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Effects of Anticancer Drug on Chromosome Instability (CIN) and New Clinical Implications for Tumor-Suppressing Therapies

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Abstract

Whole-chromosomal instability (CIN), manifested as unequal chromosome distribution during cell division, is a distinguishing feature of most cancer types. CIN is generally considered to drive tumorigenesis, but a threshold level exists whereby further increases in CIN frequency in fact hinder tumor growth. While this attribute is appealing for therapeutic exploitation, drugs that increase CIN beyond this therapeutic threshold are currently limited. In our previous work, we developed a quantitative assay for measuring CIN based on the use of a non-essential human artificial chromosome (HAC) carrying a constitutively expressed EGFP transgene. Here, we used this assay to rank 62 different anticancer drugs with respect to their effects on chromosome transmission fidelity. Drugs with various mechanisms of action such as antimicrotubule activity, histone deacetylase (HDAC) inhibition, mitotic checkpoint inhibition, and targeting of DNA replication and damage responses were included in the analysis. Ranking of the drugs based on their ability to induce HAC loss revealed that paclitaxel, gemcitabine, dactylolide, LMP400, talazoparib, olaparib, peloruside A, GW843682, VX-680, and cisplatin were the top ten drugs demonstrating HAC loss at a high frequency. Therefore, identification of currently used compounds that greatly increase chromosome mis-segregation rates should expedite the development of new therapeutic strategies to target and leverage the CIN phenotype in cancer cells.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Keywords

chromosome instability; CIN; human artificial chromosome; HAC; anticancer drugs

Introduction

An abnormal chromosome number (aneuploidy) is a feature of most solid tumors and is often accompanied by an elevated rate of chromosome mis-segregation termed chromosome instability (CIN) (1). The gain or loss of entire chromosomes leads to large-scale changes in gene copy number and expression levels. The molecular mechanisms underlying CIN include defects in chromosome cohesion, mitotic checkpoint function, centrosome copy number, kinetochore-microtubule attachment dynamics, and cell-cycle regulation. While CIN can drive cancer genome evolution and tumor progression, recent findings point to the existence of a threshold level beyond which CIN becomes a barrier to tumor growth, and therefore, can be exploited therapeutically (2–7).

Elevation of CIN as an approach to cancer therapy is an attractive strategy; however drugs known to increase CIN beyond the therapeutic threshold are currently few in number. Hence a screen of established anticancer drugs to rank their CIN potency is warranted. In our recent work, we developed a new quantitative assay for measuring CIN (8). The assay is based on the use of a non-essential human artificial chromosome (HAC) with a functional kinetochore (9). This HAC was constructed for gene delivery and contains a single gene-loading site (10,11). To adopt the HAC for CIN studies, a constitutively expressed *EGFP* transgene was inserted into the HAC. Thus, cells that inherit the HAC display green fluorescence, while cells lacking the HAC do not. This allows the measurement of HAC loss rate by routine flow cytometry (Fig. 1). There are several advantages of HAC-based assay compared to karyotype analysis and micronucleus tests that are commonly used to study CIN and its induction by environmental agents (12). Firstly, the HAC-based assay is significantly faster and less labor intensive. Secondly, the flow cytometer can readily analyze tens of thousands of cells compared to the hundred or so cells the latter two methods can analyze. Thus the measurements of our HAC-based assay are more precise. Finally, while the HAC contains a functional centromere/kinetochore, its relatively small size (~1 Mb) causes a frequency of spontaneous HAC loss roughly 10-fold higher than that of native chromosomes (8,13). This HAC feature allows detection of small differences between frequencies of chromosome loss induced by different compounds. This is important because accurate assessment of CIN is crucial to select drugs with the highest effect on chromosome transmission. In summary, our HAC based system offers sensitive, precise and simple means to measure CIN, particularly after drug treatment.

In this study, the HAC-based CIN assay has been applied for the analysis of 62 anticancer drugs corresponding to six groups of compounds with different mechanisms of action. Within each group, drugs were ranked with regards to their effect on the rate of chromosome loss. The highest rate of HAC mis-segregation (25–50-fold increase over a spontaneous frequency of HAC loss) was observed for four microtubule-stabilizing drugs (paclitaxel, dactylolide, peloruside A, and ixabepilone), inhibitors of Polo-like and Aurora kinases

(GW843682 and VX-680), two PARP inhibitors (olaparib, and talazoparib), an inhibitor of topoisomerase I (Top 1) (LMP400), a nucleoside analog (gemcitabine), and two DNA damaging agents (cisplatin and bleomycin). Ranking the analyzed compounds with respect to their effect on chromosome mis-segregation rates should expedite the development of new therapeutic strategies to target the CIN phenotype in cancer cells.

