High-throughput genotoxicity assay identifies antioxidants as inducers of DNA damage response and cell death

Jennifer T. Fox[a], Srilatha Sakamuru[b], Ruili Huang[b], Nedelina Teneva[a], Steven O. Simmons[c], Menghang Xia[b], Raymond R. Tice[d], Christopher P. Austin[b], and Kyungjae Myung[a,1]

[a]Genome Instability Section, Genetics and Molecular Biology Branch, National Human Genome Research Institute, and [b]National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, MD 20892; [c]Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, United States Environmental Protection Agency, Research Triangle Park, NC 27711; and [d]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709

Edited by Richard D. Kolodner, Ludwig Institute for Cancer Research, La Jolla, CA, and approved February 16, 2012 (received for review August 30, 2011)

Human ATAD5 is a biomarker for identifying genotoxic compounds because ATAD5 protein levels increase posttranscriptionally in response to DNA damage. We screened over 4,000 compounds with a cell-based quantitative high-throughput ATAD5-luciferase assay detecting genotoxic compounds. We identified 22 antioxidants, including resveratrol, genistein, and baicalein, that are currently used or investigated for the treatment of cardiovascular disease, type 2 diabetes, osteopenia, osteoporosis, and chronic hepatitis, as well as for antiaging. Treatment of dividing cells with these compounds induced DNA damage and resulted in cell death. Despite their genotoxic effects, resveratrol, genistein, and baicalein did not cause mutagenesis, which is a major side effect of conventional anticancer drugs. Furthermore, resveratrol and genistein killed multidrug-resistant cancer cells. We therefore propose that resveratrol, genistein, and baicalein are attractive candidates for improved chemotherapeutic agents.

chemotherapy | high-throughput screening

One distinctive characteristic of cancer cells is persistent cell division that requires DNA replication. This feature is often exploited to develop chemotherapeutic drugs because cancer cells are exquisitely sensitive to the inhibition of DNA replication by the introduction of DNA damage by radiation or genotoxic chemicals. DNA lesions resulting from exposure to genotoxic agents stall DNA replication, collapse replication forks, and produce DNA double-strand breaks (DSBs), resulting in cell death. If not repaired properly, many of these genomic insults can also induce gene mutations or chromosomal alterations that may make cells more resilient to cell-cycle checkpoints or apoptosis. Thus, cancer treatment may greatly benefit from the identification of genotoxic agents that kill rapidly dividing cells with minimal mutagenic side effects.

ATAD5 is the homolog of yeast Enhanced Level of Genome Instability Gene 1 (*ELG1*), which makes a heteropentameric alternative replication factor C complex and suppresses genomic instability and tumorigenesis (1–3). ATAD5 plays a key role in the translesion synthesis (TLS) pathway where TLS polymerases are used to bypass DNA lesions that stall or collapse DNA replication forks (4, 5). In the TLS pathway, switching from replicative polymerases to TLS polymerases is promoted through the interaction between proliferating cell nuclear antigen (PCNA) monoubiquitylated at lysine 164 and an ubiquitin-binding motif in TLS polymerases, a mechanism that is conserved from yeast to humans (6). ATAD5 is stabilized and forms nuclear foci at the site of stalled replication forks in response to DNA damage (7) and appears to participate in the removal of ubiquitin from chromatin-bound monoubiquitylated PCNA through its interaction with ubiquitin-specific peptidase 1 (5). The ATAD5-mediated deubiquitylation of PCNA allows lesion-bypassed TLS polymerases to switch back to replicative polymerases, and thereby prevents the low-fidelity TLS polymerases from causing harmful mutations or collapsed replication forks.

Because ATAD5 is stabilized in response to various types of DNA damage, we reasoned that we could identify genotoxic compounds with chemotherapeutic potential from small-molecule libraries by monitoring ATAD5 protein levels. The results of our screen not only validated our hypothesis, but also revealed several antioxidants as promising chemotherapeutic agents. Three of these antioxidants, resveratrol, baicalein, and genistein, kill rapidly dividing cells without producing the potentially deadly side-effects of chromosomal alterations and mutagenesis, and thus are potentially better chemotherapeutic agents than ones currently used for cancer treatment.

Results

Development of a High-Throughput ATAD5-Luciferase Assay. To identify compounds that enhance human ATAD5 protein levels, we created an HEK293T cell line that stably expresses luciferase-tagged ATAD5 (Fig. 1 *A*–*C*). Like native ATAD5 (7), the level of the ATAD5-luciferase fusion protein (ATAD5-luc), as monitored by Western blot, as well as the measurement of luciferase activity, was increased following treatment with the DNA alkylating agent, methyl methanesulfonate (MMS), in a dose-dependent manner (Fig. 1 *C* and *D*). ATAD5-luc formed DNA damage-induced nuclear foci following treatment with MMS and UV (Fig. S1*A*), similar to the native ATAD5 protein, and could complement the PCNA deubiquitylation defect observed upon ATAD5 knockdown (5) (Fig. S1 *B* and *C*) Expression of ATAD5-luc did not affect the DNA damage response in HEK293T cells, as evidenced by similar levels of cisplatin-induced TLS polymerase foci in the ATAD5-luc cell line compared with the unmodified cell line (Fig. S1*D*).

After optimizing the ATAD5-luc assay in a 1,536-well plate format (Fig. S2) (signal-to-background ratio = 5.6, coefficient of variation = 7.3%, and Z factor = 0.74), we screened 4,156 molecules from the compound collections of the National Toxicology Program (NTP, see http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcsubstance&term=NTPHTS for the complete list of compounds) and Tocris Biosciences (TB, see Table S1 for the complete list of compounds) in a quantitative high-throughput manner using at least seven different concentrations of each compound

Author contributions: J.T.F. and K.M. designed research; J.T.F., S.S., R.H., N.T., S.O.S., and K.M. performed research; R.H., S.O.S., M.X., R.R.T., C.P.A., and K.M. contributed new reagents/analytic tools; J.T.F., R.H., M.X., R.R.T., and K.M. analyzed data; and J.T.F. and K.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: kmyung@mail.nih.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1114278109/-/DCSupplemental.

