An Image Based, High-Throughput, Screening Assay
For Molecules That Induce Excess DNA Replication In Human Cancer Cells

Wenge Zhu¹,³,⁴, Chrissie Y. Lee¹,⁴, Ronald L. Johnson², Jennifer Wichterman², Ruili Huang², and Melvin L. DePamphilis¹,⁵

¹National Institute of Child Health and Human Development
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892-2753

²NIH Chemical Genomics Center
National Institutes of Health
9800 Medical Center Drive
Rockville, MD 20892-3370

³Current Address:
Department of Biochemistry and Molecular Biology
The George Washington University Medical School
2300 Eye Street N.W.
Washington, DC 20037

⁴These authors contributed equally to this work

⁵Corresponding Author:
E-mail depamphm@mail.nih.gov
Phone 301-402-8234
FAX 301-480-9354

Running Title: qHTS for compounds that induce excess DNA replication
SUMMARY

Previous studies have shown DNA re-replication can be induced in cells derived from human cancers under conditions in which cells derived from normal tissues are not. Since DNA re-replication induces cell death, this strategy could be applied to the discovery of potential anti-cancer 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 1280 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, as well as 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.

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 (1). 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 (2, 3). In fact, artificially inducing cells to re-replicate the same stretch of nuclear DNA before S-phase is completed (a process termed DNA re-replication) is an aberrant event that leads to DNA damage, stalled replication forks and cell death (4). For example, DNA re-replication can be induced in cancer cells either by over-expression 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 CDK•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 (2, 4).
Remarkably, cancer cells differ from normal human cells in that cancer cells rely solely on high levels of geminin to prevent DNA from reinitiating replication before the genome has been completely replicated (4). 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 from binding to Cdt1 protein in vitro have been reported (5-7). Of the three 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 or not coenzyme Q10 selectively inhibited cancer cell proliferation was not clear, and in our hands, 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 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, as well as 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 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 demonstrate the sensitivity, accuracy and reproducibility of this assay in screening for compounds that induce excess DNA replication 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 excess DNA replication. Of particular interest are two compounds that induced DNA re-replication (tetrathylthiuram disulfide 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 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.5 mg/mL penicillin and streptomycin (qHTS) or in DMEM 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 DMSO. The LOPAC1280 (Sigma Aldrich) was plated as seven five-fold interplate titrations beginning at 10 mM as described (8). For follow-up studies, compounds were plated as 16 two-fold intraplate titrations beginning at 10 mM for all compounds except taxol (50 µM) and colchicine (50 mM).

Excess DNA Replication (EDR) Assay And Quantitative High-Throughput Screen (qHTS)

SW480 or MCF10A cells were plated at 250 cells/5 µL/well into Aurora 1536-well clear bottom, black low-base plates (Nexus Biosystems) using a Multidrop Combi (Thermo Scientific). The cells were cultured for 16 h at 37°C in 5% CO₂ and then either 23 nL DMSO or test compound dissolved in DMSO were transferred to each well in the microtiter plate using an automated pin tool (Kalypsys). Thus, each well contained a final concentration of 0.4% (65 mM) DMSO. Library compounds were added to columns 5-48 and controls were added separately to columns 1-4 as follows: 16 two-fold titrations in duplicate of podophyllotoxin and SU6656...
beginning at 46 µM final concentration (columns 1 and 2 respectively), 0.4% DMSO (column 3), and 6 µM SU6656 and 3 µM podophyllotoxin to the top and bottom half, respectively, of column 4. Following 48 h 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 min. Using an automated plate washer (Kalypsys), medium was aspirated from each well, leaving a residual volume of ~2 µL, and 6 µL/well of PBS was added. Nuclei in each well were imaged and enumerated using an Acumen Explorer eX3 (TTP LabTech) plate reader at 405 nm excitation and 420-500 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 µM podophyllotoxin) activity. These normalized activities were adjusted by applying a pattern correction algorithm using 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 two days. Flow cytometry was performed as described (4) by harvesting the attached cells, staining them with propidium iodide, and then analyzing them in a FACSCalibur (Becton Dickinson) using 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 DAPI. For immuno-fluorescence, cells were grown in chamber slides, fixed with 4% paraformaldehyde for 10 min, rinsed briefly with PBS, and permeabilized for 10 min with 0.2% Triton X100. Slides were blocked with 3 % BSA, 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 using 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 one day after seeding them at ~3-6 x 10⁶ 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 excess DNA replication 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 in order to image them using 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 four 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 four sets of chromosomes (<4N) were distinguished from cells that had excessively replicated their nuclear DNA to a content greater than the amount in four 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 excess DNA replication assay (henceforth termed the 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 1536-well plate
format using 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 using the EDR assay to identify additional compounds that induced DNA re-replication. The results revealed that podophyllotoxin, an inhibitor of microtubule assembly (11), was an effective inducer of excess DNA replication in both SW480 and MCF10A cells with a low EC$_{50}$ (the concentration that produces one-half of its maximum effect) and full efficacy at concentrations spanning three 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 1280 compounds in the ‘Library of Pharmacologically Active Compounds’ (LOPAC$_{1280}$, Sigma Aldrich) using quantitative high throughput screening (qHTS). qHTS is a method whereby 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 EC$_{50}$) 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 LOPAC$_{1280}$ at seven five-fold serial dilutions beginning at 46 µM and ending at 3 nM (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 compounds were defined as 0% activity, and cells treated with podophyllotoxin at its EC$_{100}$ 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 base line 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 EC$_{50}$ values of 54±5 nM for SW480 cells and 50±11 nM for MCF10A cells (Fig. 2D, E). These values were comparable to those determined for the podophyllotoxin library sample in the qHTS runs (Fig. 2B, 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, B). $Z'$-factor is a performance measure incorporating both the S/B ratio and the well-to-well variation among control wells (12). $Z'$-factors of 0.5 or greater 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 nM range, and reproducible between separate runs on both cell lines.

