Discovery of Small-Molecule Enhancers of Reactive Oxygen Species That are Nontoxic or Cause Genotype-Selective Cell Death

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High-Throughput Screening Identifies Small-Molecule Enhancers of Reactive Oxygen Species That are Nontoxic or Cause Genotype-Selective Cell Death

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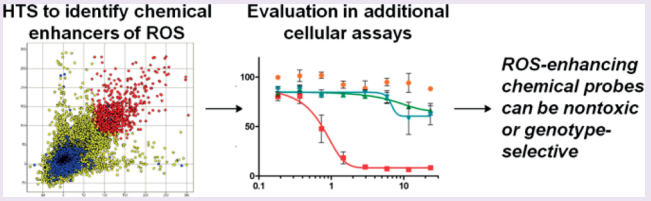
Supporting Information

ABSTRACT: Elevation of reactive oxygen species (ROS) levels has been observed in many cancer cells relative to nontransformed cells, and recent reports have suggested that small-molecule enhancers of ROS may selectively kill cancer cells in various *in vitro* and *in vivo* models. We used a high-throughput screening approach to identify several hundred small-molecule enhancers of ROS in a human osteosarcoma cell line. Only a minority of these compounds diminished the viability of cancer cell lines, indicating that ROS elevation by small molecules is generally insufficient to induce death of cancer cell lines. Three chemical probes (BRD5459, BRD56491, BRD9092) are highlighted that most strongly elevate markers of oxidative stress without causing cell death and may be of use in a variety of cellular settings. For example, combining nontoxic ROS-enhancing probes with nontoxic doses of L-buthionine sulfoximine, an inhibitor of glutathione synthesis previously studied in cancer patients, led to potent cell death in more than 20 cases, suggesting that even nontoxic ROS-enhancing treatments may warrant exploration in combination strategies. Additionally, a few ROS-enhancing compounds that contain sites of electrophilicity, including piperlongumine, showed modest selective toxicity for transformed cells over nontransformed cells in an engineered cell-line model of tumorigenesis. These studies demonstrate that cancer cell lines are more resilient to chemically induced increases in ROS levels than previously thought and highlight electrophilicity as a property that may be more closely associated with cancer-selective cell death than ROS elevation.



Reactive oxygen species (ROS) are a common byproduct of cellular metabolism and are used by cells for signal transduction and as defense agents against pathogens.[1−3] Although certain species, including nitric oxide and hydrogen peroxide, are increasingly thought to play important roles in signaling and regulation of protein function, other highly reactive species can damage cellular nucleic acids, proteins, and lipids. As a result, various mechanisms have evolved to limit undesired cellular damage and maintain redox homeostasis. Superoxide radical, which can be generated by NADPH oxidases and other enzymes or by leakage of one electron from the electron transport chain to molecular oxygen, is processed by superoxide dismutases to provide hydrogen peroxide and molecular oxygen (Figure 1A). Metalloenzymes (e.g., catalase) and enzymes that harness glutathione as a nucleophilic cofactor (e.g., glutathione peroxidase, glutathione *S*-transferase) reduce hydrogen peroxide and related cellular peroxides. Proper detoxification of superoxide and hydrogen peroxide is critical to prevent the formation of even more damaging species, including peroxynitrite (by recombination of superoxide with nitric oxide) and hydroxyl radical (by Fenton-type cleavage of peroxides). During periods of oxidative stress, several transcriptional programs, including the transcription factor NRF2, can be activated to re-establish redox homeostasis by upregulating genes bearing antioxidant response-element promoters.[4]

A role for chronic oxidative stress has been proposed in the etiology of various diseases, including diabetes,[5,6] cardiovascular disease,[7] and neurodegenerative diseases.[8,9] Accumulated cellular damage initiated by ROS has also been proposed to play a central role in the processes of aging[10,11] and tumorigenesis.[12] More recently, insights from cancer biology have suggested that *increasing* ROS levels may be a strategy for selectively targeting cancer cells while sparing nontransformed cells.[1,12−14] Many cancer cells have elevated basal levels of ROS relative to nontransformed cells,[15] often as a direct result of the activity of specific oncogenes.[16] Although this chronic oxidative stress can enhance proliferation, migration, and other cancer phenotypes, it may also leave some cancer cells vulnerable to chemical agents that further elevate ROS to levels that induce

