Quantitative Assessment of the Complex Dynamics of $G_1$, $S$, and $G_2$-M Checkpoint Activities

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Abstract

Although studies of cell cycle perturbation and growth inhibition are common practice, they are unable to properly measure the activity of cell cycle checkpoints and frequently convey misinterpretation or incomplete pictures of the response to anticancer treatment. A measure of the strength of the treatment response of all checkpoints, with their time and dose dependence, provides a new way to evaluate the antiproliferative activity of the drugs, fully accounting for variation of the cell fates within a cancer cell line. This is achieved with an interdisciplinary approach, joining information from independent experimental platforms and interpreting all data univocally with a simple mathematical model of cell cycle proliferation. The model connects the dynamics of checkpoint activities at the molecular level with population-based flow cytometric and growth inhibition time course measures. With this method, the response to five drugs, characterized by different molecular mechanisms of action, was studied in a synoptic way, producing a publicly available database of time course measures with different techniques in a range of drug concentrations, from sublethal to frankly cytotoxic. Using the computer simulation program, we were able to closely reproduce all the measures in the experimental database by building for each drug a scenario of the time and dose dependence of $G_1$, $S$, and $G_2$-M checkpoint activities. We showed that the response to each drug could be described as a combination of a few types of activities, each with its own strength and concentration threshold. The results gained from this method provide a means for exploring new concepts regarding the drug–cell cycle interaction. [Cancer Res 2009;69(12):5234–40]

Introduction

Pharmacodynamics, intended in a broad sense as the study of the efficacy of a drug in cancer or in cancer biological models, is object of research from the molecular scale, focused on inhibition of specific targets, up to large-scale tumor growth inhibition in vivo. However, only simple relationships between drug concentration and growth inhibition are usually assumed, neglecting the dynamics of cytostatic and cytotoxic effects and the complexity of their dose dependence.

Studies of the proliferation of a cancer cell population are done at an intermediate scale, distinct from the molecular level, because the behavior of a group of cells is not a straightforward consequence of how a single (or “typical” or “average”) cell functions, as studied in molecular research. This intermediate level is where in vitro toxicity tests are done and “probabilistic” quantities, such as the number of surviving cells, are measured. Noticeably, at this level of the biological scale, cytometric techniques can provide a large amount of data because they are specifically designed to retrieve the frequency distribution of the cell content of specific constituents such as DNA. In a study of the cell population response to anticancer drugs, the time course of percentages of cells in $G_1$, $S$, and $G_2$-M phases is readily measured, and this can be complemented by measures of DNA-bromodeoxyuridine (BrdUrd) and absolute cell count. However, each measure conveys only one piece of information, which may be misinterpreted if taken individually because different combinations of underlying cytostatic and cytotoxic effects can lead to the same result. The “macroscopic” variations of these measurable quantities are in fact the result of superimposition of the effects of cell cycle block and cell loss, their dynamics in the times before the measure, and the proliferation of surviving cells. Thus, rough data depend on, but do not provide a direct measure of, the activities of the molecular networks regulating $G_1$, $S$, and $G_2$-M checkpoints, which in turn are the results of complex molecular interactions studied by systems biology.

We attempted to link the molecular and cellular biological scales using a simulation approach (Fig. 1), entwining a detailed model of cell cycle kinetics with models of cytostatic and cytotoxic effects of drugs in each phase to simulate experimental results (1–3). This simulation partly bridges the gap between the molecular and cellular levels, deciphering cell cycle percentages and any other quantity measurable at the “cell population” level, in terms of parameters that directly represent the global activities of $G_1$, $S$, and $G_2$-M checkpoints, each governed by a specific molecular network. The model incorporates inter-cell heterogeneity of unperturbed and drug-perturbed proliferation as a key feature connecting the two biological scales.