Following each qHTS run, the dose response data for each compound was fitted to a curve using a custom algorithm based on a four parameter model, and the curves were categorized into five 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 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 based on two separate runs of the qHTS. Compounds that exhibited Class 1.1, 1.2 or 2.1 dose response curves in one or both runs were considered ‘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, D), demonstrating reproducibility between independent screens.

Of 1280 compounds, 15 were active and 53 were inconclusive on SW480 cells (Table I). 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 I). Thus, SW480 cells provided a selective target for identifying small molecules that can induce excess DNA replication in cancer cells by scoring only 1.2% of the screened compounds as active. MCF10A cells, on the other hand, provided a sensitive counter screen for identifying those compounds that induced excess DNA replication selectively in cancer cells.

**Confirmation of Active Compounds**
To confirm the qHTS, independent samples of eight compounds that were active on SW480 cells and five 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 two-fold dilutions beginning at 46 µM in at least two independent runs. All of the active compounds responded as expected with EC$_{50}$ values comparable to those measured in the original screens (#2-5, 7, 9, 10, and 12 in Table I, Figs. 2, 7, S1), thereby confirming the ability of the qHTS to identify compounds active in the EDR assay. Three of the five inconclusive compounds also were confirmed by this assay (#23, 25, and 40 in Table I; Figs. 9, S3). However, independent samples of two of the five compounds with inconclusive activity in the original qHTS were either marginally active or not active when tested in the EDR assay. FGIN 1-27 (#61 Table I) was only marginally active in some runs with an efficacy near threshold levels in SW480 cells, and inactive in other runs (Fig. S4). Tetraisopropyl pyrophosphoramide (iso-OMPA, #57 Table I) was not active in any run (Figs. S4). Since the LOPAC$_{1280}$ 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 two 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 µM (the EC$_{50}$ value of podophyllotoxin determined from EDR assays), 44% of the cells contained >4N DNA, whereas at 3 µM, 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 excess DNA replication 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) also were active in the FACS assay and produced similar FACS profiles (Figs. S2, and 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 excess DNA replication 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 LOPAC\textsuperscript{1280} 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 Table I) with EC\textsubscript{50} values ranging from 0.013 to 0.32 µM. All of them 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 in order 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), in order 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 two days (Table II). 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.

Three other compounds in the LOPAC\textsuperscript{1280}, rotenone, 2-methoxyestradiol and IC261, also are 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 Table I) blocks mitosis by inhibiting microtubule assembly (22, 23). Similarly, in addition to inhibiting angiogenesis, 2-methoxyestradiol (#12 Table I) also binds tubulin, alters the rate of microtubule polymerization, and inhibits endothelial cell proliferation (24, 25). The third example is IC261 (#11 Table I). 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 δ and ε which appear to regulate centrosome or microtubule function during mitosis (26). Thus, all of the compounds in the LOPAC1280 that are known to affect microtubule dynamics induced excess DNA replication in both SW480 and MCF10A cells.

Excess DNA replication also can 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 six inhibitors of CDK activity in the LOPAC1280, only CGP-74514A selectively inhibits CDK1 (29), and only CGP-74514A (#20 Table I) exhibited activity in both screens. This result was confirmed by FACS analyses using commercially available samples of CGP-7414A as well as RO3306, a selective inhibitor of CDK1 (10) that was not in the LOPAC1280 (data not shown). Other CDK inhibitors in the LOPAC1280, such as roscovitine and olomoucine, inhibit CDK2 as well as 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 eight topoisomerase inhibitors in the LOPAC1280, only etoposide (#8), ellipticine (#30) and XK469 (#40 Table I, 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 I; Fig. S3). Thus, compounds previously reported to induce excess DNA replication 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 demonstrated the accuracy of the EDR assay in identifying small molecules that can induce excess DNA replication 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, four have not been reported previously to induce excess DNA replication in mammalian cells. The A1 adenosine receptor
antagonist 7-chloro-4-hydroxy-2-phenyl-1,8-naphthyridine (CHPN, #10 Table I) 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 I, Fig. S1). Moreover, fluorescent microscopy (data not shown) and FACS assays (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 of microtubule dynamics. CHPN also produced FACS profiles comparable to those compounds (Fig. S2). These results suggested that CHPN induced excess DNA replication through mitotic slippage. The other three novel active compounds were the protein kinase C inhibitor rac-2-ethoxy-3-octadecanamido-1-propylphosphocholine (#13 Table I), a selective inhibitor of receptor-mediated and voltage-gated Ca^{2+} entry termed SKF 96365 (#14), and a NK1 tachykinin receptor antagonist termed WIN 62,577 (#15).