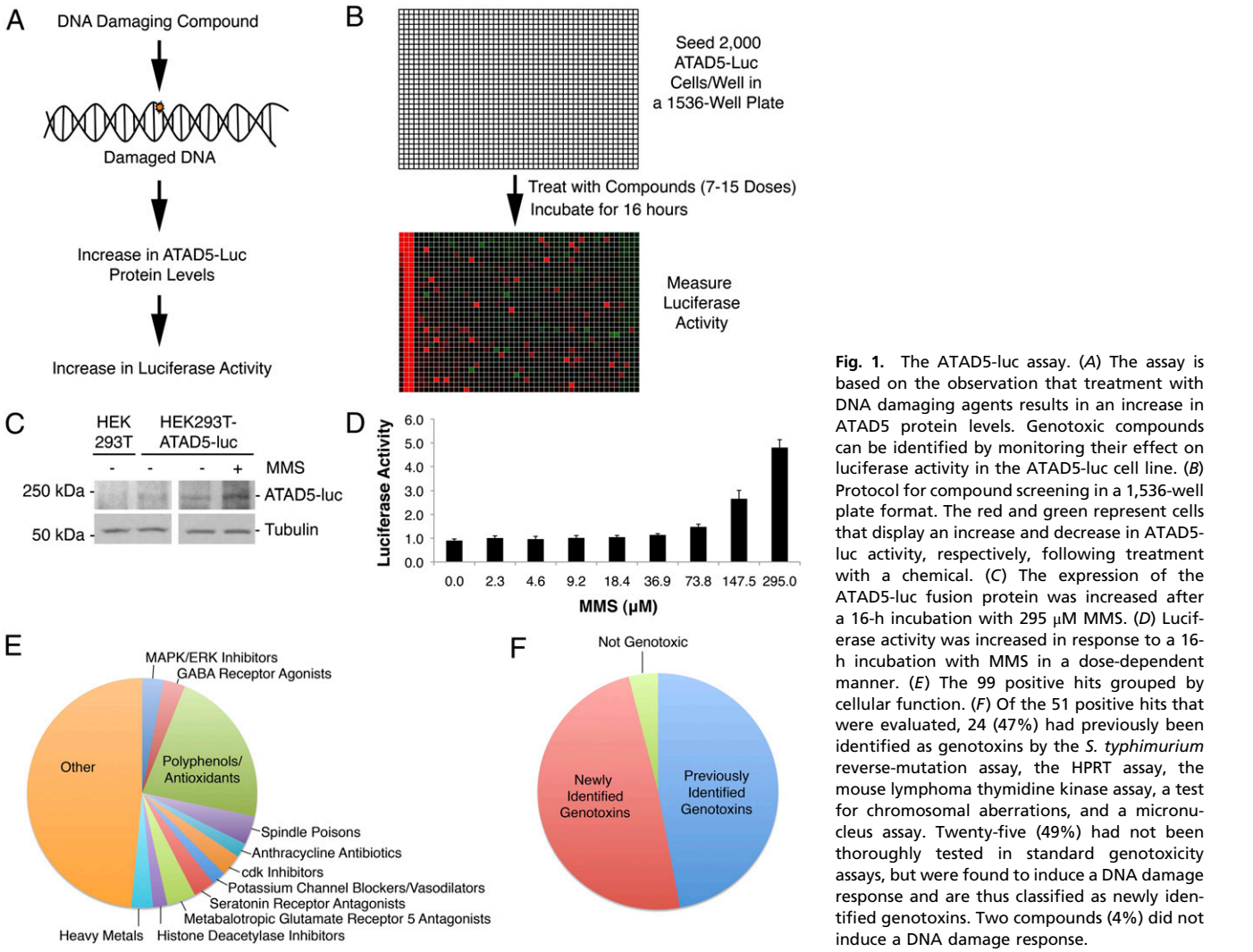

Fig. 1. The ATAD5-luc assay. (*A*) The assay is based on the observation that treatment with DNA damaging agents results in an increase in ATAD5 protein levels. Genotoxic compounds can be identified by monitoring their effect on luciferase activity in the ATAD5-luc cell line. (*B*) Protocol for compound screening in a 1,536-well plate format. The red and green represent cells that display an increase and decrease in ATAD5-luc activity, respectively, following treatment with a chemical. (*C*) The expression of the ATAD5-luc fusion protein was increased after a 16-h incubation with 295 μM MMS. (*D*) Luciferase activity was increased in response to a 16-h incubation with MMS in a dose-dependent manner. (*E*) The 99 positive hits grouped by cellular function. (*F*) Of the 51 positive hits that were evaluated, 24 (47%) had previously been identified as genotoxins by the *S. typhimurium* reverse-mutation assay, the HPRT assay, the mouse lymphoma thymidine kinase assay, a test for chromosomal aberrations, and a micronucleus assay. Twenty-five (49%) had not been thoroughly tested in standard genotoxicity assays, but were found to induce a DNA damage response and are thus classified as newly identified genotoxins. Two compounds (4%) did not induce a DNA damage response.

