An Image-Based, High-Throughput Screening Assay for Molecules that Induce Excess DNA Replication in Human Cancer Cells

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Abstract

Previous studies have shown DNA re-replication can be induced in cells derived from human cancers under conditions in which it is not possible for cells derived from normal tissues. Because DNA re-replication induces cell death, this strategy could be applied to the discovery of potential anticancer therapeutics. Therefore, an imaging assay amenable to high-throughput screening was developed that measures DNA replication in excess of four genomic equivalents in the nuclei of intact cells and indexes cell proliferation. This assay was validated by screening a library of 1,280 bioactive molecules on both normal and tumor-derived cells where it proved more sensitive than current methods for detecting excess DNA replication. This screen identified known inducers of excess DNA replication, such as inhibitors of microtubule dynamics, and novel compounds that induced excess DNA replication in both normal and cancer cells. In addition, two compounds were identified that induced excess DNA replication selectively in cancer cells and one that induced endocycles selectively in cancer cells. Thus, this assay provides a new approach to the discovery of compounds useful for investigating the regulation of genome duplication and for the treatment of cancer. Mol Cancer Res; 9(3); 1–18. ©2011 AACR.

Introduction

Proliferating mammalian cells undergo mitotic cell cycles in which their nuclear genome is duplicated once, but only once each time a cell divides. Although polyploidy does occur during mammalian development, it is a rare event that results in terminally differentiated cells that are viable but no longer proliferate. Multiple mechanisms exist in mammalian cells to prevent a second round of nuclear genome duplication from beginning until the cell has completed mitosis and cytokinesis. In fact, artificially inducing cells to re-replicate the same stretch of nuclear DNA before mitosis and cytokinesis is an aberrant event that leads to DNA damage, stalled replication forks, and cell death. For example, DNA re-replication can be induced in cancer cells either by overexpression of proteins required for loading the replicative DNA helicase onto replication origins (e.g., Cdc6 and Cdt1) or by suppression of a unique inhibitor of Cdt1 called geminin. However, in normal cells, induction of DNA re-replication requires suppression of both geminin and cyclin A, because the cyclin-dependent protein kinase CDK1/cyclin A inactivates Cdc6, Cdt1, and one of the subunits of the origin recognition complex, all of which are required to load the replicative DNA helicase onto chromatin.

Remarkably, cancer cells differ from normal human cells in that cancer cells rely solely on high levels of geminin to prevent DNA from re-initiating replication before the genome has been completely replicated. Thus, siRNA targeted against geminin induces DNA re-replication in cells derived from human cancers, but not in cells derived from normal human tissues. Mammalian cells that do not initiate DNA re-replication under these conditions, do re-replicate their DNA when both geminin and cyclin A expression are suppressed by siRNA. Therefore, normal human cells utilize multiple pathways to prevent DNA re-replication, whereas many cancer cells rely on a single pathway in which the level of Cdt1 activity limits initiation of DNA replication.

Screens for compounds that can prevent geminin protein binding to Cdt1 protein in vitro have been reported. Of the 3 inhibitors of Cdt1-geminin interaction identified by these screens, only coenzyme Q10 was reported to inhibit proliferation of human cancer cells. However, this inhibition required millimolar concentrations of coenzyme Q10 in the cell culture medium, whereas inhibition of geminin activity in vitro required only...
micromolar concentrations. Moreover, whether coenzyme Q10 selectively inhibited cancer cell proliferation was not clear, and in our previous studies, coenzyme Q10 did not induce DNA re-replication in cancer cells (data not shown). Therefore, rather than screen for molecules that interfere with geminin activity in vitro, an assay was developed to identify small molecules that induce excess DNA replication (EDR) in human cells. Such an assay would detect molecules that inhibit any pathway that restricts genome duplication to once per cell division, regardless of its mechanism of action. For example, molecules that inhibit geminin activity would be detected, so would be molecules that interfere with geminin expression or stability. Compounds would also be detected that trigger DNA re-replication by pathways not yet discovered. Compounds that trigger endoreduplication or endomitosis would also be detected. These are mechanisms used by developmentally programmed stem cells to respond to changes in mitogenic stimuli by undergoing multiple rounds of genome duplication in the absence of cell division (1), generally referred to as endocycles. This assay also records the effect of compounds on cell proliferation, so that compounds that do not induce DNA re-replication in normal cells, but the compounds that nevertheless inhibit their proliferation can be distinguished from compounds that selectively induce DNA re-replication in cancer cells without adversely affecting normal cells.

Here we show the sensitivity, accuracy, and reproducibility of this assay in screening for compounds that induce EDR in human cells. In addition, we report the identification of 68 compounds with activity on cancer cells. Only a fraction of these have been reported previously to induce EDR. Of particular interest are two compounds that induced DNA re-replication [tetraethylthiuram disulfide (Disulfiram or DSF) and 3-phenylpropargylamine hydrochloride], and one compound that induced endocycles (SU6656) selectively in cancer cells. Such molecules should prove useful in elucidating pathways that regulate genome duplication in investigating the mechanism by which mitotic cell cycles are converted into endocycles and in providing new therapies for the treatment of cancer.

**Materials and Methods**

**Cells**

Human colorectal cancer cells SW480 and human breast epithelial tissue cells MCF10A were purchased from the American Type Culture Collection. Both wild-type and p53 nullizygous human colorectal cancer cells HCT116 were kindly provided by Bert Vogelstein (Howard Hughes Medical Institute, Baltimore, MD). SW480 cells were cultured either in RPMI 1640 medium supplemented with 10% FBS, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 0.5 mg/mL penicillin and streptomycin (quantitative high-throughput screening; qHTS) or in Dulbecco’s modified Eagle’s medium with 10% FBS (FACS analysis). MCF10A cells were cultured in serum free mammary epithelial growth medium (MEGM) supplemented with MEGM Bulletkit (Lonza, cc3150) and 50 ng/mL cholera toxin. Both lines were negative for mycoplasma.

**Chemicals**

All chemicals were purchased from Sigma-Aldrich and solubilized in dimethyl sulfoxide (DMSO). The LOPAC1280 (Sigma Aldrich) was plated as 7 fivefold interplate titrations beginning at 10 mmol/L as described (8). For follow-up studies, compounds were plated as 16 twofold intraplate titrations beginning at 10 mmol/L for all compounds except taxol (50 μmol/L) and colchicine (50 mmol/L).