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 I). Thus, they did not induce excess DNA replication selectively in the cancer cell line. However, of the 53 compounds that were inconclusive on SW480 cells (#16-68 Table I), 20 were not active on MCF10A cells, suggesting that they could induce excess DNA replication 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 or not 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 about -50%, because the number of control cells generally doubled within the two-day period of the assay.

Most compounds (45/68) that induced excess DNA replication in SW480 cells reduced the cell proliferation index by 40% to 60% (Table I), consistent with the fact that excess DNA replication arrests cell proliferation. This conclusion was confirmed by measuring the cell
proliferation index of commercially available compounds (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 (Figs. 6, S5; data not shown). In general, MCF10A cells were more sensitive to inhibition of cell proliferation than SW480 cells. Virtually all of the compounds (65/68) that were either active or inconclusive on SW480 cells reduced MCF10A cell numbers by at least 40%, with most (47/68) 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, 9, S2), and fewer adherent cells were observed following compound treatment (data not shown). Thus, most of the compounds in Table I inhibited proliferation of both cancer cells and normal cells. Some of these effects, however, were observed only at high concentrations of the compound. FGIN 1-27, for example, had an EC$_{50}$ of about 30 µM, a cell proliferation index around -60%, and a cell proliferation rate of zero (#61 Table I; Figs. 6A, 6C, S4). These results were consistent with reports that FGIN 1-27 can induce apoptosis in primary chronic lymphocytic leukemia cells and in colorectal cancer cells (33, 34).

Compounds That Induce Excess DNA Replication Selectively In Cancer Cells

The EDR qHTS detected only three compounds that induced excess DNA replication in SW480 cells with little or no affect on MCF10A cell proliferation: 3-phenylpropargylamine (PPA), iso-OMPA, and tetraethylthiuram disulfide (#25, #57, #7 Table I). To confirm these observations, independent samples of each compound were subjected to the EDR, cell proliferation, and FACS assays on both SW480 and MCF10A cells. As reported above, an independent sample iso-OMPA had no demonstrable effect on either SW480 or MCF10A cells. However, an independent sample of PPA confirmed that this compound induced excess DNA replication in SW480 cells with an EC$_{50}$ of 6 - 8 µM, and that induction of excess DNA replication was accompanied by arrest of cell proliferation and apoptosis (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 two days (Fig. S5A, 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 two days (Figs. S4, 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 excess DNA replication.

Linear regression analysis of the log EC₅₀ values for the 47 compounds that were either active or inconclusive on both cell lines (Table I) revealed that they did not differ greatly between the two cell lines (0.81 slope, 0.75 r², data not shown). The one exception was tetraethylthiuram disulfide (#7 Table I), also termed Disulfiram or DSF. DSF was at least 110-fold more effective on SW480 cells than on MCF10A cells in the EDR qHTS assay (#7 Table I), a finding that was confirmed using a commercially available sample of DSF in the EDR assay (Fig. 7, upper panels). This conclusion was also evident in FACS assays when the cells were seeded in the presence of 0.2 µM DSF (Fig. 7, lower panels). Thus, DSF can induce excess DNA replication 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 µM had no affect on either proliferation (Fig. 6) or DNA replication (Fig. 8, lower panels; 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 affect. 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 excess DNA replication in MCF10A cells (compare Figs. 7 and 8, upper panels). These results demonstrate 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 panels), an effect that was accompanied by inhibition of MCF10A cell proliferation (Fig. 6C, 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 excess DNA replication by at least two mechanisms, one that involves a DSF-Cu⁺² complex and one that does not. Furthermore, DSF alone can induce excess DNA replication 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 nine members of the Src family of non-receptor 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-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine (7-CPP, #55 Table I) is a selective inhibitor of Lck, a Src family tyrosine kinase expressed primarily in T lymphocytes (39). 7-CPP was active on MCF10A cells, but it exhibited only modest activity on SW480 cells, with an EC$_{50}$ of 25 µM. SU6656 (#23 Table I) is a potent inhibitor of Yes, Lyn, Fyn and Src (40) as well as Aurora kinases B and C (41), proteins that function in the attachment of the mitotic spindle to the centromere. SU6656 has been reported to stimulate mitotic slippage (17) and induce polyploidy in lymphocytes (42) and megakaryoblasts (43). SU6656 activity in the qHTS was inconclusive on both SW480 and MCF10A cells with an EC$_{50}$ of 3-4 µM, but its activity and potency were confirmed with an independent sample (Table I; Fig. 9A). Remarkably, SU6656 induced multiple rounds of endoreduplication in SW480 cells, but not in MCF10A cells.