(Table S2). Between these two collections, 99 compounds (53 from TB, 42 from NTP, and 4 from both libraries) were considered to be positive hits because they displayed >40% of the activity of the MMS control, which corresponds to at least a twofold increase in luciferase activity compared with DMSO treatment (Fig. 1*E* and Table S3). Among these 99 compounds were putative MAPK/ERK inhibitors, GABA receptor agonists, polyphenols/antioxidants, spindle poisons, anthracycline antibiotics, cdk inhibitors, potassium channel blockers, seratonin receptor antagonists, metabalotropic glutamate receptor 5 antagonists, histone deacetylase inhibitors, and heavy metals (Fig. 1*E* and Table S3). At least one compound from each group in Table S3 (60 in total) was randomly selected and retested in the ATAD5-luc assay to evaluate the screen. Of these compounds, 56 (93%) enhanced ATAD5-luc activity in the evaluation (Fig. S3*A* and Table S3). The correlation coefficient between the log of the EC_{50} values obtained from the screen and those obtained during the evaluation was 0.83. Because direct stabilizers of luciferase can generate a false-positive signal in luciferase-based screens (8), we also tested these 56 compounds for their ability to stabilize FLAG-tagged ATAD5 at the protein level. Of the selected compounds, 51 (91%) stabilized the FLAG-ATAD5 protein (Fig. S3*B* and Table S3).

ATAD5-luc Assay Identifies Genotoxic Compounds, Including Antioxidants. Many positive hits from the ATAD5-luc screen have been reported to negatively affect genomic integrity by a variety of molecular mechanisms (Table S3, column 4). Twenty-four of the 51 compounds that stabilized the FLAG-ATAD5 protein were reported as genotoxins by several standard genotoxicity assays, including the *Salmonella typhimurium* reverse-mutation assay, the hypoxanthine-guanine phosphoribosyltransferase (HPRT) assay, the mouse lymphoma thymidine kinase assay, a test for chromosomal aberrations, and a micronucleus assay (Fig. 1*F* and Table S4) [data obtained from the Chemical Carcinogenesis Research Information System (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS), the NTP (http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm), and Leadscope toxicity databases]. Twenty-five additional compounds produced other DNA damage responses, including changes in the level of chromatin-bound ubiquitylated PCNA, and the phosphorylation of RPA32, CHK1, ATM, and H2AX in HEK293T cells, as detected by Western blotting (Figs. 1*F* and 2*A*, Fig. S4, and Table S4). In total, 49 (96%) of 51 compounds selected from the ATAD5-luc screen were positive for genotoxicity in an independent assay.

More than 20% of the positive hits from the ATAD5-luc screen were small molecules classified as polyphenols and antioxidants (Fig. 1*E* and Table S3). We should point out, though, that not all polyphenols/antioxidants present in the chemical libraries were active in the ATAD5-luc assay. For example, kaempferol, myricetin, morin, fisetin, and quercetin, which are all flavonoids, failed to stabilize ATAD5-luc. However, given the predominance of the polyphenols/antioxidants, we decided to investigate the nature of the genotoxicities induced by these compounds as well as their

