Systematic Analysis of Cell Cycle Effects of Common Drugs Leads to the Discovery of a Suppressive Interaction between Gemfibrozil and Fluoxetine

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Abstract

Screening chemical libraries to identify compounds that affect overall cell proliferation is common. However, in most cases, it is not known whether the compounds tested alter the timing of particular cell cycle transitions. Here, we evaluated an FDA-approved drug library to identify pharmaceuticals that alter cell cycle progression in yeast, using DNA content measurements by flow cytometry. This approach revealed strong cell cycle effects of several commonly used pharmaceuticals. We show that the antilipemic gemfibrozil delays initiation of DNA replication, while cells treated with the antidepressant fluoxetine severely delay progression through mitosis. Based on their effects on cell cycle progression, we also examined cell proliferation in the presence of both compounds. We discovered a strong suppressive interaction between gemfibrozil and fluoxetine. Combinations of interest among diverse pharmaceuticals are difficult to identify, due to the daunting number of possible combinations that must be evaluated. The novel interaction between gemfibrozil and fluoxetine suggests that identifying and combining drugs that show cell cycle effects might streamline identification of drug combinations with a pronounced impact on cell proliferation.

Introduction

Adjusting rates of cell proliferation is the objective of many therapeutic strategies. Most often, the goal is to impede or block cell proliferation of target cells, as with chemotherapy in cancer. In other cases, as in tissue regeneration, the goal is to promote cell proliferation. Proliferating eukaryotic cells pass through a series of highly regulated cell cycle phases, culminating with mitosis [1]. Hence, drugs that influence the timing of cell cycle transitions are useful in efforts to adjust rates of cell proliferation.

Identifying drugs that potentiate the effects of other drugs is the leading therapeutic strategy in the treatment of numerous diseases, such as cancer [2], tuberculosis [3] and HIV-AIDS [4]. Conversely, drug interactions may suppress a desired response, or even lead to a harmful outcome. Screening libraries composed of a few hundred thousand compounds for a sought-after effect of a single chemical is now common [3]. However, testing all the possible combinations, even binary ones, of these chemicals represents a formidable obstacle [6].

Here we report a systematic analysis of cell cycle progression of yeast cells exposed to a panel of FDA-approved drugs. We document novel cell cycle effects of several compounds. We also reasoned that drugs that affect cell cycle progression might be more likely to display interactions with other such drugs, and thereby greatly impact overall cell proliferation. We demonstrate one such novel drug interaction, between gemfibrozil and fluoxetine.

Results and Discussion

We used a commercially available panel of 640 FDA-approved drugs (see Materials and Methods). The target cells were Saccharomyces cerevisiae budding yeast, a model system of eukaryotic cell cycle studies [1]. We monitored the effects of each drug on cell cycle progression by measuring the DNA content of the cells by flow cytometry [7] (see Figure 1, and Materials and Methods). The G1 phase of any given cell cycle lasts from the end of the previous mitosis (M phase) until the beginning of DNA synthesis (S phase). Any drug that alters the length of the G1 phase relative to the rest of the phases of the cell cycle will alter the DNA content profile. We quantified each sample in an automated manner, recording the percentage of cells with unreplicated genome (%G1, see Materials and Methods). We did not quantify complex profiles (see Figure 2), and we excluded these drugs from further analyses. At the beginning and end of most batches of samples, we measured the reference sample (a yeast strain that lacks the multidrug transporters Pdr5p and Snq2p, mock-treated with DMSO; see Materials and Methods), which was cultured and processed along with the cultures that were treated with drugs. We evaluated each drug in at least two independent experiments. We deposited all...
Figure 1. Decision flow-chart diagram of our primary analysis. This diagram summarizes our DNA content measurements using the pdr5 Δ, snq2 Δ strain. See text for details.
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Figure 2. Representative DNA content histograms. Independent experiments of the indicated samples are shown in each case. Fluorescence is plotted on the x-axis, while the number of cells analyzed is on the y-axis. Reference samples were treated with DMSO, shown at the top. Examples of “High G1” profiles include cells treated with ketoconazole or gemfibrozil, while cells treated with fluoxetine give rise to a “Low G1” DNA content profile. At the bottom, we show a few examples of complex DNA content histograms that were unquantifiable. These include profiles of cells treated with suramin and 5-fluorouracil (antineoplastic agents), and flubendazole (a microtubule blocker used as anti-nematodal).

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raw flow cytometry data in a public database (see Dataset S1, and Materials and Methods).