After two days of exposure to SU6656, FACS assays revealed that SW480 cells accumulated as a relatively homogeneous population with >4N DNA content, indicating that the entire genome had undergone endoreduplication (Fig. 9B). 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 h 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 (44). Since compounds that induce mitotic slippage are more effective on cells with reduced Tp53 activity (45-48), 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 (49) and HCT116(Tp53-/-) cells lack a functional Tp53 gene, but otherwise retain intact DNA damage-dependent and spindle-dependent checkpoints (50). 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 (1). By coupling the EDR assay with qHTS, collections of molecules can be surveyed for both their efficacy and potency at inducing excess DNA replication. 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 (4). Since 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 two 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 excess DNA replication. 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 excess DNA replication in either cancer cells or normal cells. Out of 1280 biologically active molecules, the EDR qHTS identified 15 (1.2%) active compounds, as defined by their dose-response curves (Table I). These compounds were
identified reproducibly in two or more trials with comparable EC_{50} values, consistent signal to background ratios, and minimal well-to-well variation among control samples. Five of the active compounds (#7, 10, 13-15 Table I) have not been reported previously to induce excess DNA replication. However, the mechanism by which they induce excess DNA replication is unlikely to be related to their known biological activities. For example, the A1 adenosine receptor antagonist CHPN (#10 Table I) 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 four of the inconclusive compounds were reported previously to induce excess DNA replication in mammalian cells, thereby confirming the assay’s accuracy. For example, all of the compounds in the LOPAC^{1280} known to affect microtubule dynamics were active on both SW480 and MCF10A cells (#1-6, 9, 11, 12 Table I). 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, 40 Table I). In fact, all three inhibitors have been reported to promote the accumulation of polyploid cells (30-32). In contrast, the five LOPAC^{1280} compounds that inhibit topoisomerase I, an enzyme essential for replication fork activity, did not induce excess DNA replication, thereby confirming that only those compounds that inhibit topoisomerase II specifically induce excess DNA replication. In fact, as one would expect, none of the LOPAC^{1280} 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 LOPAC^{1280}. Selective inhibition of Cdk1, the only CDK required to initiate mitosis, triggers endocycles (27, 28) by arresting cells under conditions that allow assembly of pre-replication complexes followed by initiation of S-phase (52). Of the six compounds in this collection that inhibit CDK activities, only CGP-74514A (#20 Table I) 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.
Out of 1280 compounds in the LOPAC collection, the EDR qHTS identified only three 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 using independent samples. These results demonstrate 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. 3-Phenylpropargylamine (PPA, #25 Table I) was at least 75% as effective as podophyllotoxin, 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).

Tetraethylthiuram disulfide (DSF, #7 Table I) 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 over 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 excess DNA replication. The FACS assay did, however, confirm the ability of DSF to induce excess DNA replication when in the presence of Cu\(^{+2}\), as previously reported (35). DSF and Cu\(^{+2}\) 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 Cu\(^{+2}\) (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), BCA2 (59), ERG and minichromosome maintenance proteins (36), and the 26S proteasome (35, 60). 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 two days in the presence of 0.5 \(\mu\)M 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 Cu\(^{+2}\) 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 excess DNA replication, 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 appear 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 excess DNA replication 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 excess DNA replication in mammalian cells, regardless of their mechanism of action. When this assay was coupled with a quantitative high-throughput screening protocol, it correctly identified compounds that can induce excess DNA replication in human cells and avoided compounds previously shown to prevent DNA replication. Moreover, only 1% of the 1280 compounds screened were active, and only 0.2% induced excess DNA replication in cancer cells without inhibiting normal cell proliferation. Some of these compounds have never before been recognized as inducers of excess DNA replication. 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.

**ACKNOWLEDGEMENTS**

This work was supported by the National Institutes of Health (NIH) Roadmap for Medical Research, the intramural research programs of the National Human Genome Research Institute...
and the National Institute of Child Health and Human Development, and NIH grant 4R00CA136555 awarded to W. Z. We thank Paul Shinn and Danielle Van Leer for compound management and Rajarshi Guha for informatics support.

LITERATURE CITED


FIGURE LEGENDS

Figure 1. An image-based assay that identifies excess DNA replication within cells. SW480 cells were incubated for 48 hrs at 37°C and 5% CO₂ in medium containing 0.4% (65 mM) DMSO alone or with 3 μM podophyllotoxin. After addition of Hoechst's DNA stain and incubation for 1 h at ambient temperature, the cells were imaged using a laser scanning microtiter plate cytometer. Scans of a single well from a 1536-well plate are shown for DMSO minus or plus podophyllotoxin treated control wells. Fluorescent objects were classified and color-coded as follows: nuclei with ≤4N DNA content (dark blue, 3,000-50,000 fluorescence units [FLU], 40%-100% Gaussian shape), nuclei with >4N DNA content (red, 50,000-200,000 FLU, 40%-100% Gaussian shape), unrecognizable as single nuclei (cyan, 3,000-200,000 FLU, <40% Gaussian shape), and excluded fluorescent objects (yellow, <3,000 or >200,000 FLU). Guassian shape refers to the fit of the intensity profile to an ideal sphere (100%). Histograms using these color classifications were constructed from 16 wells each treated with DMSO alone (-podophyllotoxin) or with 3 μM podophyllotoxin (+ podophyllotoxin) to reveal the frequency of...
each type of object. The results are analogous to a FACS profile in which the relative numbers of
cells in G1, S, and G2/M phases of the cell cycle are determined.