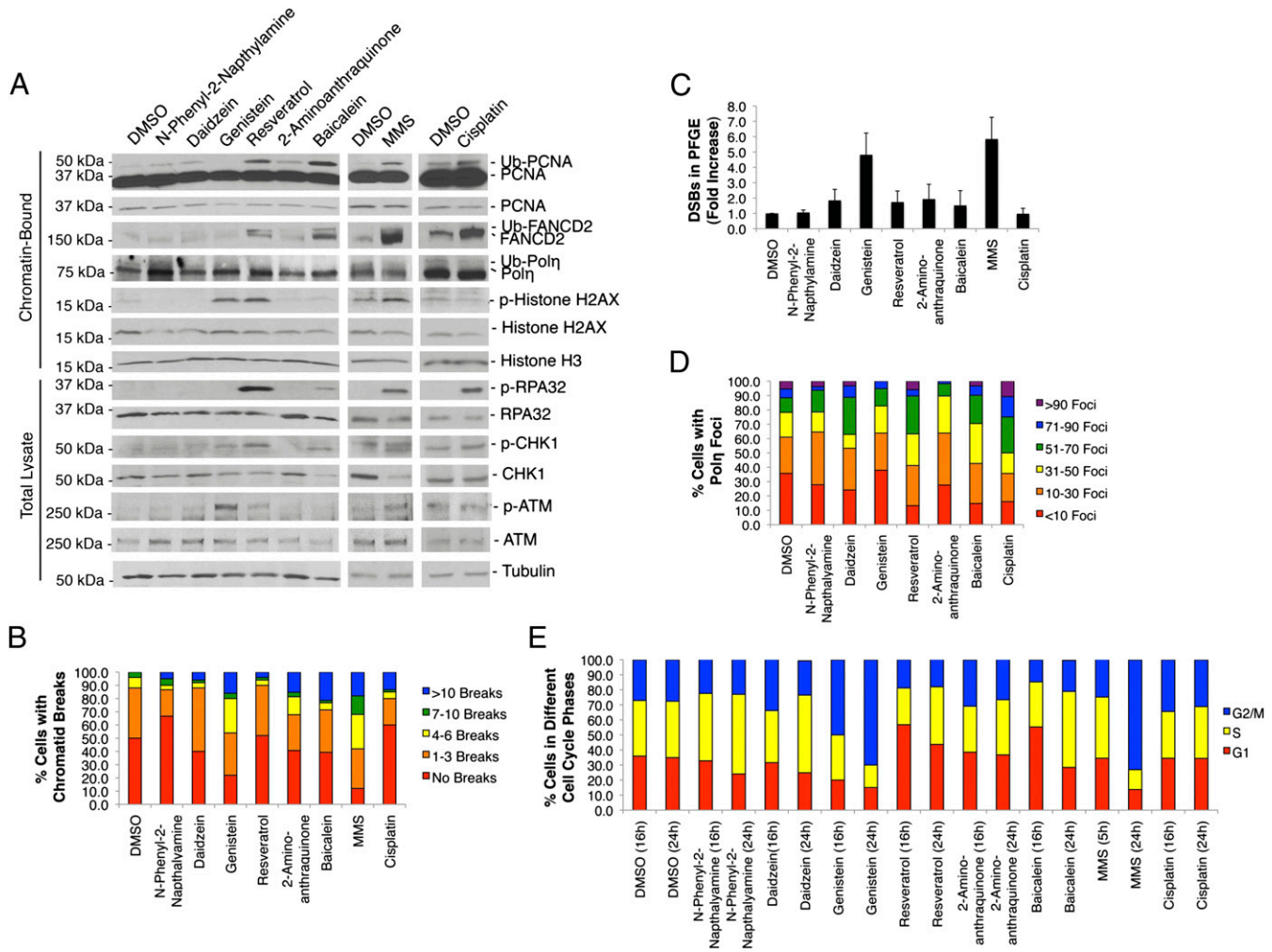

Fig. 2. Polyphenols/antioxidants identified from the ATAD5-luc screen induce genotoxicity. (*A*) HEK293T cells were treated with 92 μM polyphenols/antioxidants (the concentration that produced the greatest increase in luciferase activity in the evaluation) or 10 μM cisplatin for 16 h, or 1.18 mM MMS for 1 h followed by a 5-h recovery. The level of the indicated proteins was determined by Western blot analysis using either the chromatin-bound fraction or total cell lysate. (*B*) The percentage of HEK293T metaphase cells containing the indicated number of chromatid breaks following treatment as described in *A* was determined in at least 50 metaphases per treatment. (*C*) HEK293T cells were treated as described in *A*, and DNA DSBs were visualized by pulsed-field gel electrophoresis. The intensity of the smear produced by DNA containing DSBs was quantified using ImageJ and normalized to the DMSO control. The graph represents the average of at least three independent experiments ± SD. (*D*) YFP-Polη foci were counted in at least 60 HEK293T cells after treatment with 92 μM polyphenols/antioxidants or 10 μM cisplatin for 16 h. (*E*) Cell-cycle profiles of HEK293T cells after treatment with 92 μM polyphenols/antioxidants, 1.18 mM MMS, or 10 μM cisplatin for the indicated times (Note: the times listed for MMS are recovery times following treatment for 1 h).

chemotherapeutic potential. Six compounds were selected based on the different structure subgroups to which they belong and whether or not they have been reported to exhibit antioxidant activity. 2-Aminoanthraquinone is an anthraquinone and exhibits no antioxidant activity; the stilbene resveratrol, the flavone baicalein, and the isoflavones daidzein and genistein all have some degree of antioxidant activity; and *N*-phenyl-2-napthylamine, which is not a polyphenol, is used as an industrial antioxidant.

First, these compounds were tested for their ability to induce breaks in DNA. Treatment with all compounds increased the fraction of cells having more than 10 chromatid breaks (Fig. 2*B* and Fig. S5*A*). Treatment with genistein and resveratrol resulted in a significant induction of a DSB marker, H2AX phosphorylation (Fig. 2*A*), and the level of DSBs induced by genistein treatment could also be observed by pulsed-field gel electrophoresis (Fig. 2*C* and Fig. S5*B*). Second, we investigated the DNA damage response upon treatment with these compounds. *N*-phenyl-2-napthylamine, daidzein, and 2-aminoanthraquinone were mild inducers of a DNA damage response. Treatment with these three compounds moderately increased PCNA ubiquitylation, but did not significantly affect Fanconi anemia complementation group D type 2 (FANCD2) ubiquitylation or the phosphorylation of RPA32 or CHK1 (Fig. 2*A*). These compounds also increased the number of TLS polymerse Polη foci in cells approximately threefold compared with the DMSO control (Fig. 2*D* and Fig. S5*C*). Resveratrol and baicalein induced a robust DNA damage response. Both compounds caused significant increases in PCNA and FANCD2 ubiquitylation as well as RPA32 and CHK1 phosphorylation (Fig. 2*A*). These two compounds also resulted in approximately six times the number of TLS polymerse Polη foci compared with the DMSO control (Fig. 2*D* and Fig. S5*C*). Genistein treatment resulted in a significant decrease in the amount of unmodified and ubiquitylated PCNA bound to chromatin and induced a high level of CHK1 phosphorylation (Fig. 2*A*). Finally, all six compounds were tested for their effects on cell-cycle progression (Fig. 2*E*). The inducers of the most robust DNA damage response, resveratrol and biacalein, increased the percentage of cells in G1 after a 16-h treatment. After 24 h, resveratrol-treated cells remained in G1, whereas baicalein-treated

cells had progressed to S phase. *N*-phenyl-2-napthylamine, daidzein, and 2-aminoanthraquinone, which are inducers of a mild DNA damage response, caused an increase in the population of cells in S phase after a 16-h exposure. This effect was more drastic when the cells were treated for 24 h. Treatment with genistein caused a severe (70%) G2/M phase arrest.

Taken together, these data indicate that genistein-induced DNA DSBs arrest cells in the G2/M phase without causing a strong S phase-specific DNA damage response. In contrast, *N*-phenyl-2-napthylamine, daidzein, resveratrol, 2-aminoanthraquinone, and baicalein most likely produce DNA lesions that stall the progression of the replication fork at different levels. This process, in turn, affected the increase in the number of Polη nuclear foci in a similar manner. Even though some previous reports have suggested that polyphenols/antioxidants could produce DNA damage by acting as pro-oxidants, intercalating into DNA, inhibiting topoisomerase, and inhibiting DNA polymerase (Table S3, column 4) (9, 10), the severity of the genotoxicities induced by resveratrol, baicalein, and genistein was surprising. The level of DNA damage caused by a 16-h treatment with 92 μM resveratrol, baicalein, or genistein was comparable to that caused by treatment with the well-known genotoxins MMS and cisplatin (Fig. 2). The potent genotoxicity of MMS and cisplatin led to the successful use of these compounds to kill rapidly dividing cancer cells (11). Therefore, resveratrol, baicalein, and genistein could have chemotherapeutic potential.