Materials and Methods

Cell line

Human fibrosarcoma HT1080 cells containing the alphoid^{tetO}-HAC carrying the *EGFP* transgene cassette (EGFP-HAC) have been previously described (8). The HAC was transferred into HT1080 cells from donor hamster CHO cells using a standard microcell-mediated chromosome transfer (MMCT) protocol (14). HT1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (v/v) tet system-approved fetal bovine serum (Clontech Laboratories, Inc.) at 37°C in 5% CO₂. Blasticidin (BS) was added to the medium at a concentration of 4 µg/ml to select cells containing the EGFP-HAC.

Flow cytometry

Analysis of EGFP expression was performed on a FACS Calibur instrument (BD Biosciences) using CellQuest acquisition software and analyzed statistically with FlowJo software (8,15). The cells were harvested by trypsin-treatment. Intensities of fluorescence were determined by flow cytometry. A minimum of 4×10^4 cells was analyzed for each cell sample.

Drug treatment

The drugs used in this study are listed in Table 1. More detailed information on the drugs is summarized in Supplementary Table S1. Our experimental protocol was as follows. HT1080 cells containing the EGFP-HAC were maintained under blasticidin selection to select for the presence of HAC. Approximately 1×10^5 cells were cultured either in the presence or absence of blasticidin selection in parallel with a third culture that was exposed to the compound of interest to test its potential effect on EGFP-HAC segregation. The drug concentration applied was adjusted to the IC₅₀ level for each compound, which was determined using a proliferation assay described below. Concentrations of drugs are presented in Table 1. For each drug, the length of treatment was 20–22 hrs. Subsequently, the drug was removed by performing three consecutive medium washes and the cells were subsequently grown without blasticidin selection for 13 days. At the end of the experiment, cells were collected after trypsinization and analyzed by flow cytometry to detect the proportion of cells that retained EGFP fluorescence. This served as a measure of EGFP-HAC stability following the drug treatment. For each drug, experiments were carried out in triplicate.

γ-Irradiation

HT1080 EGFP-HAC cells were split into a 6-well plate at 1×10^5 cells/well in complete medium (DMEM, 10% fetal bovine serum, 100 U/ml penicillin-streptomycin). The next day,

cells were washed 3 times with non-selective medium. The cells were exposed to 2, 4, 6 and 8 Gy doses of ^{137}Cs γ -rays (dose rate: 2 Gy/min) in a Mark-1 γ -irradiator (JL Sherpherd & Associates, San Fernando, CA). When irradiation was combined with the drug treatment, after irradiation, cells were incubated at 37°C for 2 hrs before adding of a drug.

Calculation of the rate of HAC loss after drug treatment

To calculate the rate of HAC loss after cell treatment by a single dose of drug, we used the formula $P_n = P_0 \times (1 - R_{\text{Drug}})^{n1} \times (1 - R_{\text{Normal}})^{n2}$ where P_0 is the percentage of HAC-containing cells in the population cultured under selection before drug treatment, P_n is the percentage of HAC-containing cells after d days in culture after drug treatment in absence of selection, $n1$ is the number of cell doublings that occurs during drug treatment, $n2$ is the number of cell doublings that occurs during the culturing without selection after the drug treatment (8). CompuSyn software which is based on the median-effect method by Chou–Talalay (16) was used to identify synergy in drug combination. The combination index (CI) theorem of Chou-Talalay defines additive effect as ($\text{CI} = 1$), synergism ($\text{CI} < 1$), and antagonism ($\text{CI} > 1$) in drug combinations.

Cell viability test

MTS tetrazolium cell viability assays were done according to the manufacturer's instructions (CellTiter 96 AQueous Assay reagent; Promega). Briefly, the CellTiter 96 AQueous One Solution Reagent was added to each well and incubated at 37°C for 3 hrs. Cell proliferation was determined by measuring the absorbance at 490 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA). The half-maximal inhibitory concentration (IC_{50}) was obtained from the MTS viability curves using GraphPad Prism 5. We chose this parameter in order to normalize the results with different drugs. It was previously demonstrated that the highest rate of HAC loss was observed at the IC_{50} (8). For each drug, the experiments aimed at the detection of the IC_{50} were carried out in triplicate. These data are presented in Supplementary Table S1.