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 LOPAC1280 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, C) in which each of seven plates containing the concentration indicated in panel A are shown
for SW480 (B) and MCF10A (C) cells. Intraplate control titrations of 16 different concentrations
of podophyllotoxin (D, E) are shown for SW480 (D) and MCF10A (E) cells. Each panel
contains a dose response curve from two runs (○ dashed line, ● solid line).

Figure 3. Performance and potency comparisons of qHTS runs. (A, B) Shown are 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, D) The potencies of active and inconclusive compounds were reproducible between
qHTS runs for each cell line. Plots of log EC50 values were fitted using linear regression and
showed correlation for SW480 (r2 = 0.9, N = 68) and MCF10A (r2 = 0.81, N = 195) cells.

Figure 4. Dose-sensitive induction of excess DNA replication as detected by FACS analysis.
MCF10A or SW480 cells were cultured for 2 days either with 0.4% DMSO alone or with 0.03 or
3 μM podophyllotoxin (PPT). These were the EC50 or EC100 levels, respectively, for
podophyllotoxin on SW480 cells. Adherent cells were suspended and stained with propidium
iodide to quantify their DNA content in the FACS assay. The fraction of cells with >4N DNA is
indicated in each panel.

Figure 5. Nuclear morphology of SW480 cells treated for 2 days either with DMSO alone or
with 0.03 μM podophyllotoxin (the EC50) or 6 μM SU6656 (the EC100). Clusters of micronuclei
are indicated by arrows.
Figure 6. Cell proliferation assays for selected compounds. SW480 (A, B) and MCF10A (C, D) cells were cultured for up to five days in the presence of either 0.4% DMSO alone () or plus the EC$_{50}$ (Table I) of the indicated compound. The EC$_{100}$ was used for SU6656. Panels A and C: FGIN 1-27 (X, dashed line), rotenone (○), 2-methoxyethanol (2-ME, ◊), CHPN (Δ), podophyllotoxin (PPT, ■). Panels B and D: DSF (●), Cu$^{+2}$ (Δ), PPT (■), SU6656 (○), DSF+Cu$^{+2}$ (◊).

Figure 7. Tetraethylthiuram disulfide (DSF) can induce excess DNA replication selectively in SW480 cells. (Top Panels) Using the EDR assay, SW480 and MCF10A cells were treated for two days with the indicated concentrations of DSF alone. DSF was dissolved in DMSO. (Bottom Panels) The FACS assay confirmed that low concentrations of DSF induced excess DNA replication selectively in SW480 cells. Test cells were seeded in the presence of 0.2 µM DSF (EC$_{50}$ value on SW480 cells) and then cultured for 2 days before FACS analysis. Control cells were cultured in the presence of 0.4% DMSO alone. The fraction of cells with >4N DNA is indicated in each panel.

Figure 8. Tetraethylthiuram disulfide (DSF) plus equimolar amounts of cupric (Cu$^{+2}$) chloride or cupric acetate induced excess DNA replication and cell death in both SW480 and MCF10A cells. (Top Panels) The EDR assay using DSF with equimolar concentrations of either cupric acetate (shown) or cupric chloride. (Bottom Panels) When cells were plated 24 h before adding 0.2 µM DSF and then cultured for two days in the presence of the drug, DSF induced excess DNA replication in SW480 cells only in the presence of 0.2 µM Cu$^{+2}$. The EC$_{50}$ for DSF alone on SW480 cells was 0.2 µM (Table I). Control cells were cultured in the presence of 0.4% DMSO. The fraction of cells containing >4N DNA is indicated in each panel.

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 µM SU6656 triggered endoreduplication in SW480 cells, but DNA re-replication and apoptosis in MCF10A cells. Control cells received 0.4% DMSO.

Figure 10. SU6656 induces endocycling selectively in cancer cells. The FACS assays in figure 9 were repeated over several days of cell culture, and fluorescence was plotted on a logarithmic
scale. SU6656 triggered multiple endocycles in SW480 and Tp53-deficient HCT116 cells, but not in MCF10A cells. The three peaks evident in HCT116(p53-/-) cells after 4 days of incubation were 8N, 16N and 32N when viewed on a linear scale.

**Figure 11.** Tp53 did not prevent the induction of endocycles by SU6656. HCT116 wild-type cells (Tp53+/+) and HCT116 cells nullizygous for Tp53 (Tp53-/-) were treated with 6 µM SU6656 for 3 days before subjecting them to the FACS assay.

**SUPPLEMENTARY FIGURES**

**Figure S1.** Confirmation of selected qHTS actives using the EDR assay. Dose response curves from the qHTS on SW480 cells are shown for vinblastine, colchicine, rotenone, 7-chloro-4-hydroxy-2-phenyl-1,8-naphthyridine (CHPN), and 2-methoxyestradiol (2-ME). Commercially available samples of these compounds were then assayed in the EDR assay on both SW480 cells and MCF10A cells at 16 different concentrations. In the qHTS panels, the data points (gray) unconnected to the lines were excluded from the curve fit.

**Figure S2.** Confirmation of selected qHTS actives using the FACS assay. SW480 cells were treated for two days either with 0.4% DMSO alone (Ctrl) or with the indicated concentration of the compound. Cell suspensions were stained with propidium iodide and DNA content was measured by the FACS assay. The fraction of cells with >4N DNA is indicated in each panel.