To identify drugs that altered the cell cycle, we compared the frequency distribution of cultures treated with drugs against a normal distribution fit of the reference (n = 82) samples (Figure 3A). Samples that had a %G1 greater than or less than two standard deviations from the mean of the reference sample distribution were considered to differ significantly from the mock-treated samples (Figures 1 and 3A). Drugs that led to an increase (%G1 > 60.00%) in the percentage of cells with unreplicated DNA formed the “High G1” group, while others led to a mitotic delay and a “Low G1” (%G1 < 38.76%) DNA content (see Figure 3A, and Dataset S1). In this initial screen, we added the drugs to cultures diluted from an overnight stationary phase culture, where most cells would be in the G1 phase of the cell cycle [1]. Hence, drugs in samples with a “High G1” DNA content may have arrested cell cycle progression non-specifically. In that case, the high G1 DNA content reflected the state of the starting culture, and not cell cycle effects of the drugs. To exclude such possibilities, we re-tested the “High G1” drugs by adding them to actively dividing cells (see Figure 1). Overall, from this primary analysis we identified 27 compounds that interfered with progression in the G1 phase of the cell cycle, before initiation of DNA replication, resulting in a “High G1” DNA content (see Table S1). Another 12 drugs affected mitotic progression, resulting in a “Low G1” DNA content (see Table S2).

Along with DNA content, we also analyzed the forward scatter (FSC) from the same flow cytometry experiments (see Figure 3B). FSC values often serve as a proxy for cell size, but they are also affected by cell shape and intracellular composition [8]. We noticed that most drugs in the “Low G1” group had elevated FSC values compared to the group with no cell cycle effects (Figure 3B).

This is consistent with the notion that mitotic delay leads to an increase of cell size. It should also be noted that yeast cells in mitotic phases of the cell cycle are budded [1]. Hence, their irregular shape may also contribute to an increase in FSC values. An increase of FSC values was also evident for a significant fraction, but not all, of drugs in the “High G1” group (Figure 3B).

We are not aware of other systematic studies of drug effects on cell cycle progression measured by DNA content analyses. Our results reveal that several drugs currently and commonly used for human therapy have specific effects on the eukaryotic cell cycle. The higher number of drugs that interfered with G1 progression likely reflects the fact that cells commit to initiation of cell division in the G1 phase [1,9,10]. Among the “High G1” group, we noted antifungals that inhibit biosynthesis of ergosterol, a component of fungal membranes [11], and rapamycin, a potent inhibitor of the TOR pathway known to block G1 progression [12]. Overall, however, there was a diverse range of compounds in the “High G1” group (see Table S1). Although most drugs in the “Low G1” group have well established mitotic roles (see Table S2), we noted that the highest-ranked drug from this group was fluoxetine (brand name Prozac). To our knowledge, this is the first time that such strong cell cycle effects have been reported for fluoxetine.

Since we did our primary analysis in a sensitized pdr5Δ, snq2Δ yeast strain, we then tested the drugs that led to the “High G1” and “Low G1” groups against the PDR5Δ, SNO2Δ wild type reference strain BY4741. We found that several drugs were not effective in this case. For example, lovastatin, which leads to a G1 arrest in mammalian cells [13], had no effect in PDR5Δ, SNO2Δ yeast cells (see Table S1). This is consistent with an earlier report that yeast cells are sensitive to lovastatin in a pdr5Δ-dependent manner [14]. Nonetheless, about half of the drugs in both groups remained effective in cells with intact multidrug transporters (see Tables S1 and S2).

Among drugs that led to a “High G1” DNA content, we further examined the cell cycle effects of the potent antilipemic gemfibrozil [15], a Peroxisome Proliferator-Activated Receptor α (PPARα) agonist. To our knowledge, a G1 cell cycle role for gemfibrozil has not been reported, in any system. The High G1 DNA content could result from roles specific to G1 progression, or manifest in G1 as a “carryover” from roles in other cell cycle phases. To distinguish between these two possibilities, we added gemfibrozil to highly synchronous newborn G1 cells, obtained by centrifugal elutriation [16,17].