FISH analysis of EGFP-HAC with the PNA probe

The presence of the HAC in an autonomous form was confirmed by FISH analysis as described previously (8,10). HT1080 cells containing the HAC were grown in complete medium to 70–80% confluence. Metaphase cells were obtained by adding colcemid (Gibco) to a final concentration of 0.05 $\mu\text{g}/\text{ml}$ and incubating overnight. Medium was aspirated, and the plate washed with 1 $\times$ PBS. Cells were removed from the plate by trypsin, washed with medium, pelleted and resuspended in 10 ml of 50 mM KCl hypotonic solution for 30 min at 37°C. Cells were fixed by three washes with 10 ml of fixative solution (75% acetic acid, 25% methanol). Between washes, cells were pelleted by centrifugation at 900 rpm for 4 min. Metaphase cells were evenly spread on a microscope slide and the fixative solution was evaporated over boiling water. Dry slides were rehydrated with PBS for 15 min, and fixed in 4% formaldehyde-1 $\times$ PBS for 2 min, followed by three 5 min cycles of PBS washes followed by dehydration with ethanol. The peptide nucleic acid (PNA)-labeled probe contained a sequence from a tetO-alphoid array (FITC-OO-ACCACTCCCTATCAG) (Panagene, South Korea). 10 nM PNA probe was mixed with hybridization buffer and applied to the slide followed by denaturation at 80°C for 3 min. Slides were hybridized for 2

hrs at room temperature in the dark. Slides were washed twice with 70% formamide, 10 mM Tris pH 7.2, 0.1% BSA, followed by three washes with 1×TBS, 0.08% Tween-20. Slides were dehydrated gradually with a series of 70%, 90% and 100% ethanol washes and mounted (Vectorshield with DAPI). Images were captured using a Zeiss Microscope (Axiophot) equipped with a cooled-charge-coupled device (CCD) camera (Cool SNAP HQ, Photometric) and analyzed by IP lab software (Signal Analytics). The hybrid probe demonstrated high hybridization efficiency, staining intensity and formed a stable PNA-DNA duplex with the complementary nucleic acid. 70–150 metaphases were analyzed for each experiment (one drug treatment).

Depletion of Ska3, Mis18 and CENP-E by siRNA

Pre-validated siRNA targeting published sequences, Ska3/Ram1 (5'-AGACAAACAUGAACAUAUUAUU-3'), Mis18 (5'-AGGCAGUACUACAACCUUTT-3') and CENP-E (5'-AACACGGAUGCUGGUGACCUC-3'), were used to delete these genes (17–19). All duplexes were chemically synthesized and purchased either from Qiagen (Ska3/Ram1 and Mis18) or from Dharmacon (CENP-E). A scramble RNA duplex (5'-UUCUCCGAACGUGUCACGU-3') was used as a control (Dharmacon). For siRNA treatment, we used conditions described previously (17–19) with minor modifications. 1×10^5 /well HT1080 cells were seeded in 6 well plates before a day of the experiment. Cells were transfected two times with each siRNA using lipofectamine-RNAiMAX (Invitrogen) reagent according to the protocol provided by manufacture. Silencing efficiency of Ska3/Ram1 and Mis18 proteins was monitored by Western blot analysis. The following antibodies were used for Western blot analysis: Anti-OIP5 (Mis18) antibody (ab168516), Anti-Ska3 antibody (ab175951) and Anti-GAPDH antibody (#2118) (Cell Signaling Technology).

RESULTS

Mitotic stability of the HAC in human HT1080 cells and analysis of EGFP expression

Human fibrosarcoma HT1080 cells carrying the EGFP-HAC have been used to evaluate drug effects on the fidelity of chromosome transmission (8). Normally, the HAC is mitotically stable with no integration into host chromosomes. Furthermore in HT1080 cells, EGFP expression from the HAC is very stable under the selection conditions. No indication of epigenetic silencing of EGFP was observed after 24 months of continuous culturing under blasticidin selection. Based on the results from FISH analysis, the rate of spontaneous HAC loss was 7×10^{-3} per cell division. The rate of HAC loss measured by the accumulation of non-fluorescent cells during growth in the absence of selection for the HAC by FACS was very similar, 13×10^{-3} , indicating that non-fluorescent cells arise primarily through loss of the EGFP-marked HAC. As mentioned previously, mitotic stability of the EGFP-HAC is approximately 10-fold lower than that of natural chromosomes in HT1080 cells ($\sim 1 \times 10^{-3}$) (8,14,15) that makes the HAC a sensitive model for studying a CIN phenotype in cancer cells. The following experiment demonstrates that the loss of EGFP-HAC reflects the underlying CIN phenotype. Depletion of several genes critical for proper chromosome segregation [Ska3/Ram1 (17,20), CENP-E (18), Mis-18 (19)] results in an increased rate of EGFP-HAC loss (Supplementary Figure S1).