**Excess DNA replication assay and quantitative high-throughput screening**

SW480 or MCF10A cells were plated at 250 cells per 5 μL/well into Aurora 1,536-well clear bottom, black low-base plates (Nexus Biosystems) by a Multidrop Combi (Thermo Scientific). The cells were cultured for 16 hours at 37°C in 5% CO₂ and then either 23 mL DMSO or test compound dissolved in DMSO were transferred to each well in the microtiter plate by an automated pin tool (Kalypsys). Thus, each well contained a final concentration of 0.4% (65 mmol/L) DMSO. Library compounds were added to columns 5 to 48 and controls were added separately to columns 1 to 4 as follows: 16 twofold titrations in duplicate of podophyllotoxin and SU6656 beginning at 46 μmol/L final concentration (columns 1 and 2, respectively), 0.4% DMSO (column 3), and 6 μmol/L SU6656 and 3 μmol/L podophyllotoxin to the top and bottom half, respectively, of column 4. Following 48 hours at 37°C in 5% CO₂, each well received 1 μL Hoechst 33342 (0.56 μg/mL final concentration in PBS), and the plates were incubated at 37°C in 5% CO₂ for 40 minutes. Using an automated plate washer (Kalypsys), medium was aspirated from each well, leaving a residual volume of approximately 2 μL, and 6 μL/well of PBS was added. Nuclei in each well were imaged and enumerated by an Acumen Explorer eX3 (TTP LabTech) plate reader at 405-nm excitation and 420-to 500-nm emission. Classification of fluorescent objects is explained in Figure 1 and the Results section. The percent activity of test compounds was normalized to control wells present on each plate to establish 0% (DMSO alone) and 100% (3 μmol/L podophyllotoxin) activity. These normalized activities were adjusted by applying a pattern correction algorithm by microtiter plates in which cells were treated with DMSO alone. One such plate was placed at the beginning and end of each qHTS run. Titration–response curves were fitted and classified as described (8).

**FACS assay**

SW480 and MCF10A cells were propagated in 75 cm² flasks at 37°C with 5% CO₂ (4). When cells were approximately 30% confluent, DMSO alone or with the indicated concentration of compound was added to test flasks and the cells cultured for an additional 2 days. Flow cytometry was done as described (4) by harvesting the attached cells, staining them with propidium iodide, and then analyzing them in a
FACSCalibur (Becton Dickinson) by Cellquest software. Cells were gated to exclude aggregates. Unless otherwise stated, FACS assays were carried out after 2 days exposure of cells to the EC50 concentration of the test compound, in an effort to mimic conditions in the EDR assay.

Nuclear cytology assay

For nuclear cytology, cells were grown in chamber slides and sealed with Vectashield (Vector Labs), a fade-resistant mounting medium containing 4',6-diamidino-2-phenylin-dole (DAPI). For immunofluorescence, cells were grown in chamber slides, fixed with 4% paraformaldehyde for 10 minutes, rinsed briefly with PBS, and permeabilized for 10 minutes with 0.2% Triton X100. Slides were blocked with 3% bovine serum albumin, 0.02% Tween in PBS, rinsed, and incubated with a phosphoserine 10 Histone H3 polyclonal antibody (Abcam) for at least 1 hour at room temperature. Slides were washed and incubated with a secondary antibody conjugated with Alexa Fluor 594 (Invitrogen) for at least 1 hour. Slides were washed and then sealed with Vectashield (Vector Labs) mounting medium and examined by a fluorescence microscope (Nikon). Unless otherwise stated, nuclear cytology assays were carried out after 2 days exposure of cells to the test compound.

Cell proliferation assay

Cells were propagated in the same manner as for FACS analysis except that tissue culture dishes were used. The EC50 amount of test compound was added to cells 1 day after seeding them at approximately 3 x 10^6 to 6 x 10^6 cells per 100 x 15 mm dish. To quantify the number of cells per dish, nonattached cells were removed by washing the
dishes with PBS and then attached cells were released by trypsinization, recovered by centrifugation, and resuspended in PBS and counted in a hemocytometer.

Results

An image-based assay that measures excessive DNA replication in cells

An assay for EDR in human cells that is amenable to high-throughput screening of small molecules was developed by culturing cells in microtiter plates, and then staining them with a fluorescent dye (Hoechst) that binds DNA to image them by a laser-scanning microtiter plate cytometer (9). Only cells adhering to the well were imaged, thereby avoiding dead cells floating in the medium. Each fluorescent object was enumerated into 4 populations based on its fluorescence intensity and shape (Fig. 1). Using total fluorescence intensity as a measure of nuclear DNA content, both quiescent and proliferating cells with a DNA content less than 4 sets of chromosomes (\(<4N\)) were distinguished from cells that had excessively replicated their nuclear DNA to a content greater than the amount in 4 sets of chromosomes (\(>4N\)). Individual cells containing a single nucleus were distinguished from cell aggregates by the shape of the fluorescence emission. Objects whose fluorescence intensity was outside the range observed in nuclei of control cells were excluded as well.

This EDR assay was applied to two cell lines, one that is sensitive to suppression of geminin and one that is not. siRNA targeted against geminin induces DNA re-replication in SW480 cells derived from a human colorectal adenocarcinoma, but not in MCF10A cells derived from normal human mammary tissue (4). Consequently, MCF10A cells continue to divide under conditions where SW480 cells stop proliferating and then undergo apoptosis. These two cell lines were selected, because they adhered easily to the wells of a microtiter plate, proliferated well, and formed distinct and relatively uniform nuclei (Fig. 1). Assay conditions were optimized for the 1,536-well plate format by SW480 cells and RO3306, a small molecule inhibitor of CDK1 that induces DNA re-replication in tumor cells (10). However, RO3306 had a narrow concentration range for activity in this assay (data not shown), and therefore a library of bioactive molecules was screened by the EDR assay to identify additional compounds that induced DNA re-replication. The results revealed that podophyllotoxin, an inhibitor of microtubule assembly (11),

![Figure 2. Activity of SW480 qHTS runs and podophyllotoxin titration–response profiles of from library and control samples. A, activity heat maps of plates incubated with increasing concentrations of the LOPAC\(^{1280}\) compounds. The percent activity of each well was calculated relative to podophyllotoxin (defined as 100% activity) and DMSO (defined as 0% activity). The results are depicted here as a gradient of color where white, blue, and red indicate no, increasing, and decreasing activity, respectively, relative to DMSO-treated control wells. These data were converted into dose–response curves. Podophyllotoxin dose–response curves from the qHTS runs (B and C) in which each of 7 plates containing the concentration indicated in (A) are shown for SW480 (B) and MCF10A (C) cells. Intraplate control titrations of 16 different concentrations of podophyllotoxin (D and E) are shown for SW480 (D) and MCF10A (E) cells. Each panel contains a dose–response curve from two runs (○ dashed line, ⋄ solid line).](image-url)
was an effective inducer of EDR in both SW480 and MCF10A cells with a low EC₅₀ (the concentration that produces 50% of its maximum effect) and full efficacy at concentrations spanning 3 orders of magnitude. Therefore, podophyllotoxin was subsequently used as a standard against which the efficacy of other compounds was measured.