Although, at the present stage, the “model” is not intended for prediction of results of future experiments, it supports data interpretation and integrates all observations into a coherent scenario to comprehend the response to treatment. We applied our modeling approach on the measures taken with different techniques after treatments with five “classic” anticancer drugs, at concentrations spanning the whole range of drug efficacy, and at several times after drug exposure. Specific comments to cell cycle effects of each drug and possible connections to molecular mechanisms of action were discussed in other publications (1–5). Exploiting the fact that the cell line, the experimental
Materials and Methods

Cell Culture and Drug Treatment

IGROV1 cells were maintained as previously described (3). Exponentially growing cells were treated for 1 h with different drug concentrations of doxorubicin (generously provided by Nerviano Medical Sciences), topotecan (generously provided by Glaxo SmithKline), paclitaxel (Bristol-Myers Squibb), cisplatin, and melphalan (Sigma).

After treatment, the cells were washed twice with warm PBS and left in drug-free medium for 0, 6, 24, 48, and 72 h. At each time, cells from three replicated flasks were detached, counted, then pooled and fixed as previously described (3).

Flow Cytometric Analyses

Cells were harvested and prepared for flow cytometric analysis as previously described (3). DNA histograms were analyzed as described (6).

BrdUrd pulse-chase analysis. Short-term perturbations were investigated by BrdUrd pulse–chase analysis: 30 μmol/L BrdUrd (Sigma), which is an analogue of thymidine, was added to the cell culture during the last 20 min of treatment. After drug and BrdUrd washout, the cells were left in drug- and BrdUrd-free medium for 6 ± 1 h.

Cytochemical preparations for flow cytometric detection of DNA/BrdUrd and apoptotic cells were made as previously described (5).

Computer Simulation of Cell Cycle and Drug Effects

The cell cycle mathematical model (Fig. 2) is based on the theory of age-structured cell populations (7–10), and the underlying equations (1) describe the time course of the variables \( N_{G1}(a,t) \), \( N_{S}(a,t) \), and \( N_{G2M}(a,t) \) representing the number of cells of age \( a \) in a given cell cycle phase at time \( t \). In practice, in each phase, the simulation follows the cohorts of cells with the same age (±15 min) while they mature (by increasing their age).

The drug effects were superimposed on the cell cycling model, using specific parameters describing cell cycle arrest or delay (cytostatic effect) and cell killing (cytotoxic effect) in each phase.

Further details of the model are reported in Supplementary Methods.

Optimization and Sensitivity Analysis

We adopted a principle of parsimony for the best fitting of the experimental data with the fewest parameters. Minimal models with few parameters (such as a simple block in one or two phases) were initially tested, then the number of parameters was judiciously increased until satisfactory fits were reached, in keeping also with qualitative information on the presence or absence of cell loss and its monotonic dose dependence.

Because we could not find a satisfactory function of merit for fitting together all the measures obtained with different techniques and precision, and we wanted to take account of qualitative information too, we renounced to perform a standard nonlinear fitting and we adopted an integer penalty score with the supervised trial-and-error fitting procedure described in detail in Supplementary Methods.
Results

Experimental data. The data set used for this study are reported as Supplementary Table. It includes time courses of absolute cell counts and cell cycle percentages and short-time pulse-chase BrdUrd data. All treatments were on ovarian cancer cells (IGROV1) in standardized conditions of exponential growth. For each drug, doxorubicin, cisplatin, topotecan, paclitaxel, and melphalan, 1-hour treatment with several concentrations was analyzed to span the whole range of efficacy.

Time course plots of absolute cell number, %G1, %S, and %G2-M for each drug were reported in Supplementary Figure, together with their best-fit model parameters. BrdUrd experiments distinguish the effects specifically occurring in cells treated in S phase (BrdUrd positive), and the model can catch this additional level of complexity. Only for melphalan and topotecan, S phase-specific effects had to be considered to explain the data (discussed in refs. 3, 5), whereas for cisplatin, paclitaxel, and doxorubicin, the simple time dependence of the effect was enough for fitting.