Drugs for analysis of chromosome instability

CIN may be induced by different mechanisms and consequently we chose several groups of drugs targeting these processes. Among them are 14 antimicrotubule agents, 13 inhibitors of HDAC, 8 mitotic checkpoint inhibitors, 16 inhibitors of DNA damage repair (DDR) and DNA replication, and 11 other drugs targeting different elements involved in control of cell survival and proliferation. It was previously demonstrated that the highest rate of HAC loss is observed at the IC₅₀ (8). Treatment of the cells with higher doses of the compounds killed more cells but did not increase the rate of HAC loss. On the other hand, treatment of the cells with lower doses of the compounds induced either no or lower frequency of HAC loss. Therefore, for each compound a cell cytotoxicity assay was carried out to determine the IC₅₀ values for HT1080 cells, i.e., the conditions under which the viability of cells would be around 50%. We chose this parameter in order to normalize the results with different drugs. The complete list of drugs used in this work is presented in Table 1. Concentrations of the drugs corresponding to their IC₅₀ values are shown in Supplementary Table S1.

Effect of antimicrotubule drugs on the HAC mis-segregation rate during mitotic divisions

Over the past five decades, antimicrotubule agents, with different modes of tubulin targeting such as vinca alkaloids and taxanes, have been developed and used clinically. They have demonstrated high potency against the proliferation of various cancer cells, as well as in multidrug-resistant cancers (21,22).

In the current study, we first investigated whether the EGFP/HAC-based assay could be used to rank compounds that affect microtubule dynamics (21,22). HT1080 cells with an autonomously propagated EGFP-HAC were treated with six well established microtubule-stabilizing drugs: paclitaxel, docetaxel, peloruside A, ixabepilone, dactylolide, zampanolide, and eight microtubule-destabilizing drugs: nocodazole, vincristine, combretastatin A4, maytansine, cryptophycin, vindesine, vinorelbine, and eribuline mesylate (Table 1).

For each compound, a cell cytotoxicity assay was carried out to determine the IC₅₀ values. After treatment, the cells were grown for 13 days in the absence of selection and then the proportion of non-fluorescent cells was determined. Based on our previous studies, this is the optimal sampling time (8). The delay between HAC loss and the appearance of non-fluorescent cells is due to the persistence of the EGFP protein, which has a half-life of over a day. The rate of HAC loss induced by drug treatment was calculated from the proportion of non-fluorescent cells in the cell population as described in Materials and Methods. Notably, the data on HAC loss determined by FACS correlate with the results of quantitative FISH analyses of metaphase chromosome spreads using a HAC-specific probe (see Supplementary Table S2) (8). Figure 2 summarizes the estimated rates of HAC loss in response to different microtubule-targeting drugs. As shown in Figure 2A, all analyzed microtubule-stabilizing drugs increased the rate of HAC loss. Maximal effect was observed for taxol (paclitaxel) and dactylolide (>50-fold increase compared to untreated controls). Treatment by four other drugs, ixabepilone, peloruside A, docetaxel, and zampanolide, resulted in a lower frequency of HAC loss (~32 times higher than spontaneous HAC loss). HAC loss was also increased after treatment of cells by microtubule-destabilizing drugs (Fig. 2B). However, the rates of

HAC loss were significantly lower compared to that observed for microtubule-stabilizing compounds. Maximum effects were detected for combretastatin A4, maytansine, vinorelbine, and eribulin mesylate (~20-fold increase compared to untreated controls).

Thus, the EGFP-HAC-based assay allowed us to rank 14 antimicrotubule drugs with respect to their effect on chromosome stability. Taxol and dactylolide exhibited the highest effect on chromosome stability and may be considered as good candidates for the development of new therapeutic strategies to target the CIN phenotype in cancer cells.

Effect of inhibitors targeting mitosis

Microtubule poisons have already proven efficacy in the clinic against a broad range of malignancies, and novel targeted strategies are now evaluating the inhibition of critical activities, such as cyclin-dependent kinase 1 (CDK1), Aurora or Polo kinases, or spindle kinesins that have been identified as mitosis-specific targets developed in a search for non-microtubule targets that preserve the efficacy of microtubule-targeting drugs (23). Abrogation of the mitotic checkpoint or induction of the energetic or proteotoxic stress within aneuploid or chromosomally unstable cells may also provide further benefits by inducing lethal levels of chromosome instability.