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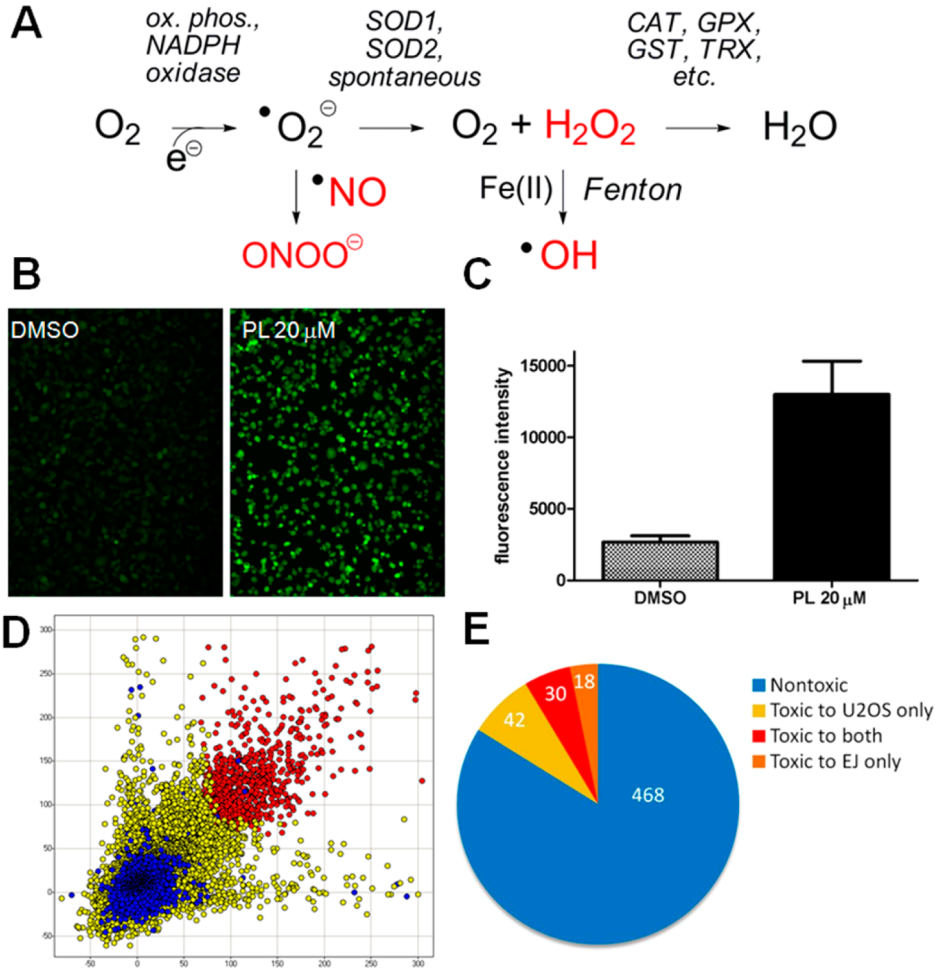

Figure 1. Identification of small-molecule enhancers of ROS and evaluation of toxicity in cancer cell lines. (A) Common pathways for the generation and metabolism of ROS. (B) U2OS cells were treated with either DMSO or 20 μM piperlongumine (PL) for 1 h, and ROS were measured using CM-H_2DCF-DA and automated fluorescence microscopy. (C) Quantification of fluorescence levels following PL treatment. Mean and standard deviation from a representative experiment are shown. (D) Summary of high-throughput screening results. Blue, negative control (DMSO); yellow, test compounds; red, "hit" compounds (903); positive control (PL), not shown. Each assay plate was normalized to DMSO = 0, PL = 100. Compounds scoring >75 in both replicates were considered "hits". (E) Occurrence of toxicity (>50% reduction in ATP at ≥20 μM after 48-h treatment) in U2OS and EJ cell lines.

cell death.[17] For several ROS-enhancing compounds, including phenethylisothiocyanate (PEITC),[18] parthenolide,[19] piperlongumine,[20] erastin,[21] and lanperisone,[22] selectivity for cancer cells over nontransformed cell types has been demonstrated in *in vitro* or *in vivo* models of cancer.