As a function of time, we then measured cell size and the percentage of budded cells [budding correlates with initiation of DNA replication in yeast [1]]. This allowed us to measure the...
length of the G1 phase accurately, by calculating two parameters:
i) the “critical size” these newborn daughter cells must attain to
initiate cell division; ii) the rate (“growth rate”) at which they grow
to their critical size. DMSO-treated cells had a critical size of
63.2±2.4 fl and a specific growth rate constant $k=0.328±0.008\ h^{-1}$ (Figure 4). Rapamycin markedly prolonged the
G1 phase, because cells had to reach a substantially larger critical
size (79.4±1.2 fl) before they could initiate DNA replication (Figure 4A). Rapamycin-treated cells also grew very
slowly ($k=0.104±0.004\ h^{-1}$, Figure 4B), although this effect was
evident ~1 h after addition of the drug (Figure S1). We found that
cells treated with gemfibrozil delayed initiation of DNA replica-
tion, not because they had altered critical size (65.4±0.6 fl,
Figure 4A), but because they reached that size slower than cells
treated with DMSO did ($k=0.287±0.07\ h^{-1}\ \ P=0.005$,
Figure 4B). In addition, from the cell size distributions of
asynchronously dividing cells, we obtained the “birth size” of
newborn cells (see Materials and Methods). While DMSO-treated
cells had a “birth size” of $40.3±2.7\ fl$ under these growth
conditions, gemfibrozil-treated newborn cells were significantly
smaller ($30.1±4.7\ fl, P=0.04$, Figure 4C). Taken together, these
data show that the smaller “birth size” and slower “growth rate”
of cells treated with gemfibrozil lengthen the G1 phase.

Next, we focused on the effects of gemfibrozil and fluoxetine on
overall cell proliferation rates. We tested these drugs alone and in
combination, at several doses (Figure 5A). We found that
gemfibrozil did not significantly affect overall cell proliferation at
the doses tested (Figure 5). Hence, the prolongation of the G1 phase by gemfibrozil is likely accompanied by compensatory
shortening of subsequent cell cycle phases, resulting in similar
overall generation time. On the other hand, fluoxetine arrested
proliferation of yeast cells at 200 $\mu$M (Figure 5A, first green bar to
the left; and Table S3, bottom left cell). To our knowledge, the
near complete inhibition of yeast cell proliferation by fluoxetine
has not been reported. Remarkably, however, addition of
gemfibrozil even at a 4-fold less molar concentration fully
suppressed the inhibitory effects of fluoxetine (see Figure 5A,
compare the left green bar to the other green bars; and Table S3,
last row).

We then added the two drugs not simultaneously, but in
different order, removing the first drug before adding the second
(Figure 5B). We found that gemfibrozil suppressed fluoxetine’s
anti-proliferative effects only if added before (representative
experiment in Figure 5B, compare the blue and yellow bars on
the right; and Table S4, compare the top and middle cells in the
3rd column), but not after fluoxetine (Figure 5B, compare the left
and middle green bars; and Table S4, compare the left and middle
cells in the 3rd row). These results suggest that the suppressive
interaction between gemfibrozil and fluoxetine is not due to
extracellular interaction or competition for transport between the
two drugs. Furthermore, the results from the order-of addition
experiment suggest that gemfibrozil acts upstream, since it does
not reverse fluoxetine’s inhibition of cell proliferation. Instead, it
appears that fluoxetine cannot inhibit cell proliferation in the
context of gemfibrozil’s prior action.

Understanding the basis of the interaction between gemfibrozil
and fluoxetine requires a mechanistic understanding of their
function in yeast cells. We examined the combined effects on cell
proliferation between gemfibrozil and fluoxetine because of the
novel cell cycle effects of each compound, affecting different
phases of the cell cycle. We would like to note, however, that the
suppressive interaction between the two compounds could be
unrelated to their cell cycle effects. For example, gemfibrozil might
induce expression of proteins that do not interfere with cell cycle
progression, but may cause fluoxetine resistance. Fluoxetine is an
anti-depressant thought to act as a serotonin-specific reuptake
inhibitor [18]. Hence, the effects we described for fluoxetine in
yeast appear to result from some other mechanism. Similarly,
nuclear receptors of the PPAR$\alpha$/RXR type, the target of
gemfibrozil, are thought to be unique to animals and sponges
[19,20], but ancestral analogs may exist in yeast [21]. Nonetheless,
although the effects of fluoxetine and gemfibrozil on yeast cells
were described above likely represent off-target modes of action, they
may act similarly in other eukaryotic organisms, including
humans. In conclusion, our results suggest that monitoring the
effects of drugs on cell cycle progression reveals unexpected
cellular roles of widely prescribed compounds. Finally, although
we did not test all possible combinations of the compounds that
affected cell cycle progression, at least in the case of gemfibrozil
and fluoxetine, our results suggest that combining such compo-
unds may also be an effective strategy to identify novel drug
interactions.