**Titration-based screen of bioactive molecules**

To validate the EDR assay’s performance, and to identify inducers of excessive DNA replication, SW480 and MCF10A cells were tested for their sensitivity to the 1,280 compounds in the "Library of Pharmacologically Active Compounds" (LOPAC¹²⁸₀; Sigma Aldrich) by qHTS. qHTS is a method by which compounds are screened at multiple concentrations to generate a dose–response curve for each compound (8). This approach assigns each compound an activity status, and for each active compound, it determines the potency (as measured by the EC₅₀) and efficacy (as measured by the percent activity). These advantages are critical to identifying compounds that are active in cancer cells but not in normal cells.

Using this assay, the two cell lines were screened against the LOPAC¹²⁸₀ at 7 fivefold serial dilutions beginning at 46 μmol/L and ending at 3 nmol/L (Fig. 2A). Two runs were carried out with each cell line. The activity of each compound was then normalized to control wells included in each plate. Cells treated with DMSO at the concentration present in each test compound were defined as 0% activity, and cells treated with podophyllotoxin at its EC₁₀₀ were defined as 100% activity.

The EDR qHTS performed well on both cell lines. First, the fraction of cells that contained >4N DNA in DMSO-treated wells varied no more than 5% between plates for each validation run (data not shown). Thus, the baseline was stable from one plate to the next and from one run to the next. Second, the titrations of podophyllotoxin carried out in each plate were reproducible, generating EC₅₀ values of 54 ± 5 nmol/L for SW480 cells and 50 ± 11 nmol/L for MCF10A cells (Fig. 2D and E). These values were comparable to those determined for the podophyllotoxin library sample in the qHTS runs (Fig. 2B and C). Finally, the signal to background (S/B) ratios and Z’-factor scores of the DMSO and podophyllotoxin control wells were sufficient and consistent between plates (Fig. 3A and B). Z’-factor is a

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**Figure 3.** Performance and potency comparisons of qHTS runs. A and B, plate performance measures of signal to background ratio (S/B) and Z’-factor based on positive (EC₁₀₀ podophyllotoxin) and negative (DMSO alone) control wells for each plate of two qHTS runs (⁎, †) of SW480 (A) and MCF10A (B) cells. The Z’-factor was calculated as previously described (8). C and D, the potencies of active and inconclusive compounds were reproducible between qHTS runs for each cell line. Plots of log EC₅₀ values were fitted by linear regression and showed correlation for SW480 (r² = 0.9, n = 68) and MCF10A (r² = 0.81, n = 195) cells.
Table 1. LOPAC1280 compounds with activity on SW480 cancer cells

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<th>EC50 (μmol/L)b</th>
<th>EC50 (μmol/L)c</th>
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<td>MCF10A</td>
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(Continued on the following page)
Z'-factors of 0.5 or more are considered excellent assay performance, and most plates scored as such for each of the runs. These results confirmed that this image-based screening assay was stable, sensitive into the nanomolar range, and reproducible between separate runs on both cell lines.

Following each qHTS run, the dose–response data for each compound were fitted to a curve by a custom algorithm based on a 4-parameter model, and the curves were categorized into 5 groups (8). Class 1.1 curves exhibited both upper and lower asymptotes, were well fit ($r^2 \geq 0.9$), and had an efficacy greater than 80% (Fig. 2B–E). Class 2.1 curves were similar to class 1.1 curves, except that they exhibited only one asymptote. Compounds that produced well-fit curves ($r^2 \geq 0.9$) with an efficacy between threshold (~30%) and 80% were termed class 1.2, if they exhibited two asymptotes, and class 2.2 if they exhibited one.

<table>
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<th>#</th>
<th>Compound Name</th>
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<td>I</td>
<td>28</td>
<td>18</td>
<td>−43</td>
</tr>
<tr>
<td>59</td>
<td>8-Cyclopentyl-1,3-dipropylxanthine</td>
<td>I</td>
<td>28</td>
<td>16</td>
<td>−49</td>
</tr>
<tr>
<td>60</td>
<td>GBR-12909 dihydrochloride</td>
<td>I</td>
<td>28</td>
<td>18</td>
<td>−62</td>
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<tr>
<td>61</td>
<td>FGIN 1-27</td>
<td>I</td>
<td>28</td>
<td>28</td>
<td>−62</td>
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<tr>
<td>62</td>
<td>Tyrophostin B48</td>
<td>I</td>
<td>32</td>
<td>11</td>
<td>−40</td>
</tr>
<tr>
<td>63</td>
<td>3-Tropanylindole-3-carboxylate methiodide</td>
<td>I</td>
<td>32</td>
<td>18</td>
<td>−45</td>
</tr>
<tr>
<td>64</td>
<td>R(+)-6-Bromo-APB hydrobromide</td>
<td>I</td>
<td>32</td>
<td>32</td>
<td>−66</td>
</tr>
<tr>
<td>65</td>
<td>(¿)-Chloro-APB hydrobromide</td>
<td>I</td>
<td>32</td>
<td>32</td>
<td>−33</td>
</tr>
<tr>
<td>66</td>
<td>Doxycycline hydrochloride</td>
<td>I</td>
<td>32</td>
<td>32</td>
<td>−57</td>
</tr>
<tr>
<td>67</td>
<td>Tyrophostin AG 537</td>
<td>I</td>
<td>32</td>
<td>32</td>
<td>−24</td>
</tr>
<tr>
<td>68</td>
<td>Tyrophostin AG 808</td>
<td>I</td>
<td>32</td>
<td>32</td>
<td>−52</td>
</tr>
</tbody>
</table>

$^a$Active (A) compounds exhibited class 1.1, 1.2, or 2.1 dose–response curves in one or both qHTS runs. Those with class 2.2 or 3 curves in both runs were scored as inconclusive (I). The remainder was judged not active (N). Results on SW480 cells are listed from lowest to highest EC$_{50}$ values for active and then for inconclusive compounds.

$qHTS$ EC$_{50}$ values were averaged from 2 separate runs for active or inconclusive compounds, but not shown for nonactive compounds.

Independent samples were tested in the excessive DNA replication assay at 16 concentrations, and the EC$_{50}$ values were averaged from 2 separate runs. A hyphen (-) indicates the compound was tested and scored as not active; an asterisk (*) indicates the compound showed weak activity near threshold levels.