To explore the generality of these observations of selective killing of cancer cells, we used a high-throughput screening approach to identify a large set of small molecules that enhance ROS levels in a cancer cell line. Surprisingly, only a minority of ROS-enhancing compounds lowered the viability of a panel of cancer cell lines, demonstrating that increasing ROS levels was frequently insufficient to initiate cell death. However, cells treated with nontoxic ROS-enhancing small molecules appeared dependent on glutathione synthesis for survival, as co-treatment with nontoxic doses of glutathione synthesis inhibitor L-buthionine sulfoximine (BSO) led to potent cell death. Selective killing of cancer cells, a property of several known ROS-enhancing small molecules, was modest and limited to several electrophilic small molecules. The divergent cellular outcomes observed for small-molecule enhancers of ROS suggest that cancer cells may be vulnerable to certain specific ROS-elevating treatments, in particular electrophilic small molecules, while distinctly resistant to others.

RESULTS AND DISCUSSION

High-Throughput Screening and Evaluation of Cellular Viability. To identify novel small-molecule enhancers of ROS levels, we adapted a high-throughput assay for ROS levels in myotubes[23] for use in the human osteosarcoma cell line U2OS (Figure 1B,C). To detect ROS we used CM-H_2DCF-DA, a cell-permeable, nonfluorescent compound that is oxidized by hydroxyl radical, peroxynitrite, and other reactive oxygen species (sometimes with transition metal ion catalysts) to a fluorescein derivative. Though it does not distinguish between multiple species, CM-H_2DCF-DA remains a leading approach to measuring highly reactive species that may be most likely to initiate cancer cell death. Piperlongumine, a naturally occurring small molecule previously demonstrated to enhance ROS levels in U2OS cells,[20] served as positive control. Typically automated fluorescence microscopy was used as the detection method due to its optimal sensitivity (Figure 1B,C). However, during high-throughput screening, a fluorescence

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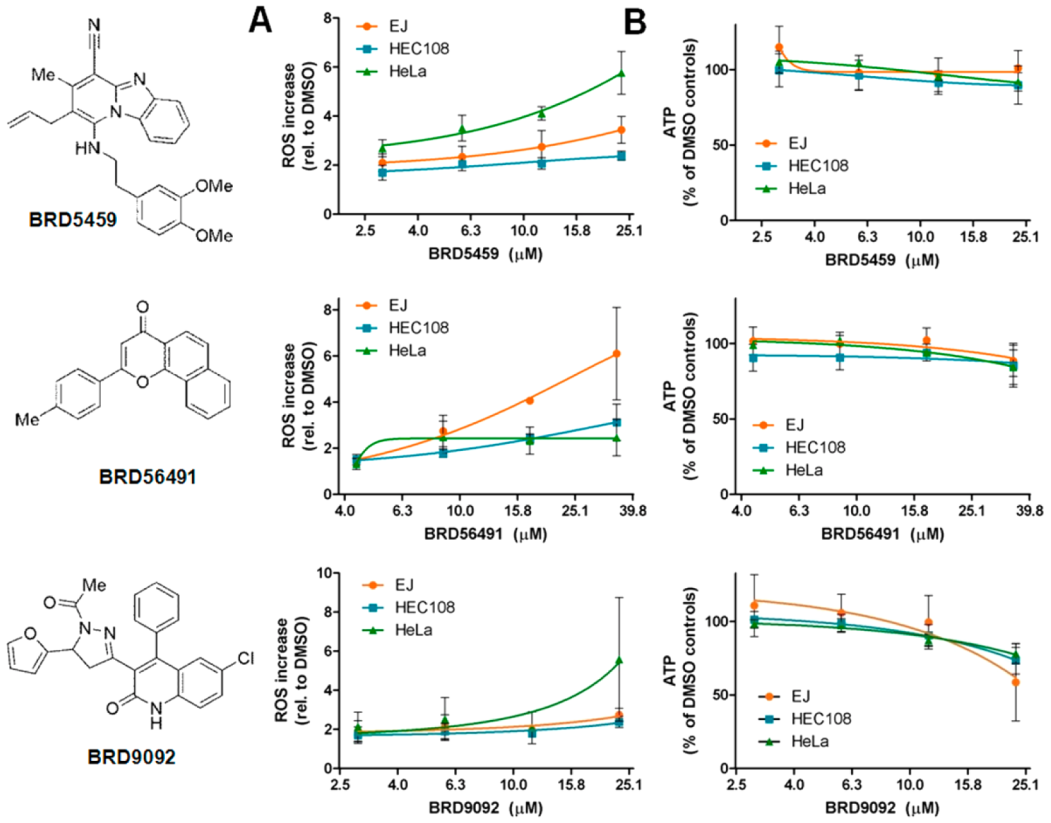