Materials and Methods

Yeast strains

For our primary analysis, we used the S. cerevisiae strain JTY2953
(MATa $pdr3::TRP1\ upg2::hisG\ ade2-104\ his3-$\Delta$00\ leu2-$A1$\ lys2-$B1$\ ura3-$C2$; a generous gift from Dr. Paul deFigueiro-
edo, Texas A&M University). For the elutriation experiments in
Figure 4 we used the diploid strain BY4743 (MATa/\ his3A1\ leu2A0/leu2A0\ lys2A0/lyS2\ MET15/met15A0\ ura3A0/ura3A0; commercially available from Open Biosystems). For all
other experiments, we used the haploid strain BY4741 (MATa
his3A1\ leu2A0\ met15A0\ ura3A0; commercially available from Open
Biosystems).

Media and culture conditions

In all experiments, strains were cultured at 30°C in YPD (1%
yeast extract, 2% peptone, 2% dextrose). For our primary analysis
with the JTY2953 strain, overnight cultures were diluted 1:200
and aliquoted into 96-well plates, 198 $\mu$L per well. To each well we
then added 2 $\mu$L of a drug stock solution (2 mg/ml in DMSO),
resulting in a final drug concentration of 20 $\mu$g/ml. At the four
corner wells of each 96-well plate, the cultures were treated with
DMSO only. These cultures served as the mock-treated reference
samples. The plates were then placed at 30°C and incubated for
6–7 h. Each of the 200 $\mu$L cultures were then transferred to
microcentrifuge tubes containing 500 $\mu$L ethanol, and sonicated for
5 s. For the experiments where the drugs were added in dividing
JTY2953 cells, the overnight cultures were diluted 1:400 and
incubated for 3 h at 30°C. We then added the drugs of interest and
incubated the plates at 30°C for another 6 h before fixing the
samples in ethanol. For DNA content measurements in BY4741
cells, which proliferate faster than JTY2953 cells do, overnight
cultures were diluted 1:400, cultured for 2.16 h before we added the
drugs of interest, and then cultured for another 4.33 h before
they were fixed in ethanol.

Cell size determinations

To obtain size distributions from asynchronous cultures,
onight cultures of BY4743 cells were diluted 1:500 in fresh
medium, and incubated for 2 h at 30°C. We then added the drugs
of interest and incubated at 30°C for another 4 h. Cell size was
then measured with a Beckman Z2 Channelizer. For each sample
we analyzed, we obtained size distributions from two different
dilutions of cells. The average of the geometric mean of each size

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distribution was recorded. We used the Accucomp Beckman software package to obtain the statistics of each size distribution.

Measurements of critical size and growth rate from elutriated cultures

For isolation of early G1 daughter cells, cultures were grown in YPD at 30°C to a density of \(\sim 1 \times 10^7\) cells/ml, then fractionated with a Beckman JE-5.0 elutriator as described previously [16]. Early fractions containing predominantly (>95%) small unbudded cells were collected by centrifugation, re-suspended in fresh medium and aliquoted in three separate flasks. To each flask, we then added as indicated rapamycin (at 0.1 µg/ml), gemfibrozil (at 50 µg/ml), or DMSO alone. After testing several doses of each drug and measuring the DNA content, we decided to use these concentrations because they were the lowest ones that resulted in consistently pronounced effects in this strain background. The cultures were incubated at 30°C. Every 20 min we monitored the percentage of budded cells and cell size. The “critical size” is the size at which 50% of the cells have budded in these experiments, and it was calculated as we described elsewhere [16]. To calculate “growth rate” assuming exponential growth, we plotted the natural log (ln) of cell size as a function of time (in h), see Figure S1. We fit the data to a straight line using the regression function in Microsoft Excel. From the slope of the line, we obtained the specific rate of cell size increase constant \(k_{inh}\). The average of all experiments (n = 3) for each treatment was then calculated, along with the associated standard deviation.

Staining for DNA content analyses

Fixed cells were stored at 4°C overnight to 14 days. Cells were collected by centrifugation and stained overnight in 0.5 ml staining solution containing 50 mM sodium citrate pH 7.0,
0.25 mg/ml RNaseA, and 1 μM SYTOX Green (Molecular Probes). Samples were stored at 4°C overnight in opaque containers. Cell suspensions were sonicated briefly at the fixing and staining steps and immediately before flow cytometry.