The percentage of cells present at the maximum efficacy of each compound was determined by the qHTS relative to the DMSO control, which was defined as 100%. The values are an average of 2 separate runs.
asymptote. Class 3 compounds had curves that were either poorly fitted ($r^2 < 0.9$) or active only at the highest concentration tested. Therefore, their activity was judged inconclusive. Class 4 compounds either did not yield a significant curve fit or the efficacy of the curve fit was below threshold. Therefore, class 4 compounds were considered inactive. Consensus activities were determined for each compound on the basis of two separate runs of the qHTS. Compounds that exhibited class 1.1, 1.2, or 2.2 dose–response curves in one or both runs were scored "active." Those with class 2.2 or 3 curves in both runs were scored "inconclusive," and the remainder was judged "not active." The potencies of compounds classified as active or inconclusive in both qHTS runs correlated well for both cell lines (Fig. 3C and D), showing reproducibility between independent screens.

Of the 1,280 compounds, 15 were active and 53 were inconclusive on SW480 cells (Table 1). All 15 of the compounds that were active on SW480 cells were either active or inconclusive on MCF10A cells. In contrast, 67 compounds were active and 130 compounds were inconclusive on MCF10A cells. Of the 67 compounds that were active on MCF10A cells, only 34 were active or inconclusive on SW480 cells (Table 1). Thus, SW480 cells provided a selective target for identifying small molecules that can induce EDR in cancer cells by scoring only 1.2% of the screened compounds as active. MCF10A cells, on the contrary, provided a sensitive counter screen for identifying those compounds that induced EDR selectively in cancer cells.

**Confirmation of active compounds**

To confirm the qHTS, independent samples of 8 compounds that were active on SW480 cells and 5 that were inconclusive were obtained from a commercial source and tested in the EDR assay on both SW480 and MCF10A cells. Each compound was titrated as 16 twofold dilutions beginning at 46 µmol/L in at least 2 independent runs. All of the active compounds responded as expected with EC50 values comparable to those measured in the original screens (#2–5, 7, 9, 10, and 12 in Table 1; Figs. 2 and 7; Supplementary Fig. S1), thereby confirming the ability of the qHTS to identify compounds active in the EDR assay. Of the 5 inconclusive compounds, 3 were also confirmed by this assay (#23, 25, and 40 in Table 1; Fig. 9; Supplementary Fig. S3). However, independent samples of 2 of the 5 compounds with inconclusive activity in the original qHTS were either marginally active or not active when tested in the EDR assay. N,N-Dihexyl-2-(4-fluorophenyl)indole-3-acetamide (FGIN) 1–27 (#61 in Table 1) was only marginally active in some runs with an efficacy near threshold levels in SW480 cells, and inactive in other runs (Supplementary Fig. S4). Tetraisopropyl pyrophosphoramide (iso-OMPA; #57 in Table 1) was not active in any run (Supplementary Fig. S4). Because the LOPAC1280 samples of FGIN 1–27 and iso-OMPA exhibited significant activity on SW480 cells only at the highest concentration tested, one or both LOPAC samples may have contained an impurity that was absent from the independent samples.

To determine the accuracy of the EDR assay, the activity of each of the 13 independent samples was assayed on both SW480 and MCF10A cells by fluorescence activated cell sorting (FACS). Cells were cultured for 2 days with one of the compounds, and then the attached cells were subjected to the FACS assay to determine the fraction of cells with >4N DNA content. For example, only 2% to 4% of cells treated with DMSO contained >4N DNA; the remainder displayed two major peaks of fluorescence that corresponded to diploid (2N) and tetraploid (4N) cells (Fig. 4). In contrast, podophyllotoxin-treated SW480 cells showed a marked shift in DNA fluorescence that corresponded to a decrease in cells with 2N and 4N DNA content together with the concurrent appearance of cells containing >4N DNA (Fig. 4). The magnitude of this shift was concentration dependent. At 0.03 µmol/L (the EC50 value of podophyllotoxin determined from EDR assays), 44% of the cells contained >4N DNA, whereas at 3 µmol/L, 88% of the cells contained >4N DNA. A similar, but less pronounced, effect was observed with MCF10A cells (Fig. 4). These results were characteristic of cells undergoing DNA re-replication; the EDR occurred randomly throughout their chromosomes rather than a complete duplication of the entire set of chromosomes a second or third time. All of the compounds that were active on SW480 cells in the EDR assay (Table 1) were also active in the FACS assay and produced similar FACS profiles (Supplementary...
Fig. S2; data not shown). Conversely, compounds that were either marginally active (e.g., FGN 1–27) or not active (e.g., iso-OMPA) in the EDR assay were not active in the FACS assay (Fig. 8; data not shown). These results confirm that the EDR assay accurately identified small molecules that can induce EDR in human cells, and that it was more sensitive to changes in nuclear DNA content than FACS assays.

**Known compounds that induce DNA re-replication in both cancer and normal cells**

All of the compounds in the LOPAC1280 that are known to affect microtubule dynamics were active on SW480 and MCF10A in the EDR assay, both during the qHTS and in the subsequent confirmation assays. The most potent of these were vincristine, taxol, podophyllotoxin, vinblastine, colchicine, and nocodazole (\#1–6 in Table 1) with EC50 values ranging from 0.013 to 0.32 \( \mu \text{mol/L} \). These bind tubulin and arrest cells in metaphase by preventing either the assembly or the disassembly of microtubules during mitosis (11, 13–16). Furthermore, depending on experimental conditions, cells treated with one of these compounds can escape the metaphase block, initiate S-phase without undergoing cytokinesis, and thereby become aneuploid or polyploid as well as micronucleated in a process termed "mitotic slippage" (17, 18).

Cells were stained with DAPI to visualize the effects of compounds on nuclear DNA compaction and to distinguish interphase cells from metaphase and anaphase cells. Cells were stained with antibodies specific for phosphorylated histone H3, a biomarker for mitotic cells (19), to determine the fraction of prophase cells. The results confirmed that these compounds caused mitotic slippage. For example, the fraction of mitotic cells in cultures treated with podophyllotoxin decreased at least 8-fold in 2 days (Table 2). In addition, podophyllotoxin induced chromatin condensation and formation of micronuclei (Fig. 5), both of which are characteristic of cells exposed to inhibitors of microtubule dynamics (17, 20, 21). Moreover, the fraction of micronucleated cells increased with the concentration of podophyllotoxin and with increasing length of exposure to this compound (data not shown). Taken together with the results from FACS assays, these results confirmed that podophyllotoxin allowed cells to slip through mitotic arrest and reinitiate DNA replication without undergoing either chromosome segregation or cell division. Similar results were obtained with each of the other compounds that were active on SW480 cells.