**Figure S3.** Topoisomerase II inhibitors cause excess DNA replication. All three topoisomerase II specific inhibitors in the LOPAC1280 qualified as inconclusive dose response curves in the qHTS on SW480 cells. Testing of a commercially available sample of XK469 in the EDR assay confirmed activity. For etoposide, the data points at the second highest concentration were excluded from the curve fit.

**Figure S4.** Activity of commercially available samples of FGIN 1-27, 3-phenylpropargylamine (PPA) and iso-OMPA in the EDR assay. SW480 and MCF10A cells were cultured for two days in the presence of the EC_{50} concentration of the indicated compound. The fraction of nuclei with >4N DNA and the cell proliferation index are shown.
Figure S5. 3-Phenylpropargylamine (PPA) and tetraisopropyl pyrophosphoramide (Iso-OMPA) inhibit cell proliferation and induce excess DNA replication. (A) SW480 cells were cultured for the time indicated with 0.4% DMSO or with EC$_{50}$ concentrations (Table I) of PPA or iso-OMPA in the cell proliferation assay. (B) Cells treated as in panel A for the times indicated were subjected to the FACS assay. The fraction of cells with >4N DNA is indicated in each panel.
### Table I. LOPAC\textsuperscript{1280} compounds with activity on SW480 cancer cells