In separate experiments, we checked several inhibitors targeting mitosis (24,25), including four inhibitors of Aurora kinases (VX-680, MLN8737, reversine, ZM-447439), two inhibitors of Polo kinases (GW843682, SBE13) and two inhibitors of mitotic kinesin (S-trityl-L-cysteine, monastrol). All inhibitors of Aurora kinases induced ~30-fold increase of HAC loss (Fig. 3A). A slightly higher effect was observed for one of the Polo kinase inhibitors, GW843682. Treatment of the cells with inhibitors of mitotic kinesin resulted in a 5–15× increase of HAC loss. To summarize, inhibitors targeting mitosis also induce a significant increase of chromosome instability.

Effect of histone deacetylase (HDAC) inhibitors

HDAC inhibitors are emerging as promising anticancer drugs, which possess tumor-selective cytotoxicity. HDAC inhibitors have been demonstrated to promote growth arrest, differentiation, and apoptosis of cancer cells, with minimal effects on normal tissue and four HDAC inhibitors have been approved by the US Food and Drug Administration for the treatment of hematologic malignancies (26–28). It has also been proposed that inhibition of histone deacetylase activity could increase chromosomal instability by the aberrant regulation of mitotic checkpoint activation. HDAC inhibitors comprise structurally diverse anticancer agents and clinical studies demonstrate that combination of HDAC inhibitors with some anticancer drugs may have synergistic or additive effects (29,30).

Here, we investigated how structurally diverse HDAC inhibitors affect chromosome stability. The HT1080 cells with an autonomously propagated EGFP-HAC were treated with 13 well-known HDAC inhibitors: trichostatin A, romidepsin, mocetinostat, MS275, SAHA, niacinamide, panobinostat, apicidin, tubastatin A, belinostat, bufexamac, cambinol, and sirtinol. Figure 3B illustrates effects of these inhibitors on the rate of HAC loss. Only four drugs, trichostatin A, romidepsin, MS275, and mocetinostat, were capable of significantly increasing the level of spontaneous HAC loss. Notably, the effect of these inhibitors was

lower (10–20×) than those demonstrated by the antimicrotubule drugs (Fig. 2). No statistically significant increase of HAC loss was detected with the other analyzed HDAC inhibitors.

Effect of inhibitors targeting DNA damage response/replication

Induction of DNA damage and inhibition of specific DNA damage response (DDR) pathways are features of most chemotherapeutic agents. In separate experiments, we analyzed a large group of DNA-damaging agents and targeted DDR inhibitors for their effect on the HAC propagation. Specifically, three inhibitors of poly(ADP-ribose) polymerase (PARP) (31,32) [talazoparib, olaparib and ABT888 (veliparib)], four inhibitors of Topoisomerases I and II (33,34), [(camptothecin, LMP400, doxorubicin and VP16 (etoposide)], three inhibitors of DNA synthesis (35,36) [gemcitabine, methotrexate and cytarabine (AraC)], four inhibitors of DDR (37) (AZ6738 and VE821 targeting ATR, and AZD7762 and PV1162 targeting Chk1 and Chk2, respectively), as well as two drugs inducing DNA damage (38) (cisplatin and bleomycin) were included in the analysis. The effect of these inhibitors on the rate of HAC loss is summarized in Figure 4A. Most of the analyzed drugs dramatically increased the frequency of HAC loss. The highest increase of chromosome instability (35–45 fold) was observed for five drugs: LMP400 (or indotecan), gemcitabine, talazoparib, olaparib, and cisplatin. It is worth noting that the HAC-destabilizing effect of gemcitabine is as great as the strongest microtubule-stabilizing agents, taxol. Further analysis may be required to clarify whether this drug disrupts only DNA replication or also impairs proper chromosome segregation.