**Table 2. Fraction of nuclei in metaphase or anaphase**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Metaphase + anaphase/total nuclei</th>
<th>Metaphase + anaphase cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>15/296</td>
<td>5.1</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>1/180</td>
<td>0.6</td>
</tr>
<tr>
<td>Rotenone</td>
<td>0/149</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>DSF + Cu(^{2+})</td>
<td>0/178</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>SU6656</td>
<td>2/230</td>
<td>0.9</td>
</tr>
</tbody>
</table>

NOTE: SW480 cells were cultured for 2 days in the presence of the indicated compound. All samples contained 0.4% DMSO.
Three other compounds in the LOPAC\textsuperscript{1280}–rotenone, 2-methoxyestradiol, and IC261–are also known to affect microtubule dynamics, and they were identified as active compounds on SW480 cells, as well. In addition to inhibiting mitochondrial electron transport, preventing NADH from producing ATP, and inhibiting proteasome activity, rotenone (#9 in Table 1) blocks mitosis by inhibiting microtubule assembly (22, 23). Similarly, in addition to inhibiting angiogenesis, 2\textsuperscript{a}methoxyestradiol (#12 in Table 1) also binds tubulin, alters the rate of microtubule polymerization, and inhibits endothelial cell proliferation (24, 25). The third example is IC261 (# 11 in Table 1). At low concentrations, IC261 causes a transient mitotic arrest in cells with low levels of p53 (26). The cells then slip through mitosis into S-phase with concomitant formation of micronuclei. IC261 is an inhibitor of casein kinases \(\delta\) and \(\varepsilon\) which seem to regulate centrosome or microtubule function during mitosis (26). Thus, all the compounds in the LOPAC\textsuperscript{1280} that are known to affect microtubule dynamics induced EDR in both SW480 and MCF10A cells.

EDR can also be induced in the form of endocycles by inhibiting cyclin-dependent protein kinase 1 (CDK1), an enzyme required to initiate mitosis (27, 28). Of the 6 inhibitors of CDK activity in the LOPAC\textsuperscript{1280}, only CGP-74514A selectively inhibits CDK1 (29), and only CGP-74514A (#20 in Table 1) exhibited activity in both screens. This result was confirmed by FACS analyses using commercially available samples of CGP-7414A and RO3306, a selective inhibitor of CDK1 (10) that was not in the LOPAC\textsuperscript{1280} (data not shown). Other CDK inhibitors in the LOPAC\textsuperscript{1280}, such as roscovitine and olomoucine, inhibit CDK2 and CDK1, and therefore inhibit initiation of S-phase as well as mitosis (27, 28). These compounds were not active in the EDR assay on either SW480 or MCF10A cells.

Selective inhibitors of topoisomerase II, an enzyme essential for separation of newly replicated chromatids, promote the accumulation of tetraploid and polyploid cells (30–32). Of the 8 topoisomerase inhibitors in the LOPAC\textsuperscript{1280}, only etoposide, ellipticine, and XK469 (#8, 30, and 40, respectively, in Table 1; Supplementary Fig. S3) specifically inhibit topoisomerase II, and only these topoisomerase inhibitors were active in the EDR assay (data not shown). XK469 activity was confirmed from a commercially available sample (Table 1; Supplementary Fig. S3). Thus, compounds previously reported to induce EDR in mammalian cells were active in the EDR assay, both in the qHTS and in subsequent confirmation assays, whereas compounds known to inhibit DNA replication were not. These results showed the accuracy of the EDR assay in identifying small molecules that can induce EDR in human cells.

**Novel compounds that induce excess DNA replication in both cancer and normal cells**

Of the 15 compounds that were active on SW480 cells, 4 have not been reported previously to induce EDR in mammalian cells. The A1 adenosine receptor antagonist 7-chloro-4-hydroxy-2-phenyl-1,8-naphthyridine (CHPN; #10 in Table 1) was active on SW480 and MCF10A cells in the EDR assay, both from the qHTS and in subsequent confirmation assays with an independent sample (Table 1; Supplementary Fig. S1). Moreover, fluorescent microscopy (data not shown) and FACS assays (Supplementary Fig. S2) revealed that CHPN induced formation of giant nuclei and micronuclei that were indistinguishable from those induced by podophyllotoxin (Fig. 5) and other inhibitors

![Figure 6](image-url). Cell proliferation assays for selected compounds. SW480 (A, B) and MCF10A (C, D) cells were cultured for up to 5 days in the presence of either 0.4% DMSO alone (\(\square\)) or plus the EC\(_{50}\) (Table 1) of the indicated compound. The EC\(_{100}\) was used for SU6656. A and C, FGIN 1–27 (x, dashed line), rotenone (\(\ast\)), 2-methoxyethanol (2-ME, \(\&\)), CHPN (\(\ast\)), PPT (\(\&\)), SU6656 (\(\square\)), DSF + Cu\(^{2+}\) (\(\square\)).
of microtubule dynamics. CHPN also produced FACS profiles comparable to those compounds (Supplementary Fig. S2). These results suggested that CHPN induced EDR through mitotic slippage. The other 3 novel active compounds were the protein kinase C inhibitor rac-2-ethoxy-3-octadecanamido-1-propylphosphocholine (#13 in Table 1), a selective inhibitor of receptor-mediated and voltage-gated Ca\(^{2+}\) entry termed SKF 96365 (#14 in Table 1), and a NK1 tachykinin receptor antagonist termed WIN 62,577 (#15 in Table 1).

**Effect of compounds on cell proliferation**

All 15 of the compounds that were active on SW480 cells were also active or inconclusive on MCF10A cells (Table 1). Thus, they did not induce EDR selectively in the cancer cell line. However, of the 53 compounds that were inconclusive on SW480 cells (#16–68 in Table 1), 20 were not active on MCF10A cells, suggesting that they could induce EDR selectively in cancer cells. Alternatively, inactivity in the EDR assay could result from inhibition of cell proliferation or induction of apoptosis by means other than induction of DNA re-replication. To determine whether these compounds inhibited proliferation of cancer cells selectively, a cell proliferation index was calculated from the EDR qHTS data by comparing the number of nuclei present at the maximum effective concentration of each test compound relative to the number of nuclei in the DMSO-treated controls. Thus, compounds that did not affect cell division would have a cell proliferation index of 0%. Compounds that stimulated cell proliferation would have a positive index, and compounds that inhibited cell proliferation would have a negative index. Compounds that induced apoptosis (cells lysed or were released from the plate) would have an index of −100%. Compounds that arrested cell division but did not alter cell viability would have an index of approximately −50%, because the number of control cells generally doubled within the 2-day period of the assay.