<table>
<thead>
<tr>
<th>#</th>
<th>Compound Name</th>
<th>Dose Response Curve\textsuperscript{1}</th>
<th>EC\textsubscript{50} (µM)\textsuperscript{2}</th>
<th>EC\textsubscript{50} (µM)\textsuperscript{3}</th>
<th>Proliferation Index\textsuperscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SW480 MCF10A</td>
<td>SW480 MCF10A</td>
<td>SW480 MCF10A</td>
<td>SW480 MCF10A</td>
</tr>
<tr>
<td>1</td>
<td>Vincristine sulfate</td>
<td>A</td>
<td>A</td>
<td>0.013</td>
<td>0.018</td>
</tr>
<tr>
<td>2</td>
<td>Taxol</td>
<td>A</td>
<td>A</td>
<td>0.016</td>
<td>0.022</td>
</tr>
<tr>
<td>3</td>
<td>Podophyllotoxin (PPT)</td>
<td>A</td>
<td>A</td>
<td>0.028</td>
<td>0.032</td>
</tr>
<tr>
<td>4</td>
<td>Vinblastine sulfate salt</td>
<td>A</td>
<td>A</td>
<td>0.028</td>
<td>0.020</td>
</tr>
<tr>
<td>5</td>
<td>Colchicine</td>
<td>A</td>
<td>A</td>
<td>0.063</td>
<td>0.040</td>
</tr>
<tr>
<td>6</td>
<td>Nocodazole</td>
<td>A</td>
<td>A</td>
<td>0.13</td>
<td>0.32</td>
</tr>
<tr>
<td>7</td>
<td>Tetraethylthiuram disulfide (DSF)</td>
<td>A</td>
<td>I</td>
<td>0.20</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>Etoposide</td>
<td>A</td>
<td>A</td>
<td>0.32</td>
<td>1.1</td>
</tr>
<tr>
<td>9</td>
<td>Rotenone</td>
<td>A</td>
<td>A</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>7-Chloro-4-hydroxy-2-phenyl-1,8-naphthyridine (CHPN)</td>
<td>A</td>
<td>A</td>
<td>2.5</td>
<td>7.1</td>
</tr>
<tr>
<td>11</td>
<td>IC 261</td>
<td>A</td>
<td>I</td>
<td>2.8</td>
<td>5.6</td>
</tr>
<tr>
<td>12</td>
<td>2-methoxyestradiol</td>
<td>A</td>
<td>A</td>
<td>4.5</td>
<td>2.8</td>
</tr>
<tr>
<td>13</td>
<td>rac-2-Ethoxy-3-octadecanamido-1-propylphosphocholine</td>
<td>A</td>
<td>A</td>
<td>6.3</td>
<td>1.8</td>
</tr>
<tr>
<td>14</td>
<td>SKF 96365</td>
<td>A</td>
<td>I</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>WIN 62,577</td>
<td>A</td>
<td>A</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>16</td>
<td>Ammonium pyrrolidinedithiocarbamate</td>
<td>I</td>
<td>N</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Diphenyleneiodonium chloride</td>
<td>I</td>
<td>A</td>
<td>1.4</td>
<td>5.0</td>
</tr>
<tr>
<td>18</td>
<td>Z-L-Phe chloromethyl ketone</td>
<td>I</td>
<td>A</td>
<td>2.5</td>
<td>3.2</td>
</tr>
<tr>
<td>19</td>
<td>5-Azacytidine</td>
<td>I</td>
<td>A</td>
<td>2.8</td>
<td>4.0</td>
</tr>
<tr>
<td>20</td>
<td>CGP-74514A hydrochloride</td>
<td>I</td>
<td>A</td>
<td>2.8</td>
<td>3.2</td>
</tr>
<tr>
<td>21</td>
<td>R(-)-N-Allylnorapomorphine hydrobromide</td>
<td>I</td>
<td>N</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Quinacrine dihydrochloride</td>
<td>I</td>
<td>A</td>
<td>4.5</td>
<td>2.8</td>
</tr>
<tr>
<td>23</td>
<td>SU 6656</td>
<td>I</td>
<td>I</td>
<td>4.5</td>
<td>3.2</td>
</tr>
<tr>
<td>24</td>
<td>Emetine dihydrochloride hydate</td>
<td>I</td>
<td>I</td>
<td>5.0</td>
<td>0.45</td>
</tr>
<tr>
<td>25</td>
<td>3-Phenylpropargylamine hydrochloride (PPAA)</td>
<td>I</td>
<td>N</td>
<td>6.3</td>
<td>7.6</td>
</tr>
<tr>
<td>26</td>
<td>Cyclosporin A</td>
<td>I</td>
<td>A</td>
<td>6.3</td>
<td>7.9</td>
</tr>
<tr>
<td>27</td>
<td>A23187</td>
<td>I</td>
<td>A</td>
<td>7.1</td>
<td>1.8</td>
</tr>
<tr>
<td>28</td>
<td>Prochlorperazine dimaleate</td>
<td>I</td>
<td>I</td>
<td>7.9</td>
<td>14</td>
</tr>
<tr>
<td>29</td>
<td>DL-Stearoylcarnitine chloride</td>
<td>I</td>
<td>I</td>
<td>8.9</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>Ellipticine</td>
<td>I</td>
<td>A</td>
<td>10</td>
<td>7.1</td>
</tr>
<tr>
<td>31</td>
<td>Reserpine</td>
<td>I</td>
<td>A</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>32</td>
<td>Caffeic acid phenethyl ester</td>
<td>I</td>
<td>A</td>
<td>11</td>
<td>5.0</td>
</tr>
<tr>
<td>33</td>
<td>4-Phenyl-3-furoxancarbonitrile</td>
<td>I</td>
<td>A</td>
<td>11</td>
<td>5.6</td>
</tr>
<tr>
<td>34</td>
<td>Phenamil methanesulfonate</td>
<td>I</td>
<td>A</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>35</td>
<td>Cortexolone maleate</td>
<td>I</td>
<td>I</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Source</td>
<td>IS</td>
<td>IC50</td>
<td>Kd</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------------------------------------</td>
<td>--------</td>
<td>-----</td>
<td>-------</td>
<td>-----</td>
</tr>
<tr>
<td>36</td>
<td>1-Phenyl-3-(2-thiazolyl)-2-thiourea</td>
<td>I</td>
<td>N</td>
<td>13</td>
<td>-74</td>
</tr>
<tr>
<td>37</td>
<td>rac-2-Ethoxy-3-hexadecanamido-1-propylphosphocholine</td>
<td>I</td>
<td>A</td>
<td>14</td>
<td>3.2</td>
</tr>
<tr>
<td>38</td>
<td>U-83836 dihydrochloride</td>
<td>I</td>
<td>A</td>
<td>14</td>
<td>6.3</td>
</tr>
<tr>
<td>39</td>
<td>Chlorpromazine hydrochloride</td>
<td>I</td>
<td>I</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td>40</td>
<td>XK469</td>
<td>I</td>
<td>A</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>41</td>
<td>JWH-015</td>
<td>I</td>
<td>N</td>
<td>16</td>
<td>-48</td>
</tr>
<tr>
<td>42</td>
<td>ODQ</td>
<td>I</td>
<td>I</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>43</td>
<td>(+)-Bromocriptine methanesulfonate</td>
<td>I</td>
<td>I</td>
<td>18</td>
<td>7.1</td>
</tr>
<tr>
<td>44</td>
<td>Trifluromazine hydrochloride</td>
<td>I</td>
<td>N</td>
<td>18</td>
<td>-44</td>
</tr>
<tr>
<td>45</td>
<td>Tyrphostin 25</td>
<td>I</td>
<td>N</td>
<td>18</td>
<td>-41</td>
</tr>
<tr>
<td>46</td>
<td>Tyrphostin AG 698</td>
<td>I</td>
<td>A</td>
<td>20</td>
<td>3.5</td>
</tr>
<tr>
<td>47</td>
<td>Dihydroergotoxine mesylate</td>
<td>I</td>
<td>I</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>48</td>
<td>Dihydrexidine hydrochloride</td>
<td>I</td>
<td>N</td>
<td>20</td>
<td>-51</td>
</tr>
<tr>
<td>49</td>
<td>5-(N-Methyl-N-isobutyl)amiloride</td>
<td>I</td>
<td>N</td>
<td>20</td>
<td>-29</td>
</tr>
<tr>
<td>50</td>
<td>Tyrphostin AG 528</td>
<td>I</td>
<td>N</td>
<td>20</td>
<td>-44</td>
</tr>
<tr>
<td>51</td>
<td>GW2974</td>
<td>I</td>
<td>A</td>
<td>22</td>
<td>3.5</td>
</tr>
<tr>
<td>52</td>
<td>Mifepristone</td>
<td>I</td>
<td>I</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td>53</td>
<td>N-Methyldopamine hydrochloride</td>
<td>I</td>
<td>N</td>
<td>22</td>
<td>-36</td>
</tr>
<tr>
<td>54</td>
<td>Daphnetin</td>
<td>I</td>
<td>N</td>
<td>22</td>
<td>-17</td>
</tr>
<tr>
<td>55</td>
<td>7-Cyclopentyl-5-(4-phenoxy)phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamine (7-CPP)</td>
<td>I</td>
<td>A</td>
<td>25</td>
<td>3.5</td>
</tr>
<tr>
<td>56</td>
<td>SKF 95282 dimaleate</td>
<td>I</td>
<td>N</td>
<td>25</td>
<td>-29</td>
</tr>
<tr>
<td>57</td>
<td>Tetraisopropyl pyrophosphoramid (Iso-OMPA)</td>
<td>I</td>
<td>N</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>58</td>
<td>Prazosin hydrochloride</td>
<td>I</td>
<td>A</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>59</td>
<td>8-Cyclopentyl-1,3-dipropylxanthine</td>
<td>I</td>
<td>I</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>GBR-12909 dihydrochloride</td>
<td>I</td>
<td>I</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td>61</td>
<td>FGIN 1-27</td>
<td>I</td>
<td>N</td>
<td>28</td>
<td>*</td>
</tr>
<tr>
<td>62</td>
<td>Tyrphostin B48</td>
<td>I</td>
<td>A</td>
<td>32</td>
<td>11</td>
</tr>
<tr>
<td>63</td>
<td>3-Tropanylindole-3-carboxylate methiodide</td>
<td>I</td>
<td>A</td>
<td>32</td>
<td>18</td>
</tr>
<tr>
<td>64</td>
<td>R(+)6-Bromo-APB hydrobromide</td>
<td>I</td>
<td>N</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>65</td>
<td>(c)-Chloro-APB hydrobromide</td>
<td>I</td>
<td>N</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>Doxycycline hydrochloride</td>
<td>I</td>
<td>N</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>67</td>
<td>Tyrphostin AG 537</td>
<td>I</td>
<td>N</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>68</td>
<td>Tyrphostin AG 808</td>
<td>I</td>
<td>N</td>
<td>32</td>
<td>-</td>
</tr>
</tbody>
</table>
1 - 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 EC50 values for active and then for inconclusive compounds.