Resveratrol and Genistein Selectively Kill Multidrug-Resistant Cancer Cells. To investigate the chemotherapeutic potential of resveratrol, baicalein, and genistein further, we tested their ability to selectively kill KB-V1 cells. KB-V1 cells are derived from the human KB-3-1 cell line and exhibit resistance to multiple anticancer drugs, including colchicine, vincristine, vinblastine, adriamycin, actinomycin D, and puromycin (12). Despite their multidrug-resistance phenotype, KB-V1 cells were more sensitive than KB-3-1 cells to resveratrol and genistein (Fig. 3 *A* and *B*). In contrast, both cell lines exhibited a similar response to baicalein and MMS treatment (Fig. 3 *C* and *D*). Thus, resveratrol and genistein may be especially useful in treating cancers that have developed drug resistance to conventional chemotherapeutic agents.

Resveratrol, Baicalein, and Genistein Are Not Mutagenic. A major problem associated with conventional genotoxic chemotherapeutic agents, such as cisplatin, is the induction of mutagenesis at the chromosomal and nucleotide levels. Based on our observations that resveratrol, baicalein, and genistein are potent genotoxins, we hypothesized that these compounds might also produce mutations similar to MMS or cisplatin. Surprisingly, in contrast to the five- to sixfold increase in mutation frequency observed following treatment with either MMS or cisplatin, resveratrol, baicalein, and genistein did not increase mutagenesis in the *SupF* plasmid mutagenesis assay, even though the doses of these compounds were as cytotoxic as the doses of MMS and cisplatin used in this study (Fig. 4*A*). Similarly, there were no significant increases in the forward canavanine resistance mutagenesis frequency in yeast (Fig. S6*A*) or in a bacterial reverse-mutation assay (13). However, we need to point out that there are reports showing a slight increase in HPRT mutagenesis after genistein treatment (14, 15).

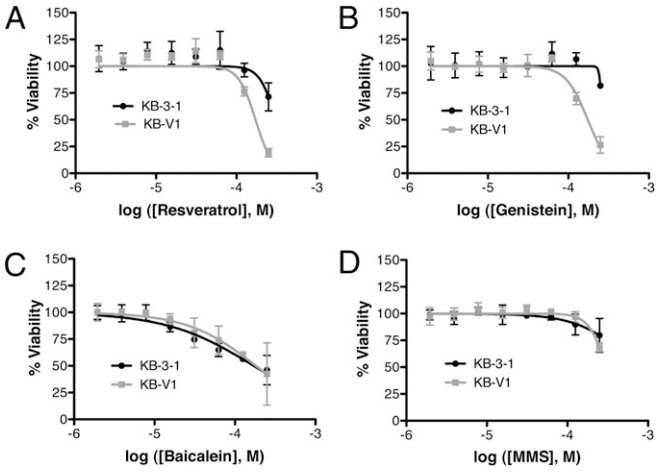

Fig. 3. Viability of multidrug resistant (KB-V1) and parental (KB-3-1) cells following a 24-h treatment with 0–250 μM of (*A*) resveratrol, (*B*) genistein, (*C*) baicalein, and (*D*) MMS. Viability was determined using CellTiter-Glo immediately following treatment. The data represent the average of three independent experiments ± SD.

Polη, the defects of which cause a variant form of the skin cancer-prone syndrome Xeroderma Pigmentosum (16, 17), can bypass DNA damage in a fairly error-free fashion (17, 18). Therefore, we hypothesized that the above results were because of the Polη-dependent bypass of DNA lesions generated by resveratrol and baicalein. Consistent with our hypothesis, we observed the induction of mutagenesis by resveratrol and baicalein, but not by MMS or cisplatin, when the expression of Polη was reduced by siRNA (Fig. 4*A*). Compared with MMS and cisplatin treatments, treatment with resveratrol or baicalein also resulted in significant Polη deubiquitylation (Fig. 2*A* and Fig. S6*B*), which is necessary for the binding of Polη to monoubiquitylated PCNA and subsequent activation of the polymerase (19). Therefore, DNA lesions produced by resveratrol and baicalein appear to be bypassed mainly by Polη in an error-free manner.

In contrast to MMS, resveratrol, baicalein, and genistein also did not significantly increase the frequency of gross chromosomal rearrangements (Fig. 4*B*) or recombination (Fig. 4*C*) in yeast, even though the doses of all four compounds used in these assays generated similar viabilities compared with the dose of MMS used. Although one report showed the induction of sister chromatid exchanges by resveratrol in a Chinese hamster lung cell line (13), there was no significant increase in sister chromatid exchanges in retinal pigment epithelium cells following resveratrol treatment (Fig. S6*C*). Collectively, these data indicate that although resveratrol, baicalein, and genistein inflict the same amount of DNA damage and cause a similar level of cell death as MMS and cisplatin, they do not cause serious genomic instabilities either at the chromosomal or nucleotide levels, in contrast to MMS and cisplatin.

Discussion

There are currently several high-throughput screening methods to assess the genotoxicity of pharmaceutical and environmental chemicals in vitro. However, it is difficult to determine the possible genotoxicity of chemicals with a single assay. Therefore, to assess the genotoxicity of a compound accurately, it is necessary to use a battery of diverse assays (20). In this study, we developed a high-throughput ATAD5-luc assay that will be a valuable addition to this battery. The ATAD5-luc assay is very efficient both in time and cost. It can be completed in less than 24 h, and because of the miniaturization in a 1,536-well plate format, it requires only a minimal volume of reagents and test chemicals. Above all, the ATAD5-luc assay identifies genotoxins with a low false-positive rate. More than 80% of the selected hits from the ATAD5-luc assay induced genotoxicity in at least one other independent assay.

