance, Taxol has been shown to accumulate inside cells to concentrations exceeding extracellular levels by 10–100 fold [23]. From this time, it is maintained at near identical concentrations in tumor cells *in vitro* following 16 hour exposure [31] as tumors *in vivo* after standard infusion [28] out to at least 5 days, suggesting modeling drug exposure times on drug half-lives may recapitulate what occurs *in vivo*. This appears to be conserved with other drugs, such as with doxorubicin which concentrates up to 95.1% of intratumoral doxorubicin into cell nuclei; a phenomena that would be retained *in vitro* [30].

Our results suggest that longer-term analysis coupled with mimicking *in vivo* exposure times could help improve drug discovery efforts. In example, when comparing carboplatin and cisplatin across 36 cell lines *in vitro* Lancaster and colleagues found that all cell lines were more sensitive to cisplatin, often by orders of magnitude [32] even though the two drugs show no difference in *in vivo* efficacy [33]. At early time scales, we too observed this order of magnitude difference, but at late time scales the drugs showed no difference in potency (Figure 2E, Figure S2B). Carboplatin was adapted as a primary treatment for ovarian cancer due to its decreased systemic toxicity relative to cisplatin [33], so selection of drugs with more delayed effect profiles could present additional novel therapeutics with more tolerable side effects. While feeding slightly improved the efficacy of DNA targeting drugs (Figure 2B–C), it decreased the efficacy of other drugs such as 5-fluorouracil (5-FU, Figure 2D). The fluoropyrimidine 5-FU is an uracil analog that induces cytotoxicity either through misincorporation into RNA or inhibition of thymidylate synthase [34]. Drug removal may allow for recovery of normal uracil levels decreasing the efficacy of 5-FU. Notably, 5-FU also had one of the shortest half-lives of drugs evaluated here (Table 1). Though 5-FU has been extremely successful therapeutically, efficacy may be further improved by incorporation of controlled release strategies to increase exposure time.

Another potential source of error in current drug discovery techniques is the reliance on IC_{50} as the sole measure of drug potency. While we found that maximum effect and IC_{50} generally trended together, in some cases such as microtubule targeting drugs they had nearly opposite profiles (Figure 3G–H). Recent work has shown that the maximum drug effect and slope of the dose-response curve may be more informative than IC_{50} when designing new drugs [35]. Moreover, we found that the coefficient of variation (standard deviation divided by the mean) was universally smaller for E_{max} than IC_{50} . With the observed large lab-to-lab discrepancies in IC_{50} values inclusion of these additional parameters may improve the repeatability of these assays allowing for broader interpretation of the results [36].

Finally, analysis of the time-course of drug action allowed for direct implementation of optimal drug scheduling to maximize therapeutic efficacy. The scheduling of chemotherapeutic administration has been shown to contribute to acquisition of a drug-resistant phenotype [37]. Optimal timing of imatinib based on mathematical models may also improve CML patient outcomes [38]. Here, we found that by co-administering carboplatin and Taxol together to carboplatin-resistant MDA-MB-231 cells we were able to reduce viability to $75 \pm 4\%$, which closely

matches the $80 \pm 6\%$ viability that would be achieved by simple additive effects. Optimally scheduling these two agents by applying slower-acting carboplatin before the faster-acting Taxol this effect was improved by 44% reducing viability to $54 \pm 5\%$, inducing the same amount of cell death as the parental MDA-MB-231 cells (Figure 4). This increase in toxicity is most likely not due to simple changes in intracellular drug removal as Taxol and carboplatin are removed through distinct mechanisms, the former through P-gp pumps and the latter through enzymatic degradation [39]. The knowledge gained from current molecular profiling of chemoresistant cell lines [40,41,42] may allow for timed administration of inhibitors to target the affected pathways as a method to increase therapeutic efficacy.

5. Conclusion

This work shows how recapitulating *in vivo* drug exposure times along with longer-term analysis of cell viability may improve the reliability of *in vitro* drug screening. We demonstrate that drug exposure time causes changes in cell viability that vary with drug mechanism of action, as does the time course of drug potency. This pharmacodynamic information can be implemented to increase the efficacy of therapeutics, even regaining chemosensitivity to a drug-resistant cell line. Implementation of this knowledge along with *in vivo* pharmacokinetics could allow for a mathematical model to optimize drug administration schedules which should be tested in future studies *in vivo*.

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Conflicts of Interest

The authors declare no commercial or financial conflict of interest.

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