Most compounds (45/68) that induced EDR in SW480 cells reduced the cell proliferation index by 40% to 60% (Table 1), consistent with the fact that EDR arrests cell proliferation. This conclusion was confirmed by measuring the cell proliferation index of commercially available compounds (Supplementary Fig. S4; data not shown) and by a cell proliferation assay in which the rate of change in cell number was measured as a function of the time cells were exposed to the test compound (Fig. 6; Supplementary Fig. S5; data not shown). In general, MCF10A cells were more sensitive to inhibition of cell proliferation than SW480 cells. Virtually all of the compounds (65 of 68) that were either active or inconclusive on SW480 cells reduced MCF10A cell numbers by at least 40%, with most (47 of 68) of them greater than 60% (Table 1). The increased sensitivity of MCF10A cells was caused in part by the induction of apoptosis. FACS analyses revealed increased fractions of MCF10A cells with <2N DNA content (Figs. 4, 8, and 9; Supplementary Fig. S2), and fewer adherent cells were observed following compound treatment (data not shown). Thus, most of the compounds in Table 1 inhibited proliferation of both cancer cells and normal cells. Some of these effects, however, were observed only at high concentrations of the compound. FG1N 1–27, for example, had an EC\(_{50}\) of approximately 30 µmol/L, a cell proliferation index of approximately −60%, and a cell proliferation rate of zero (#61 in Table 1; Fig. 6A and C; Supplementary Fig. S4). These results were consistent with reports that FG1N 1–27 can induce apoptosis in primary chronic lymphocytic leukemia cells and in colorectal cancer cells (33, 34).
cells with an EC$_{50}$ of 6 to 8 µmol/L, and that induction of EDR was accompanied by arrest of cell proliferation and apoptosis (Supplementary Fig. S4). This conclusion was confirmed by FACS and cell proliferation assays, although the effects of PPA in these assays were not discernable until the cells had been exposed to PPA for at least 2 days (Supplementary Fig. S5A and B). Under the same conditions, PPA had no effect on MCF10A cells. Similarly, an independent sample of iso-OMPA exhibited activity only when cells were incubated for longer than 2 days (Supplementary Figs. S4 and S5A), even in the presence of higher concentrations. These results not only confirmed the accuracy of the EDR assay, but also revealed that it was more sensitive than standard FACS and cell proliferation assays in detecting compounds that induce EDR.

Linear regression analysis of the log EC$_{50}$ values for the 47 compounds that were either active or inconclusive on both cell lines (Table 1) revealed that they did not differ greatly between the two cell lines (0.81 slope, $r^2 = 0.75$; data not shown). The one exception was DSF (#7 in Table 1). DSF was at least 110-fold more effective on SW480 cells than on MCF10A cells in the EDR qHTS assay (#7 in Table 1), a finding that was confirmed by a commercially available sample of DSF in the EDR assay (Fig. 7, top). This conclusion was also evident in FACS assays when the cells were seeded in the presence of 0.2 µmol/L DSF (Fig. 7, bottom). Thus, DSF can induce EDR selectively in cancer cells. However, this result was affected strongly by experimental conditions.

When DSF was added to cell cultures 24 hours after seeding, DSF concentrations ranging from 0.2 to 10 µmol/L had no effect on either proliferation (Fig. 6) or DNA replication (Fig. 8, bottom; data not shown) unless accompanied by equimolar amounts of Cu$^{2+}$, as reported in previous studies (35, 36). Treatment of cells with Cu$^{2+}$ alone had no significant effect. Under these conditions, the fraction of SW480 cells with >4N DNA increased by 6-fold, and most of these cells eventually underwent apoptosis, as revealed by the cell proliferation. Addition of Cu$^{2+}$ did not increase the potency of DSF on SW480 cells in the EDR assay, but it did induce EDR in MCF10A cells (compare Figs. 7 and 8, top). These results show that the EDR assay was more sensitive than the FACS assay in detecting compounds that induce DNA re-replication, and emphasize the fact that potentially useful compounds can be sensitive to experimental conditions.

The sensitivity of MCF10A cells to DSF depended on the same variables as SW480 cells, but MCF10A cells did not respond in the same manner. The potency of DSF on MCF10A cells in the EDR assay increased 83-fold in the presence of Cu$^{2+}$ (compare Figs. 7 and 8, top), an effect that was accompanied by inhibition of MCF10A cell proliferation (Fig. 6C and D; data not shown). Remarkably, DSF + Cu$^{2+}$ rapidly induced apoptosis in these cells, as judged by the FACS assay (Fig. 8), although the cells remained attached to the dish, a phenomenon observed previously with these cells (37). Taken together, these results reveal that DSF can induce EDR by at least two mechanisms, one that involves a DSF–Cu$^{2+}$ complex and one that does not. Furthermore, DSF alone can induce EDR in cancer cells under conditions where it does not affect normal cells. Under these conditions, addition of Cu$^{2+}$ stimulates the potency of DSF only in normal cells, thereby reducing the potential effectiveness of DSF in chemotherapy.

**Compounds that induce endocycles selectively in cancer cells**

Mammalian cells contain 9 members of the Src family of nonreceptor tyrosine kinases that coordinate multiple signaling pathways known to be involved in different events of tumor progression, such as proliferation, survival, motility, angiogenesis, cell–cell communication, adhesion, and invasion (38). The LOPAC$^{1280}$ contains two Src kinase inhibitors. 7-Cyclopentyl-5-(4-phenoxy)phenyl-

![Figure 8. DSF plus equimolar amounts of cupric (Cu$^{2+}$) chloride or cupric acetate induced EDR and cell death in both SW480 and MCF10A cells. Top, the EDR assay by DSF with equimolar concentrations of either cupric acetate (shown) or cupric chloride. Bottom, when cells were plated 24 hours before adding 0.2 µmol/L DSF and then cultured for 2 days in the presence of the drug, DSF induced EDR in SW480 cells only in the presence of 0.2 µmol/L Cu$^{2+}$. The EC$_{50}$ for DSF alone on SW480 cells was 0.2 µmol/L (Table 1). Control cells were cultured in the presence of 0.4% DMSO. The fraction of cells containing >4N DNA is indicated in each panel.](Image)
lymphocytes and megakaryoblasts. SU6656 stimulates mitotic slippage and induces polyploidy in the spindle to the centromere. SU6656 has been reported to inhibit the activity of proteins that function in the attachment of the mitotic spindle, such as Lyn, Fyn, and Src, as well as Aurora kinases B and C. SU6656 (#23 in Table 1) is a potent inhibitor of Yes, a tyrosine kinase expressed primarily in T lymphocytes. 7-CPP (Table 1) is a selective inhibitor of Lck, a Src family tyrosine kinase.