The ATAD5-luc assay is not without its limitations, however. Although many of the 1,408 compounds in the NTP library are known or expected to be genotoxic, only 46 compounds from the NTP collection enhanced ATAD5-luc activity >40% of the MMS control, and another 139 compounds enhanced ATAD5-luc activity <40% of the MMS control. Together, these 185 compounds represent only 13% of the total. It is possible that the

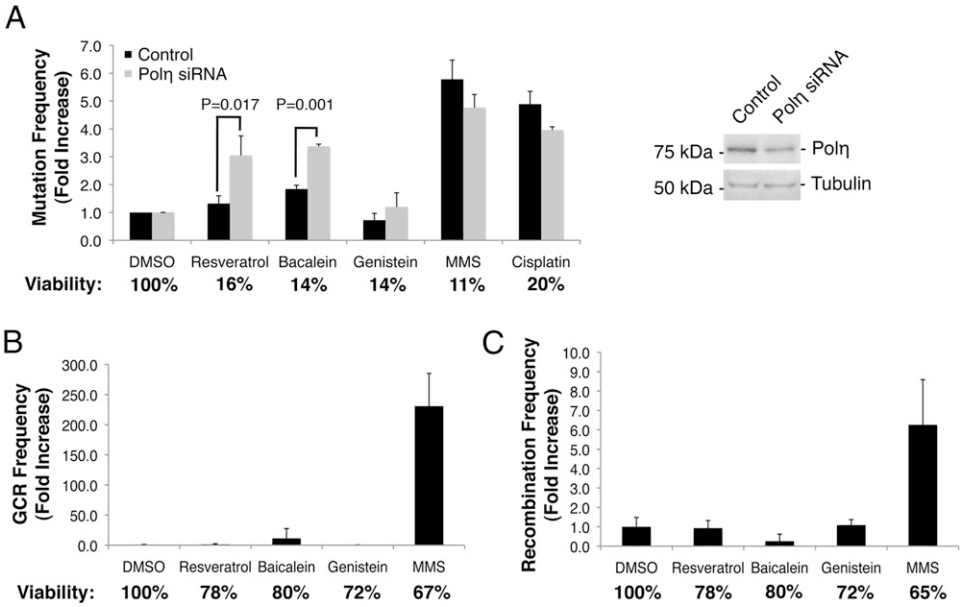

Fig. 4. Resveratrol, baicalein, and genistein do not cause mutagenesis or chromosomal rearrangements. (*A*) HEK293T cells transfected with either control or Polη siRNA were treated with 230 μM resveratrol, baicalein, or genistein for 16 h, 10 μM cisplatin for 16 h, or 1.18 mM MMS for 1 h. Mutation frequency was determined by the *SupF* plasmid mutagenesis assay with DMSO control normalization. The numbers below the graph indicate the percentage of viable cells 7 d after treatment as determined by the colony formation assay. The expression of Polη after siRNA knockdown was determined by Western blotting. (*B*) Log-phase cultures of *Saccharomyces cerevisiae* (RDKY3615) were treated with 1 mM resveratrol, baicalein, or genistein, or 2.39 mM MMS for 4 h. Gross chromosomal rearrangement (GCR) frequency was determined by counting colonies resistant to canavanine and 5-fluoroorotic acid. The fold-induction of GCR frequency was normalized to the DMSO control. (*C*) Log-phase cultures of *S. cerevisiae* (M136-11B) were treated as described in *B*, and the recombination frequency was determined by counting colonies grown on a plate without histidine and normalized to the DMSO control. Each graph in *A*, *B*, and *C* represents the average of at least two experiments ± SD. The numbers below the graph in *B* and *C* indicate the percentage of viable cells following treatment as determined by the yeast survival assay.

cytotoxicity of the other 87% of the compounds precludes the ability to measure their genotoxicity in vitro, or that the concentrations of some compounds applied to the ATAD5-luc assay may not have been high enough to produce an effect. In addition, the HEK293T cells used for the ATAD5-luc assay lack any appreciable xenobiotic-metabolizing activity, which is required for some compounds to be genotoxic. All cell-based genotoxicity assays share these limitations and consequently suffer in terms of sensitivity. For example, when seven isogenic DNA repair-deficient chicken DT40 cell lines were used to screen all 1,408 compounds from the NTP library, only 42 compounds (3%) were identified as exhibiting possible genotoxic activity (21). Similarly, when the GreenScreen HC GADD45a-GFP, CellCiphr p53, and CellSensor p53-bla high-throughput genotoxicity assays were used to screen a collection of 320 predominantly pesticide active compounds being tested in phase I of the United States Environmental Protection Agency's ToxCast research project, only 10%, 9%, and 12% of the analyzed compounds, respectively, were identified as positive hits (20). Thus, although the ATAD5-luc assay fails to pick up many genotoxins, it is no less sensitive than other cell-based assays that are currently available.

Among the compounds that the ATAD5-luc screen did identify from both the NTP and TB libraries were 22 polyphenols and antioxidants. We verified that several of these compounds, including resveratrol, genistein, and baicalein, are indeed potent DNA damaging agents at concentrations of 92 μM. Although Western blot analysis did not detect DNA damage responses to concentrations of resveratrol, genistein, and baicalein that could be obtained from oral dosing (2–5 μM) (Fig. S7*A*) (22, 23), the ATAD5-luc assay identified these compounds as genotoxins at concentrations as low as 2 μM (Fig. S7*B*). These findings were surprising given that resveratrol and genistein are currently being tested in clinical studies as treatments for cardiovascular disease, type 2 diabetes, osteopenia, and osteoporosis (24, 25); resveratrol has gained notoriety for its potential antiaging effects; and baicalein is the major constituent of a Japanese herbal remedy for chronic hepatitis (26). The genotoxicity caused by these compounds raises concerns regarding their use for such conditions and suggests a reevaluation of their safety.