G1 checkpoint activity. Figure 3 shows the time course of checkpoint activity after treatment with the five drugs, as measured by the model parameter pBlG1 (i.e., the fraction of cells actually intercepted and blocked among those crossing the G1 checkpoint between two experimental time points).

There were two bursts of G1 checkpoint activity in the time dependence of the parameters: one short-term, immediately after treatment (in the 0–6 hour interval), and the second after 24 hours. At suitable concentrations, we observed both kinds of G1 block with doxorubicin and topotecan, only the short-term one with cisplatin and only the long-term one with paclitaxel, whereas G1 activity remained weak in melphalan-treated cells. Considering cell kinetics, this first burst (0–6 hours) mainly involved cells treated in G1 and some that were in G2-M; no cell treated in S had time to reach the G1 checkpoint in 6 hours. Thus, the results indicate that cisplatin, doxorubicin, and topotecan were able to damage...
cells in G₁ and rapidly activate the G₁ checkpoint. In contrast, G₁ cells treated with melphalan and paclitaxel gave only a weak response, and most of them were able to proceed into S phase.

Figure 3 also shows the dose dependence of the short-term (C) and long-term G₁ block (D). All dose responses were sigmoid and were fitted using a Hill function. Fitting the short-term block indicated a 10-fold concentration range before this effect reached its maximum, whereas the long-term block gave a steep increase, strongly suggesting a threshold concentration. Two, not exclusive, explanations of the gradual increase of the short-term block are possible. The first is that at increasing drug concentrations, more and more cells were damaged enough to trigger the checkpoint. The second is that the checkpoint is triggered at the beginning of the increase, but the blocking activity is reduced by the presence of cells exiting the block before 6 hours, possibly due to repair. In this view, the efficacy of the repair is progressively lost as the concentration increases, but remains detectable over a wide range of concentrations.

**G₂-M checkpoint activity.** The model parameter pBlG₂M represented the probability that the cells crossing G₂-M checkpoint were intercepted and remained blocked there instead of dividing and entering G₁ phase. In samples treated with low drug concentrations (Fig. 4A), G₂-M blocking activity reached its maximum or near maximum in the first interval (0–6 hours), decreasing and exhausting itself by 6 or 24 hours. When the drug concentration was higher, the blocking in this phase became stronger and longer (Fig. 4B). With melphalan, the onset of the block was delayed, indicating that cells treated in G₂-M or late S, which reached the G₂-M checkpoint in the first 6 hours, were not blocked. Instead, cells treated in earlier cell cycle phases, reaching the checkpoint later, were blocked and >50% of cells crossing G₂-M continued to be intercepted there up to the end of the observation.

With regard to concentration dependence, the fitting indicated that in most cases, the long-term G₂-M response was much steeper than the short-term one (Fig. 4C and D), with different behavior only in cells treated with topotecan.

**S checkpoint activity.** The model parameter associated with the S checkpoint is the fractional reduction of the average DNA synthesis rate (pDS) compared with untreated cells. Figure 5 shows the time course and the concentration dependence of pDS.

At low concentrations (A), S phase was not immediately delayed, but after 6 or 24 hours, the DNA synthesis rate was reduced with most drugs. At higher drug concentrations (B), DNA synthesis was...
immediately reduced with cisplatin, doxorubicin, and topotecan, whereas the short-term S-response remained weak for melphalan and paclitaxel. S-phase checkpoint activity was sustained after 24 hours with all drugs.

The shape of the concentration dependence of this parameter (C and D) was similar to that observed for pBlG1 and pBlG2M, with a gradual dose-dependent increase at short times after treatment, and a steep dose response 24 hours from the end of treatment.

Order of checkpoint activation. Table 1 shows the threshold concentrations necessary to trigger G1/S/G2-M responses, the 4 panels reporting the responses 0 to 6, 6 to 24, 24 to 48, and 48 to 72 hours after treatment. This enabled us to follow the order in which the different responses were activated, from sublethal to lethal drug concentrations.