2 - qHTS EC50 values were averaged from two separate runs for active or inconclusive compounds, but not shown for non active compounds.

3 - Independent samples were tested in the excessive DNA replication assay at 16 concentrations, and the EC50 values were averaged from two 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.

4 - 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 two separate runs.
Table II. 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 + Cu²⁺</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>

SW480 cells were cultured for 2 days in the presence of the indicated compound. All samples contained 0.4% DMSO.
Fig. 2 (Zhu et al)

(A) LOPAC<sup>1280</sup> Concentration

Run

3 nM | 14.7 nM | 73.6 nM | 0.37 μM | 1.84 μM | 9.2 μM | 46 μM

(B) SW480 cells

Class 1.1
EC<sub>50</sub> = 28 nM

(C) MCF10A cells

Class 1.1
EC<sub>50</sub> = 32 nM

(D) SW480 cells

Class 1.1
EC<sub>50</sub> = 54 nM

(E) MCF10A cells

Class 1.1
EC<sub>50</sub> = 50 nM
Fig. 4 (Zhu et al.)

SW480

MCF10A

2N 4N

2N 4N

2% DMSO

4% DMSO

44% + 0.03 μM PPT

12% + 0.03 μM PPT

88% + 3 μM PPT

33% + 3 μM PPT

Cell Number (x 10^6)

Fluorescence Units (x 10^6)
Fig. 5 (Zhu et al.)

SW480 Cells

DMSO

+ podophyllotoxin
0.03 μM

+ SU6656, 6 μM
Fig. 7 (Zhu et al.)

SW480

MCF10A

Activity (%) vs Tetraethylthiuram Disulfide (μM)

EC_{50} = 0.09 μM

EC_{50} = 15 μM

2N 4N

DMSO 2%

DSF 25%

Cell Number (x 10^6)

Fluorescence Units (x 10^3)
Fig. 8 (Zhu et al.)

SW480

EC<sub>50</sub> = 0.07 μM

MCF10A

EC<sub>50</sub> = 0.18 μM

Tetraethylthiuram Disulfide + Cu<sup>2+</sup> (μM)

2N 4N

2% DMSO

5% + DSF

1% + Cu<sup>2+</sup>

33% + DSF + Cu<sup>2+</sup>

<0.1% apoptosis

Cell Number (x 10<sup>5</sup>)

Fluorescence Units (x 10<sup>5</sup>)
Fig. 10 (Zhu et al.)

MCF10A  
Control 4 days  
+ SU6656 1 day  
+ SU6656 2 days  
+ SU6656 3 days  
+ SU6656 4 days

SW480  
2N 4N

HCT116(Tp53-)  
2N 4N

Fluorescence Units (x 10^4)
An Image Based, High-Throughput, Screening Assay For Molecules That Induce Excess DNA Replication In Human Cancer Cells


*Mol Cancer Res* Published OnlineFirst January 21, 2011.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-10-0570</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://mcr.aacrjournals.org/content/suppl/2011/02/07/1541-7786.MCR-10-0570.DC1">http://mcr.aacrjournals.org/content/suppl/2011/02/07/1541-7786.MCR-10-0570.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.