Although the ability to induce DNA damage is not a favorable quality for drugs used to treat the above-mentioned diseases, it is the primary mechanism by which many conventional chemotherapeutic agents act. Our findings thus raised the possibility that resveratrol, baicalein, and genistein could be better used as cancer drugs. In support of this hypothesis, resveratrol, baicalein, and genistein have been reported to selectively kill cancer cells (27–29), induce apoptosis (27, 29, 30), and reduce tumors in mice (31–34), and resveratrol and genistein are currently in clinical trails for the treatment of colon, breast, prostate, bladder, and pancreatic cancers (www.clinicaltrials.gov). The data presented in this study further add to the attractiveness of these compounds as chemotherapeutic agents because resveratrol, baicalein, and genistein can kill rapidly dividing cells without causing potentially detrimental genomic instabilities. Additionally, resveratrol and genistein can sensitize cells that have developed drug resistance to conventional chemotherapeutic agents.

Collectively, we deliver several major health implications in this study: (*i*) a re-evaluation of the safety of on-going clinical trials that use resveratrol, genistein, and baicalein for the treatment or prevention of cardiovascular disease, type 2 diabetes, osteopenia, aging, and chronic hepatitis; (*ii*) a precaution for the unguided medical use of antioxidants by the public; and (*iii*) an alternative, but more efficient use of antioxidants for chemotherapy.

Materials and Methods

Generation of the ATAD5-luc Cell Line. The cDNA of human *ATAD5* was inserted in-frame downstream of the firefly luciferase gene in pTRED-CMV-HA-Luc and named as pKJM1333. The recombinant lentivirus harboring the

ATAD5-luciferase fusion gene (ATAD5-luc) was produced by transfecting pKJM1333 as well as packaging plasmids expressing viral REV, GAG/POL, and VSV-G proteins into HEK293T cells. Harvested lentivirus was then used to transduce new HEK293T cells. Cells were harvested after a 2-d transduction and plated at low density. Individual transduced HEK293T cell colonies were picked and expression of the ATAD5-luc protein and the induction in the level of the ATAD5-luc protein following treatment with MMS was tested by both luciferase assay and Western blot analysis.

Quantitative High-Throughput Screening. Compound formatting and quantitative high-throughput screening were performed as described previously (35, 36). ATAD5-luc cells were dispensed at 5 μL per well (~2,000 cells) in tissue culture-treated 1,536-well white, solid-bottom assay plates (Greiner Bio-One North America) using a Flying Reagent Dispenser (FRD) (Aurora Discovery) and then incubated at 37 °C for ~5 h. Compound plates containing small molecules from the NTP or TB were prepared as interplate titrations of 7–15 dilutions in columns 5–48 of 1,536-well compound plates. Next, 23 nL of each compound from the compound plates was transferred via pin tool (Kalypsys) to columns 5–48 of the assay plates, resulting in final compound concentrations ranging from 0.6 nM to 0.092 mM. As controls, a MMS concentration-response curve, 0.7 mM MMS, 0.6 mM MMS, and 0.46% DMSO were also included in columns 1, 2, 3, and 4 of each plate, respectively. After compound addition, the assay plates were incubated at 37 °C for 16 h. Next, 5 μL of the ONE-Glo luciferase reagent (Promega) was then added to each well using the FRD. The luminescence intensity of the assay plates was quantified using a ViewLux CCD-based plate reader (PerkinElmer) after a 30-min incubation at room temperature. Raw plate reads for each titration point were normalized to MMS (0.7 mM = 100%) and DMSO (0%) controls, and then corrected by applying a pattern-correction algorithm using compound-free control plates (DMSO plates). Concentration-response titration points for each compound were then fitted to the Hill equation.

Protein Analysis. HEK293T cells were grown in 10-cm tissue-culture plates to ~90% confluence and treated with various compounds as indicated. To obtain total lysate, the cells were resuspended in lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, protease inhibitors (Roche)] and lysed on ice for 30 min. Chromatin-bound fractions were isolated as described previously (5). Proteins in the total lysate or the chromatin-bound fraction were separated by SDS-PAGE using a 4–15% Tris-glycine gel (Bio-Rad) and transferred to a Polyvinylidene difluoride membrane. Proteins in the membrane were detected by the ECL Western Blotting Detection System (GE Healthcare). When necessary, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce). Mouse anti-luciferase, HRP-conjugated anti-PCNA, mouse anti-CHK1 (G-4), mouse anti-ATM, and mouse anti-FANCD2 antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-phospho-RPA32 (S4/S8), and rabbit antiphospho-CHK1 (S317) antibodies were purchased from Bethyl Laboratories. Rabbit anti-Polη, rabbit antihistone H2AX, and HRP-conjugated anti–β-tubulin antibodies were purchased from Abcam. Rabbit antihistone H3 antibody was purchased from Upstate. Mouse anti-RPA32 (Ab-3) antibody was purchased from Calbiochem. Mouse anti-phospho-histone H2AX (S140, 3F2) antibody was purchased from GeneTex. Rabbit antiphospho-ATM (S1981) antibody was purchased from R&D Systems.

ACKNOWLEDGMENTS. We thank S. Anderson in the FACS core, A. Dutra in the cytogenetics core, and D. Bodine, F. Candotti, P. Schwartzberg, and Y. Yang of the National Human Genome Research Institute for helpful discussions and comments on the manuscript; M. Gottesman and C. Cardarelli of the National Cancer Institute for cell lines; and K.M. especially thanks E. Cho. This research was supported by the Intramural Research Programs (Interagency Agreement Y2-ES-7020-01) of the National Toxicology Program, National Institute of Environmental Health Sciences, and the National Human Genome Research Institute, National Institutes of Health, and by Grant R03 MH092164-01 (to K.M and M.X.).