Sublethal (<10 μmol/L) cisplatin concentrations immediately activated the checkpoints in all phases (A), then cells probably repaired their damage before division because no cell kill was observed. Much higher concentrations of cisplatin were required to sustain the response for >24 hours in S and G2-M (C and D). Cell kill occurred at the same concentrations.

Melphalan triggered short-term G2-M block and persistent S delay at sublethal concentrations (between 5 and 10 μmol/L), but these responses were selective for BrdUrd-negative cells, whereas BrdUrd-positive cells were not delayed. At moderately lethal concentrations (20–40 μmol/L), long-term G2-M block and S delay were active (C and D). Very high concentrations (>100 μmol/L) were required to trigger a G1-S short-term response, and long-term G1 block was never observed.

With paclitaxel, a short-term G2-M block was triggered at very low concentrations (<0.01 μmol/L; A). At higher but still sublethal concentrations (0.02–0.05 μmol/L), a G1 block and an S delay were also triggered, which, differently from G2-M, persisted for a long time. At concentrations of at least 0.1 μmol/L, a G2-M block was active between 6 and 48 hours and cell kill occurred.

In doxorubicin-treated cells, sublethal concentrations (<0.5 μmol/L) only activated a temporary S delay and G1 block in the 6- to 24-hour interval. Then, all main responses were activated in a short concentration range (1–4 μmol/L), producing moderate lethality.

Topotecan induced a short-term S/G2-M response at very low concentrations (<0.05 μmol/L; A). The effects were restricted to BrdUrd-positive cells. Then, at ~0.1 μmol/L, S delay became long-lasting and a long-term G1 block appeared. At immediately higher but still sublethal topotecan concentrations (0.25 μmol/L), we observed a short-term G1 block of BrdUrd-negative cells. Then, at ~1 μmol/L, cell kill came in play. At higher concentrations, we observed additional cytostatic effects in both BrdUrd-negative and BrdUrd-positive cells.
Discussion

We adopted an interdisciplinary approach to measure the dynamics of checkpoint activities, joining information from independent experimental platforms and interpreting all data univocally with a simple mathematical model of cell cycle proliferation.

The model belongs to the class of age- and phase-structured models with compartments of cells out of cycle (8, 9, 11, 12). Other authors designed specific models of the anticancer therapy (13, 14), some theoretical, others oriented to fit data, and some including a modelization of cell proliferation through the cell cycle.

Data-oriented models were used to interpret tumor growth/regression curves (15), pulse-labeled mitosis (16), or flow cytometric experiments (12, 17, 18). Several researchers used such models to evaluate the response to treatment at different scales and with or without cell age or $G_1/S/G_2-M$ structure (19–24). Our data-oriented modeling approach was initially developed as a tool to support a more correct interpretation of flow cytometric DNA histograms, where the presence of $G_2-M$, but not $G_1$, block can be easily detected and the heterogeneity of the response can be overlooked. Despite several refinements, our model is based on the simple concept of cells flowing through cell cycle phases (of known duration), which encounter a checkpoint where a fraction of them is stopped. As we deal with age cohorts of thousands of cells, this fraction gives the probability that cells are intercepted while crossing the checkpoint, thus measuring checkpoint activity at a specific time. In other words, the activity of a checkpoint is high when the probability to arrest cells is close to 1 (almost all cells crossing the checkpoint are arrested) and low when it approaches zero. Then, such stopped cells may reenter in cycle or die, with given probabilities. The computer program we use is a flexible framework where the user can design simple as well as quite sophisticated models, simulate the cell cycle proliferation, and evaluate outputs corresponding to different kinds of experimental data.

The value of this descriptive approach is not only a correct data interpretation. Modeling a phenomenon using parameters with a clear biological meaning (the functioning of $G_1$, $S$, $G_2-M$ checkpoints in our case) leads to a quantitative understanding of the behavior of specific “modules” (e.g., checkpoint networks) of the system, which is beyond direct experimental observation capabilities.