Effect of inhibitors of cell proliferation, apoptosis and necroptosis

Other compounds with different targets are also currently used for cancer therapy. As they may affect chromosome stability, we have included in our analysis 13 additional drugs that act as inhibitors of cell proliferation inducing apoptosis and necroptosis. They are: i) protein phosphatase kinase inhibitors, lasonolide A (PKA) (39), PD-0325901 (Akt/PKB) and MK-2206 (MAPK); ii) bromodomain inhibitors, JQ1 and I-BET; iii) inhibitors of HSP90 (40), 17-AAG and SNX212; inhibitors of necroptosis and apoptosis, Nec1, Nec1-S and SMAC; and iv) ATR inhibitors, AZ6738, and VE-821. Figure 4B summarizes data on the HAC stability in the cells treated by these drugs. While a statistically significant increase (3–10-fold) of HAC loss was observed for most of the analyzed inhibitors, their effect was much lower compared to those observed for antimicrotubule drugs or DDR/replication inhibitors and may not be a clinically relevant mode of action for these drugs.

Effects of treatment by combinations of drugs that elevate CIN

Combination therapies are commonly used for cancer therapy. Typically, the use of combinations of chemotherapy medications allows oncologists to use some of the drugs at lower doses, and hence reduce the likelihood of toxic effects. For example, combinations of PARP inhibitors with cytotoxic agents such as chemotherapy or radiation therapy has led to synergistic effects in many preclinical models (41,42). In our work, we first investigated two combinations of the three drugs exhibiting the highest effects on chromosome instability: taxol plus gemcitabine and gemcitabine plus LMP400. As illustrated in Figure 5A, combining drugs at concentrations significantly lower than the IC50 values resulted in a 40–

45-fold increase in HAC loss. This effect is very close to that observed for the cell treatment with paclitaxel alone at the IC50 concentration. Synergism was observed with the combination of LMP400 and gemcitabine (CI=0.690). Synergy was also observed for combinations of romidepsin with inhibitors of mitosis and necroptosis, Nec-1, Nec-1s, SMAC, and Z-VAD (CI=0.030, CI=0.013, CI=0.019, CI= 0.029, respectively) (Supplementary Figure S2). However, for these treatments, the rates of HAC loss were not exceptionally high. Notably, the increase of HAC loss was accompanied by an increase in cytotoxicity (data not shown).

In parallel experiments, we demonstrated that the rate of HAC loss was greatly increased when treatment with paclitaxel or gemcitabine at lower concentration was combined with γ -irradiation (Fig. 5B). Synergetic effects were detected for treatment with paclitaxel combined with a relatively high dose of γ -irradiation, 6 Gy or 8 Gy (CI=0.624 and CI=0.378, respectively). Similar synergetic effect was observed when both paclitaxel and gemcitabine was used in combination with γ -irradiation, 6 Gy or 8 Gy (CI=0.796 and CI=0.526, respectively). It is worth noting that lower doses of irradiation did not synergistically induce HAC destabilization.

It is known that hypothermia has the potential to modify the cytotoxicity of anticancer drugs (43). One possible mechanism for this action is that the microtubular spindle is very sensitive to cooling and is rapidly depolymerized even after a slight reduction in temperature. Spindle disassembly is dependent on the extent of temperature decrease and its duration. After rewarming, the recovery is far from complete (44) and therefore may affect CIN. Also it has been demonstrated that hyperthermia may lead to damage and death of cancer cells, enhancing the effectiveness of cancer treatments (45,46). Therefore in separate experiments we checked if such treatment might increase CIN induced by anticancer drugs. For this purpose, the cells were treated by a low dose of an antimicrotubule drug (paclitaxel) or Top I inhibitor (LMP400) and then were exposed to cold shock (4°C) or heat shock (42°C). Based on the analysis, neither cold shock nor hyperthermia increased the HAC loss induced by the treatment of cells with these drugs (Supplementary Figure S3).

To conclude, these experiments indicate that drugs that increase CIN can be combined to achieve the highest effects using lower drug concentrations.

Discussion

Targeting CIN in cancer therapy requires quantitative measurement of the chromosome transmission accuracy. At present, a variety of methods are used to study chromosome instability (CIN) and its induction by environmental agents (47). The micronuclei (MNi) formation test is the most widely used method for large-scale detection of broken or lagging chromosomes [(12) and references therein]. However, the origins and fates of MNi have not been completely elucidated (48,49) and intra- and inter-laboratory variability in scoring is still common (48), and complicates the development of a standard protocol for quantitative measurement of chromosome loss rates based on the appearance of MNi. It is also noteworthy that the MNi assay does not measure the fraction of drug-arrested cells that undergo mitosis and form viable aneuploid cells.