1. Banerjee S, Sikdar N, Myung K (2007) Suppression of gross chromosomal rearrangements by a new alternative replication factor C complex. *Biochem Biophys Res Commun* 362:546–549.
2. Majka J, Burgers PM (2004) The PCNA-RFC families of DNA clamps and clamp loaders. *Prog Nucleic Acid Res Mol Biol* 78:227–260.
3. Bell DW, et al. (2011) Predisposition to cancer caused by genetic and functional defects of mammalian Atad5. *PLoS Genet* 7:e1002245.
4. Fox JT, Lee KY, Myung K (2011) Dynamic regulation of PCNA ubiquitylation/deubiquitylation. *FEBS Lett* 585:2780–2785.
5. Lee KY, et al. (2010) Human ELG1 regulates the level of ubiquitinated proliferating cell nuclear antigen (PCNA) through Its interactions with PCNA and USP1. *J Biol Chem* 285:10362–10369.
6. Friedberg EC, Lehmann AR, Fuchs RP (2005) Trading places: How do DNA polymerases switch during translesion DNA synthesis? *Mol Cell* 18:499–505.
7. Sikdar N, et al. (2009) DNA damage responses by human ELG1 in S phase are important to maintain genomic integrity. *Cell Cycle* 8:3199–3207.
8. Auld DS, et al. (2008) Characterization of chemical libraries for luciferase inhibitory activity. *J Med Chem* 51:2372–2386.
9. Ferguson LR (2001) Role of plant polyphenols in genomic stability. *Mutat Res* 475(1-2): 89–111.
10. Stopper H, Schmitt E, Kobras K (2005) Genotoxicity of phytoestrogens. *Mutat Res* 574(1-2):139–155.
11. Cohen SM, Lippard SJ (2001) Cisplatin: From DNA damage to cancer chemotherapy. *Prog Nucleic Acid Res Mol Biol* 67:93–130.
12. Akiyama S, Fojo A, Hanover JA, Pastan I, Gottesman MM (1985) Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. *Somat Cell Mol Genet* 11(2):117–126.
13. Matsuoka A, Furuta A, Ozaki M, Fukuhara K, Miyata N (2001) Resveratrol, a naturally occurring polyphenol, induces sister chromatid exchanges in a Chinese hamster lung (CHL) cell line. *Mutat Res* 494(1-2):107–113.
14. Kulling SE, Metzler M (1997) Induction of micronuclei, DNA strand breaks and HPRT mutations in cultured Chinese hamster V79 cells by the phytoestrogen coumoestrol. *Food Chem Toxicol* 35:605–613.
15. Tsutsui T, et al. (2003) Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. *Int J Cancer* 105:312–320.
16. Johnson RE, Kondratick CM, Prakash S, Prakash L (1999) hRAD30 mutations in the variant form of xeroderma pigmentosum. *Science* 285:263–265.
17. Masutani C, et al. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature* 399:700–704.
18. Johnson RE, Prakash S, Prakash L (1999) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. *Science* 283:1001–1004.
19. Bienko M, et al. (2010) Regulation of translesion synthesis DNA polymerase eta by monoubiquitination. *Mol Cell* 37:396–407.
20. Knight AW, et al. (2009) Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast chemicals. *Regul Toxicol Pharmacol* 55:188–199.
21. Yamamoto KN, et al. (2011) Characterization of environmental chemicals with potential for DNA damage using isogenic DNA repair-deficient chicken DT40 cell lines. *Environ Mol Mutagen* 52:547–561.
22. la Porte C, et al. (2010) Steady-State pharmacokinetics and tolerability of trans-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects. *Clin Pharmacokinet* 49:449–454.
23. Metzner JE, Frank T, Kunz I, Burger D, Riegger C (2009) Study on the pharmacokinetics of synthetic genistein after multiple oral intake in post-menopausal women. *Arzneimittelforschung* 59:513–520.
24. Atteritano M, et al. (2007) Effects of the phytoestrogen genistein on some predictors of cardiovascular risk in osteopenic, postmenopausal women: A two-year randomized, double-blind, placebo-controlled study. *J Clin Endocrinol Metab* 92:3068–3075.
25. Marini H, et al. (2007) Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women: A randomized trial. *Ann Intern Med* 146: 839–847.
26. Hirayama C, et al. (1989) A multicenter randomized controlled clinical trial of Shosaiko-to in chronic active hepatitis. *Gastroenterol Jpn* 24:715–719.
27. Aziz MH, Nihal M, Fu VX, Jarrard DF, Ahmad N (2006) Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3′-kinase/Akt pathway and Bcl-2 family proteins. *Mol Cancer Ther* 5: 1335–1341.
28. Baxa DM, Luo X, Yoshimura FK (2005) Genistein induces apoptosis in T lymphoma cells via mitochondrial damage. *Nutr Cancer* 51(1):93–101.
29. Chen CH, et al. (2000) Baicalein, a novel apoptotic agent for hepatoma cell lines: A potential medicine for hepatoma. *Nutr Cancer* 38:287–295.
30. Ouyang G, et al. (2009) Genistein induces G2/M cell cycle arrest and apoptosis of human ovarian cancer cells via activation of DNA damage checkpoint pathways. *Cell Biol Int* 33:1237–1244.
31. Constantinou AI, Krygier AE, Mehta RR (1998) Genistein induces maturation of cultured human breast cancer cells and prevents tumor growth in nude mice. *Am J Clin Nutr* 68(6, Suppl):1426S–1430S.
32. Garvin S, Ollinger K, Dabrosin C (2006) Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo. *Cancer Lett* 231(1):113–122.
33. Miocinovic R, et al. (2005) In vivo and in vitro effect of baicalein on human prostate cancer cells. *Int J Oncol* 26:241–246.
34. Zhou HB, Chen JJ, Wang WX, Cai JT, Du Q (2005) Anticancer activity of resveratrol on implanted human primary gastric carcinoma cells in nude mice. *World J Gastroenterol* 11:280–284.
35. Inglese J, et al. (2006) Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci USA* 103:11473–11478.
36. Xia M, et al. (2008) Compound cytotoxicity profiling using quantitative high-throughput screening. *Environ Health Perspect* 116:284–291.