Figure 2. ROS-enhancing, nontoxic compounds. (A) Elevation of ROS for the indicated concentrations of each compound after 1-h treatment in three cell lines. (B) ATP levels after 48-h treatment in the same cell line panel. All data are expressed as mean ± SD, n = 3.

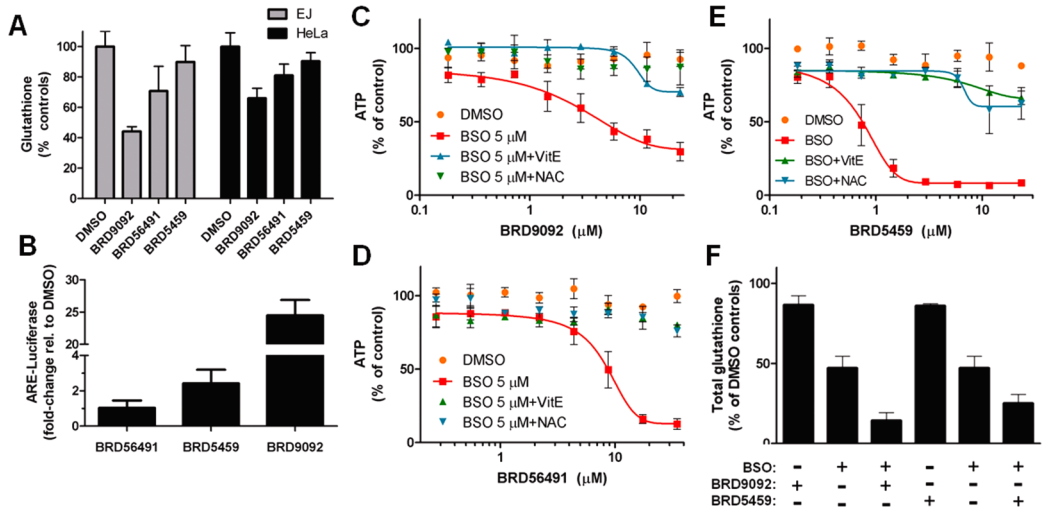

Figure 3. Cellular effects of ROS-enhancing, nontoxic compounds. (A) Total cellular glutathione after treatment with the indicated compounds (BRD9092, 23.2 μM; BRD56491, 35 μM; BRD5459, 11.7 μM) was measured in EJ and HeLa cells. (B) BRD9092 and BRD5459, but not BRD56491, elevate antioxidant response element (ARE) promoter transcription in a luciferase-based reporter-gene assay in IMR-32 cells. Data are expressed as mean ± SD, n = 3 (ARE reporter assay, n = 4). (C–E) Three ROS-enhancing nontoxic compounds were tested for viability in the presence of a nontoxic dose (5 μM) of BSO (a glutathione synthesis inhibitor), 200 μM vitamin E, or 5 mM *N*-acetyl cysteine (NAC) in EJ cells. ATP values were calculated relative to control wells lacking the indicated BRD compound but containing BSO and antioxidant when applicable. All treatments were nontoxic individually (Supporting Figure 4). (F) Pairing of BRD5459 (2.9 μM) or BRD9092 (11.6 μM) with BSO (5 μM) leads to enhanced depletion of glutathione. All data are expressed as mean ± SD, n = 3.

plate reader (FLiPR, Molecular Devices) was used to enhance assay throughput.