In the previous work we described a new assay for measuring CIN in response to drug treatment that overcomes the limitations of the current approaches (8). The assay is based on quantitative measurements of the mitotic loss rates of a nonessential human artificial chromosome (HAC) carrying a transgene expressing the enhanced green fluorescent protein (EGFP). The HAC contains a functional kinetochore and its behavior during mitotic divisions does not differ from that of normal chromosomes (8–11,14), suggesting that destabilization of natural chromosomes in response to drug treatment will be increased proportionally to that observed for the HAC. It is worth noting that the earlier development of conceptually simple color colony assays in yeast provided a powerful genetic tool to assess the rates of chromosome mis-segregation and to identify mutants deficient in this process (50–53).

In this study, the EGFP-HAC-based assay was applied to analyze 14 antimicrotubule drugs, 13 HDAC inhibitors, 8 mitotic checkpoint inhibitors, 16 drugs that target DDR and DNA replication, and 11 compounds used for targeting cell proliferation and apoptosis. For each drug, the rate of HAC loss was quantified and within each analyzed group, the compounds were ranked according to their HAC destabilizing potency. The most interesting result obtained from these experiments is that compounds with the proposed similar mechanisms of action and cytotoxicity may greatly differ from each other by their effect on mitotic stability of the non-essential human artificial chromosome. For example, paclitaxel and docetaxel have the same binding site on the microtubules (21) and exhibit the same cytotoxicity, but the rates of HAC loss differ 5–7 times. The highest rate of HAC mis-segregation was observed for the microtubule-stabilizing drugs (paclitaxel, dactylolide, and peloruside A), inhibitors of Polo-like and Aurora kinases (GW843682 and VX-680), PARP inhibitors (olaparib and talazoparin), inhibitors of Top 1 (LMP400 or indotecan), inhibitor of DNA synthesis (gemcitabine), and a DNA crosslinking agent (cisplatin). These compounds may be recommended as the first choice when CIN is considered as a target for cancer therapy. It is interesting that the top ten drugs include taxol, gemcitabine and cisplatin used as the front-line chemotherapeutic drugs for many types of cancer for decades.

The combination of drugs with different mechanisms of action resulting in chromosome destabilization also may be considered for new clinical trials. Such an approach may decrease the concentration of drugs used for treatment and thereby reduce the risk of possible side effects and may result in development of “synthetic lethal” therapeutic strategies. It is worth noting that some drugs inducing the highest rates of HAC loss have been already used in combining therapies. Specifically, cisplatin, and paclitaxel are two established chemotherapeutic drugs used in combination for the treatment of many cancers, including ovarian cancer (54,55). Also, the combination of gemcitabine plus taxol has been used for treatment of metastatic pancreas cancer (56,57). The clinical promise of targeting cancers by two drugs that increase CIN by different mechanisms warrants more study. It is worth noting that the EGFP-HAC-based assay may be used to choose an optimal drug for treatment of cancer cells carrying specific mutations. As a first attempt, human cells with transiently enhanced CIN were created with siRNA knockdown of genes known to induce chromosome instability, SKA3/RAMA1 and MIS18 (17,19,20). These CIN cells, which also carried EGFP-HAC, were then treated with either taxol or gemcitabine at concentrations lower than IC₅₀ (see Supplementary Figure S1C). As seen, combination of gene depletion

and drug treatment results in a great HAC loss. At present, we are working on developing knockouts of genes in HT1080 cells that are frequently mutated in tumors. Isogenic pairs of these cell lines will be analyzed on sensitivity to different drugs and drug combinations.

To conclude, this study represents the first systematic analysis of the chromosome destabilizing potency of drugs used in cancer therapy. A new EGFP-HAC-based assay for measuring CIN is the most sensitive system developed so far that can be applied to identify new compounds that elevate chromosome mis-segregation and drive lethal aneuploidy. New and potentially less toxic agents that selectively elevate CIN in cancer cells to promote cancer cell death identified with this new screening tool could lay the foundation for new treatment strategies for cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Scheme of assay used to measure chromosome instability (CIN) based on HAC containing the *EGFP* transgene. Cells that inherit the HAC display green fluorescence, while cells that lack it do not. It is expected that the control population of untreated cells should show uniform green fluorescence, cell population that have lost HAC after drug treatment should be highly variable in fluorescence. The actual number and percentage of cells with the *EGFP*-HAC can be measured by FACS. Thus, the compounds, which increase HAC loss and therefore increase spontaneous chromosome mis-segregation rates, may be identified.