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect on SW480 Cells</th>
<th>Effect on MCF10A Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>43%</td>
<td>27%</td>
</tr>
<tr>
<td>+SU6656</td>
<td>4%</td>
<td>3%</td>
</tr>
</tbody>
</table>

**Figure 9.** SU6656 induces endoreduplication in SW480 cells, but not in MCF10A cells. A, an independent sample of SU6656 was active in the EDR assay on both SW480 and MCF10A cells. B, the FACS assay revealed that 6 µmol/L SU6656 triggered endoreduplication in SW480 cells, but DNA re-replication and apoptosis in MCF10A cells. Control cells received 0.4% DMSO.

7H-pyrol[2,3-d]pyrimidin-4-ylamine (7-CPP; #55 in Table 1) is a selective inhibitor of Lck, a Src family tyrosine kinase expressed primarily in T lymphocytes. 7-CPP was active on MCF10A cells, but it exhibited only modest activity on SW480 cells, with an EC₅₀ of 25 µmol/L. SU6656 (#23 in Table 1) is a potent inhibitor of Yes, Lyn, Fyn, and Src as well as Aurora kinases B and C, proteins that function in the attachment of the mitotic spindle to the centromere. SU6656 has been reported to stimulate mitotic slippage and induce polyploidy in lymphocytes and megakaryoblasts. SU6656 activity in the qHTS was inconclusive on both SW480 and MCF10A cells with an EC₅₀ of 3 to 4 µmol/L, but its activity and potency were confirmed with an independent sample (Table 1; Fig. 9A). Remarkably, SU6656 induced multiple rounds of endoreduplication in SW480 cells, but not in MCF10A cells.

After 2 days of exposure to SU6656, FACS assays revealed that SW480 cells accumulated as a relatively homogeneous population with >4N DNA content, indicative of DNA re-replication. In contrast, MCF10A cells cultured under the same conditions exhibited cells with a broad range of >4N DNA content, indicative of DNA re-replication, as well as cells that contained <2N DNA, characteristic of apoptosis. Further characterization confirmed that SU6656 induced multiple rounds of endoreduplication (endocycles) both in SW480 cells and in the colorectal cancer cell line HCT116, but not in MCF10A cells (Fig. 10). When the FACS data were plotted on a log scale and samples were taken at 24-hour intervals, endocycles were evident as evenly spaced peaks of increased fluorescence that shifted with time as cells increased their DNA content. These endocycles were accompanied by the appearance of enlarged nuclei whose size increased with time of exposure to SU6656, rather than the clusters of micronuclei observed during mitotic slippage (Fig. 5). In contrast, the 2N and 4N peaks of MCF10A cells persisted, although their relative sizes changed over time due to inhibition of cell proliferation and induction of apoptosis.

The tumor suppressor protein Tp53 is a component of the G1-phase checkpoint that prevents cells with DNA damage from entering S phase. Because compounds that induce mitotic slippage are more effective on cells with reduced Tp53 activity, one explanation for the distinction between normal cells and cancer cells in their response to SU6656 was that normal human cells contain Tp53, whereas SW480 cells contain a mutated Tp53 and HCT116(Tp53⁻/⁻) cells lack a functional Tp53 gene, but otherwise retain intact DNA damage-dependent and spindle-dependent checkpoints. Therefore, wild-type and p53 nullizygous HCT116 cells were treated in parallel with SU6656. The results revealed that SU6656 induction of endocycles in cancer cells was independent of Tp53 (Fig. 11). Therefore, SU6656 induction of endocycles in cancer cells was distinct from the mitotic slippage mechanism induced by compounds that interfere with microtubule dynamics.

**Discussion**

The EDR assay described here provides a novel approach to identifying molecules that trigger excess nuclear DNA replication in human cells, regardless of whether it stems from DNA re-replication, endoreduplication, or endomitosis. By coupling the EDR assay with qHTS, collections of molecules can be surveyed for both their efficacy and potency at inducing EDR. The rationale behind this assay was the recent discovery that suppression of geminin expression rapidly induced DNA re-replication in cells derived from human cancer tissues, but not in cells derived from normal human tissues. Because DNA re-replication triggers the cell's DNA damage response, agents that induce DNA re-replication selectively in cancer cells should selectively target them for eventual apoptosis. To facilitate identification of such agents, exposure of cells to the test compound was limited to 2 days so that only the most potent molecules were active, and the cell proliferation index identified compounds that were toxic to normal cells, even if they did not induce EDR. Compounds that induce DNA re-replication selectively in cancer cells without affecting proliferation of normal cells would have obvious advantages over drugs currently in use that either inhibit DNA synthesis or induce mitotic slippage. In addition, any compound that interferes with the cell's ability to restrict
genome duplication to once per cell division is potentially useful for analyzing the regulation of genome duplication.

The results presented here validate the assay’s accuracy, reproducibility, and sensitivity in identifying compounds that can induce EDR in either cancer cells or normal cells. Of the 1,280 biologically active molecules, the EDR qHTS identified 15 (1.2%) active compounds, as defined by their dose–response curves (Table 1). These compounds were identified reproducibly in 2 or more trials with comparable EC50 values, consistent signal to background ratios, and minimal well-to-well variation among control samples. Five of the active compounds (#7, 10, 13–15 in Table 1) have not been reported previously to induce EDR. However, the mechanism by which they induce EDR is unlikely to be related to their known biological activities. For example, the A1 adenosine receptor antagonist CHPN (#10 in Table 1) was active on both SW480 and MCF10A cells, but the fact that CHPN activity in the EDR assay was unique among the 43 adenosine receptor antagonists screened strongly suggests that its ability to induce DNA re-replication in human cells is not related to its known affects on cAMP levels.