We analyzed the results obtained with our model with five drugs belonging to different classes of molecular mechanism of action. The cell cycle effects of these drugs have been studied for a long time now (25). Our results were not conflicting with most of the literature on the cell cycle effects of the drugs, but describe together many of the phenomena in play, whereas other published works often focus only on some of them. For instance, doxorubicin was shown to produce cell cycle perturbations in all phases by several authors, stressing $G_2$-phase (26, 27), $G_1$-phase (28, 29), or S-phase effects (30, 31). However, previous studies lack in the description of the time and dose dependence of these effects and how they combine, which is the aim of our work.

Comparing the five drugs, we found that it is possible to define a few types of response. In general, the time dependence highlighted two waves of checkpoint activity, one short term and the other long term, in each phase. The dose response in most cases was fitted well by Hill-type functions, consistent with the concept that this kind of function may be a general form of response in complex enzymatic systems (32). The overall response of each drug is made up of the combination of these activities, which operate at different strengths, and with specific drug concentration thresholds. However, we did not find a common phase sequence for checkpoint activation, and in most cases, the response involved different cell cycle phases, and more than one checkpoint may be sequentially or simultaneously activated, depending on drug concentration (33). These findings also suggest that individual cells undergo multiple checks in subsequent cell cycle phases. Thus, the association between maintenance of cells in a single blocking phase and completion of the repair process may be less strong than was commonly believed, eventually weakening the concept of checkpoint itself.

According to this analysis, statements such as “the drug acts by blocking in phase $G_1$, $S$, or $G_2-M$” may be inadequate to describe the response to a drug challenge. This does not mean to deny the concept of phase specificity, but this study should help to take account of the complexity of the cell kinetics and of the dynamics of checkpoint activities, avoiding too simplistic interpretation of experimental data (3, 34). Moreover, only a fraction of cells is intercepted and blocked at checkpoints, the others go further, and then blocked cells repair and recycle with different rates, a fraction being eventually committed to death. Inter-cell heterogeneity of response has been a somewhat neglected issue in vivo [as recently pointed out by Minchinton and Tannock (35)] and has been even...
more neglected by researchers dealing with ideally "homogeneous" cell lines in vitro growing in a homogeneous environment. However, this is not really surprising, considering that in vitro, too, the intracellular levels of a drug vary widely among cells of the same culture (36), and so do cellular levels of proteins (37–39), including those relevant to checkpoint functioning, repair, and apoptosis.

The probabilistic meaning of the parameters describing checkpoint activities reflects this heterogeneity in our mathematical model. Different models can be drawn in principle to mimic the heterogeneity in different mathematical ways, and other optimization procedures, such as EM methods and genetic algorithm, could be used to mine these data, which were not yet explored. We hope that public access to our database (Supplementary Table) will contribute to further theoretical investigations, stimulating the search for alternative models to find meaningful characteristics of the response besides those emerging from the present study, reaching new insights in these phenomena.

Although the reported measures of checkpoint activities should be considered specific for IGROV1, there is no reason to believe that cell cycle perturbations are any simpler with other cell lines. Each cell line may use the same bricks to build up the drug response with different strength, or not use one brick at all if the line is defective for important proteins of the associated network. Similar studies with other cell lines have qualitatively confirmed the responses with IGROV1 (5) and revealed the contribution of particular molecular defects in each checkpoint activity (4).

This article addresses cell cycle responses to short (1 hour) drug treatments. From here, more complicated issues can be addressed on solid ground toward understanding dose-response relationships with any drug scheduling, with drug combinations, and the transfer of these concepts to the interpretation of in vivo experimental models. The model also offers a first step to connect the "cell population" level of research to the more microscopic molecular level.

The dynamics of checkpoint activity shown here constitute a challenge for molecular systems biology to integrate molecular interaction schemes with quantitative descriptions of the activity of the specific molecular networks.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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