Figure 2.
A, Effect of microtubule-stabilizing drugs on HAC mis-segregation rate. HT1080 cells were treated by six different compounds, as described in Materials and Methods. The rate of HAC loss per cell division was calculated based on the ration HAC-positive, HAC-negative cells and the average time per cell division. The drug concentrations used were at IC50 for HT1080 cells. The strongest CIN inducer among the microtubule-stabilizing drugs tested was taxol. B, Effect of microtubule-destabilizing drugs on HAC mis-segregation rate. HT1080 cells were treated by eight microtubule-destabilizers and the rate of HAC loss per

cell division calculated as described in Materials and Methods. The control corresponds to the frequency of spontaneous loss of the EGFP-HAC in human HT1080 cells. Combretastatin A4 was the strongest CIN inducer among the microtubule-destabilizing drugs tested.

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Figure 3. A, Effect of inhibitors targeting mitosis on the rate of HAC loss. HT1080 cells were treated by the compounds named and the rate of HAC loss per generation was calculated as described in Materials and Methods. Bars with same color correspond to inhibitors with the same mechanism of action. GW843682 was the strongest CIN inducer among the analyzed inhibitors targeting mitosis. B, Effect of histone deacetylase (HDAC) inhibitors on the rate of HAC loss. HT1080 cells were treated by 13 different HDAC inhibitors and the rate of HAC loss per generation was calculated as described in Materials and Methods. The control

corresponds to a frequency of spontaneous loss of the EGFP-HAC in human HT1080 cells. Trichostatin A was the strongest CIN inducer among the HDAC inhibitors tested.

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Figure 4.

A, Effect of inhibitors targeting DNA damage response/replication on the rate of HAC loss. HT1080 cells were treated by the compounds as described in Materials and Methods. A rate of HAC loss per generation was calculated based on the ration HAC-positive and HAC-negative cells. Bars with same color correspond to inhibitors with the same mechanism of action. The control corresponds to a frequency of spontaneous loss of the EGFP-HAC in human HT1080 cells. B, Effect of inhibitors of cell proliferation, apoptosis and necroptosis. HT1080 cells were treated by the compounds as described in Materials and Methods. A rate

of HAC loss per generation was calculated based on the ration HAC-positive and HAC-negative cells. Bars with same color correspond to inhibitors with the same mechanism of action. The control corresponds to a frequency of spontaneous loss of the EGFP-HAC in human HT1080 cells.

Figure 5.

Effect of cells treatment by combinations of drugs and combination of a drug and γ -irradiation. A, HT1080 cells were treated either a low concentration of an inhibitors or by combination of three compounds, taxol, gemcitabine and LMP400. Bars with mixed colors correspond to double drug treatment. B, HT1080 cells were γ -irradiated by different doses then incubated for 2 hrs and treated by inhibitors at lower concentrations. Bars with mixed

colors correspond to the combining drug treatment with irradiation. The control corresponds to a frequency of spontaneous loss of the EGFP-HAC in human HT1080 cells.

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Table 1

The list of drugs used in this study

Target or targeting pathway	Compounds
Microtubule (MT stabilizing agent)	Ixabepilone, Peloruside A, Taxol, Dactylolide, Zampanolide, Docetaxel
Microtubule (MT destabilizing agent)	Vincristine, Nocadazole, Combretastatin A4, Maytansine, Cryptophycin 1, Vindesine, Vinorelbine, Eribuline mesylate
Mitosis, chromosome condensation (Aurora kinases; Polo-kinases; Mitotic kinesin)	VX-680, MLN8737, Reversine, ZM-447439; GW843682, SBE13, S-trityl-L-cysteine, Monastrol
Histone Deacetylases (HDAC)	Trichostatin A, Romidepsin, Mocetinostat, Entinostat, SAHA, Panobinostat, Apicidin, Tubastatin A, Belinostat, Buxefamac, Cambinol, Sirtinol, Niacinamide.
DNA damage response/replication (PAPR; Top1 and Top2; dNTP synthesis; Checkpoint, Chk1, Chk2; DNA damage)	Talazoparib, Olaparib, Veliparib ; Camptothecin, LMP400, Doxorubicin, Etoposide; Gemcitabine, Methotrexate, Cytarabine (AraC); AZD7762, PV1162; Cisplatin, Bleomycin
Cell survival and proliferation (Mek1/2; Caspase; Necroptosis and apoptosis, HSP90, Bromodomain, PKC, ATR)	MK-2206; Z-VAD; PD-0325901, SMAC; Nec1, Nec1-s; SNX-2112, 17-AAG; JQ1, I-BET; Lasonolide A; AZ6738, VE821.