Eleven of the active compounds and 4 of the inconclusive compounds were reported previously to induce EDR in mammalian cells, thereby confirming the assay’s accuracy. For example, all of the compounds in the LOPAC1280 known to affect microtubule dynamics were active on both SW480 and MCF10A cells (#1–6, 9, 11, and 12 in Table 1). Disruption of microtubule assembly or disassembly both inhibits mitosis and induces DNA re-replication and apoptosis in mammalian cells (51). These compounds activate the “spindle assembly checkpoint” that prevents premature entry into anaphase by suppressing the ubiquitin ligase activity of the anaphase-promoting complex (APC). However, cells eventually “slip through” this block by reactivating the APC despite continued inhibition of microtubule dynamics (17, 18, 20). For similar reasons, compounds that inhibit topoisomerase II, an enzyme essential for separation of newly replicated chromatids, also exhibited some activity on both SW480 and MCF10A cells (#8, 30, and 40 in Table 1). In contrast, the 5 LOPAC1280 compounds that inhibit topoisomerase I, an enzyme essential for replication fork activity, did not induce EDR, thereby confirming that only those compounds that inhibit topoisomerase II specifically induce EDR. In fact, as one would expect, none of the LOPAC1280 compounds known to inhibit DNA replication were active.
in the EDR assay. Yet another example of the EDR assay’s specificity was its ability to correctly identify the single CDK1-specific inhibitor in the LOPAC1280. Selective inhibition of CDK1, the only CDK required to initiate mitosis, triggers endocycles (27, 28) by arresting cells under conditions that allow assembly of prereplication complexes followed by initiation of S-phase (52). Of the 6 compounds in this collection that inhibit CDK activities, only CGP-74514A (#20 in Table 1) selectively inhibits CDK1, and only CGP—74514A was selected by the EDR qHTS. Taken together, these results clearly validate the efficacy of this assay, and justify its application to large libraries of uncharacterized compounds.

Of the 1,280 compounds in the LOPAC collection, the EDR qHTS identified only 3 that could induce DNA re-replication selectively in the SW480 cancer cell line without inhibiting cell proliferation in the MCF10A normal cell line (iso-OMPA, PPA, and DSF). Two of them were subsequently confirmed by independent samples. These results show that the EDR assay can identify compounds that selectively inhibit cancer cell proliferation and target them for destruction, in spite of their very low frequency. PPA (#25 in Table 1) was at least 75% as effective as podophylo-toxin, but its potency was at least 200-fold less than compounds such as etoposide (a derivative of podophyllotoxin), vinblastine (Velban), vincristine (Oncovin), and taxol (Paclitaxel/Abraxane) that are currently used to treat a variety of cancers. The potency of PPA in the EDR assay, however, was comparable to that of 2-methoxyestradiol (Panzem), which is currently undergoing clinical trials (53–55).

DSF (#7 in Table 1) was originally identified as a potential cancer chemotherapeutic agent in a screen for small molecules that inhibit proliferation of prostate cancer cells (36). Although DSF inhibited cells derived from normal as well cancer tissues, it has been used successfully to treat metastatic melanoma (56) and is currently in clinical trials for various other cancers (ClinicalTrials.gov; “Disulfiram”). In the EDR assay, DSF was greater than 100-fold more potent in SW480 cells than in MCF10A cells. Moreover, DSF was detected by the EDR assay under conditions in which it was not detected by the FACS assay, thereby revealing that the EDR assay is a more sensitive method for detecting EDR. The FACS assay did, however, confirm the ability of DSF to induce EDR when in the presence of Cu²⁺, as previously reported (35). DSF and Cu²⁺ together arrested cell proliferation and induced apoptosis in both SW480 and MCF10A cells. DSF alone may retard tumor growth, because tumors contain high levels of intracellular Cu²⁺ (35, 36). The basis for the difference between the sensitivity of the EDR assay and that of the FACS assay is under investigation.

The mechanism of action for DSF is not clear. It has been reported to inhibit many proteins, including aldehyde dehydrogenase (57), DNA topoisomerase (58), breast cancer-associated gene 2 (59), E26 transforming sequence-related gene and minichromosome maintenance proteins (36), and the 26S proteasome (35, 36). Although transient treatment of cells with MG132, a specific inhibitor of the 26S proteasome, can induce DNA re-replication in some mammalian cells (61), culturing SW480 or MCF10A cells for 2 days in the presence of 0.5 μmol/L MG132 caused them to accumulate with 4N DNA content, but not greater, and higher levels of MG132 induced apoptosis (data not shown). Therefore, the ability of DSF plus Cu²⁺ to induce DNA re-replication is not due simply to inhibiting the 26S proteasome. We suggest that DSF selectively targets cancer cells for apoptosis by inducing DNA re-replication, although how it does this remains to be determined.

SU6656 stands out among the compounds that induced EDR, because it induced endocycles rather than DNA re-replication; it did so only in cancer cells regardless of the presence or absence of Tp53, and it did not result in micronucleated cells. Most, perhaps all, of the small molecules characterized so far as inducers of DNA re-replication seem to induce mitotic slippage, a mechanism that produces micronucleated cells and that is strongly influenced by the tumor suppressor protein Tp53 (17). The presence of Tp53 in normal cells inhibits mitotic slippage from driving cells into S-phase, although it does not prevent the induction of apoptosis. However, most cancer cells lack Tp53 activity. Therefore, compounds that induce mitotic slippage in cancer cells are likely to induce DNA replication as well. Thus, the characteristics of SU6656 induction of EDR suggest that its mechanism of action is not through the arrest of cells during mitosis, but on some other pathway that prevents entrance into mitosis under conditions that allow the induction of multiple rounds of endoreduplication. The ability of cancer cells to change from mitotic cell cycles to endocycles is a characteristic they share in common with normal cells that are developmentally programmed to differentiate into polyploid cells that remain viable but that no longer proliferate (1). SU6656 should prove useful in identifying the pathway involved, a pathway that distinguishes cancer cells from all other normal cells.

In summary, we have established an image-based assay for molecules that induce EDR in mammalian cells, regardless of their mechanism of action. When this assay was coupled to the EDR assay, DSF was greater than 100-fold more potent in SW480 cells than in MCF10A cells. Therefore, the ability of DSF to induce EDR when in the presence of Cu²⁺, as previously reported (35). DSF and Cu²⁺ together arrested cell proliferation and induced apoptosis in both SW480 and MCF10A cells. DSF alone may retard tumor growth, because tumors contain high levels of intracellular Cu²⁺ (35, 36). The basis for the difference between the sensitivity of the EDR assay and that of the FACS assay is under investigation.

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In summary, we have established an image-based assay for molecules that induce EDR in mammalian cells, regardless of their mechanism of action. When this assay was coupled
with a qHTS protocol, it correctly identified compounds that can induce EDR in human cells and avoided compounds previously shown to prevent DNA replication. Moreover, only 1% of the 1,280 compounds screened were active, and only 0.2% induced EDR in cancer cells without inhibiting normal cell proliferation. Some of these compounds have never before been recognized as inducers of EDR. The results suggest that future screens of libraries of uncharacterized molecules will yield compounds that mimic the effects of siRNA against geminin by selectively killing cancer cells without harming normal cell proliferation.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

References


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