**Flow cytometry data acquisition, deposition and analysis**

Stained cells were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems) flow cytometer, using CellQuest (version 3.3; Becton Dickinson Immunocytometry Systems) acquisition software. Sytox Green fluorescence was collected through a 515/30-nm bandpass filter, and list mode data were acquired for 10,000 cells defined by a dot plot of FSC versus SSC. Prior to each experiment, standard beads (Cyto-Cal Multifluor Intensity Beads, Thermo Scientific) were used to calibrate the flow cytometer, and photomultiplier tube voltages were adjusted to place the highest intensity bead in the same channel. FACS files were archived at Cytobank. Automated quantification of the DNA content histograms was done with FlowJo 7.5 software. To exclude particulate non-yeast events, which had both very low forward scatter (FSC) and low fluorescence (FL2-A), asymmetrical gates were fitted with the autogating tool. Gates were centered near FSC ~100 and FL2-A

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**Figure 5. A novel interaction between gemfibrozil and fluoxetine.** A. Fluoxetine strongly inhibits yeast cell proliferation, but it is suppressed by gemfibrozil. We added to freshly reseeded wild type haploid yeast (BY4741) cells DMSO, fluoxetine and gemfibrozil at the binary combinations and concentrations shown. We then monitored cell proliferation hourly, for 8 h (see Materials and Methods). The specific growth rate constant (k) for each combination is shown. The errors associated with these measurements are shown in Table S2. B. DMSO, fluoxetine and gemfibrozil were added to dividing cells at 200 μM in binary combinations, sequentially, in the order shown. Cell proliferation was monitored for 6 h as in a, with the specific growth rate constant (k) for each combination shown. Data from one representative experiment is shown. Suppressive effects of gemfibrozil on fluoxetine arising from order of addition were assessed by calculating growth rate constant (k) folds for gemfibrozil treatment over DMSO control for all experiments, initial treatment with gemfibrozil yielding a fold of 2.51 +/- 0.25, versus final treatment, 0.71 +/- 0.21, P-value = 0.000146.

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Drugs that lead to a High G1 DNA content.

Fluoxetine strongly inhibits yeast cell proliferation, but it is suppressed by gemfibrozil.

Drugs that lead to a Low G1 DNA content.

Gemfibrozil suppresses fluoxetine’s anti-proliferative effects only if added before, but not after, fluoxetine.

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Further divided for treatment with the second drug, as above, resulting in nine total temporal combinations of vehicle, gemfibrozil, and fluoxetine. Growth constants were calculated as above from 0 h through 6 h.

Drugs

The FDA-approved library was purchased from Enzo (Cat. #: BML-2841). Artemisinin was from Enzo (Cat. #: ALX-350-219), gemfibrozil from Sigma (Cat. #: G9518), while chlorpromazine (Cat. #: 101077-482), fluoxetine (Cat. #: 89160-860) and clinafloxacin (Cat. #: 39150-368) were purchased through VWR International. All drug stock solutions were in DMSO.

Supporting Information

Figure S1 Determining the length of G1. Left, Graphs from which we determined the specific rate of cell size increase constant k, shown in Figure 4, from the same elutriation experiments. The natural log cell size (y-axis) is plotted against time (shown in hours, x-axis). Right, Graphs of the fraction of budded cells (y-axis) as a function of cell size (in fl, x-axis), from the same elutriation experiments. The data points shown were used to estimate the critical size for division we show in Figure 4A. In A, the cells were treated with DMSO, in B with rapamycin (at 0.1 μg/ml), and in C with gemfibrozil (at 50 μg/ml).

Table S1 Drugs that lead to a High G1 DNA content.

Table S2 Drugs that lead to a Low G1 DNA content.

Table S3 Fluoxetine strongly inhibits yeast cell proliferation, but it is suppressed by gemfibrozil.

Table S4 Gemfibrozil suppresses fluoxetine’s anti-proliferative effects only if added before, but not after, fluoxetine.

Dataset S1 Searchable spreadsheet of all the primary data, arranged in different worksheets.

Author Contributions

Conceived and designed the experiments: MP. Performed the experiments: SAH CD IM SE SCS SSD EMH KED RS MP. Analyzed the data: SAH CD IM SE SCS RS MP. Contributed reagents/materials/analysis tools: SAH RS MP. Wrote the paper: SAH RS MP.

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