We screened 41,000 small molecules, including natural products, bioactive compounds, commercial compounds, and products of diversity-oriented synthesis, to identify enhancers of ROS in U2OS cells. To minimize identification of compounds for which ROS elevation might be a result of ongoing cell death, ROS was detected 1 h after compound treatment. We identified 903 compounds that increased ROS levels to 75% of positive control levels in both assay replicates

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(Figure 1D). Many compounds outperformed the positive control, including 38 compounds that elevated ROS to levels more than double those of piperlongumine. Retesting hit compounds in dose using automated fluorescence microscopy confirmed 2-fold ROS enhancement for 558 compounds (1.4% confirmed hit rate) and also identified 14 autofluorescent compounds that were excluded from further analysis.

Previous reports have suggested that cancer cells may be particularly sensitive to ROS-modulating small molecules.[1,12,13,17] To explore this concept more generally, we measured the sensitivity of cancer cell lines to the confirmed ROS-enhancing small molecules arising from our screen, using cellular ATP levels to assess the effect of compounds on cell growth and viability. In U2OS cells, only 72 compounds reduced ATP levels more than 50% at ≥20 μM after a 48-h treatment (Figure 1E). As a larger impact on growth and viability was expected, a second cell line (EJ) was also tested. Similar to U2OS, only 48 compounds diminished viability at ≥20 μM. A total of 90 compounds were able to decrease ATP by at least 50% in one or both cell lines, less than 20% of confirmed ROS-enhancing compounds. Even for the 17 compounds that enhanced ROS more than 6-fold, only six lowered the viability of U2OS cells. Elevation of ROS to levels attainable with small molecules may in general be insufficient to initiate cancer-cell death.

Further Evaluation of Nontoxic Screening Hits. Since ROS-enhancing small molecules unexpectedly had minimal effects on the growth and viability of cancer cell lines, we prioritized 80 nontoxic screening hits that strongly enhanced ROS levels in U2OS for deeper characterization in cellular viability and oxidative stress assays. In three additional cancer cell lines, compound treatment elevated ROS levels without apparent loss of viability (Figure 2, Supporting Figure 1). Additionally, although ROS levels were often maximal at our standard 1-h measurement, ROS levels were still greatly elevated after 8- and 24-h treatment in many cases (Supporting Figure 2A). Likewise, little effect on growth and viability for these compounds was observed even after 5 days of treatment (Supporting Figure 2B). These data suggest that the persistence of cell viability in the face of elevated ROS levels was not simply due to choice of cell line or treatment length.

To provide additional evidence that ROS-enhancing small molecules identified using CM-H_2DCF-DA were indeed causing increased levels of functional ROS in cells, we measured their effects on additional markers of cellular oxidative stress. Treatment with these nontoxic, ROS-enhancing compounds resulted in varying levels of decrease in total cellular glutathione (Figure 3A). We also used a reporter-gene assay measuring transcription from an antioxidant response element (ARE)-containing promoter in IMR-32 cells[24] as a surrogate measure for the activity of the redox-sensitive transcription factor NRF2. Although some nontoxic ROS-enhancing compounds had little to no effect on ARE transcription, others led to strong activation of an ARE promoter (BRD9092, Figure 3B). These studies suggest that elevation of oxidative stress by small molecules need not lead to cancer-cell death and highlight several specific chemical probes that most strongly and generally elevate ROS levels and other markers of oxidative stress without loss of cellular viability. Such compounds may elevate specific ROS that are less effective at inducing cell death or may induce oxidative stress in subcellular compartments that are less susceptible to lethal damage. Alternatively, the elevated ROS levels resulting from compound treatment may still be below a threshold required to initiate cell death.

Although many ROS-enhancing small molecules do not affect cancer cell growth and viability as single agents, we hypothesized that co-treatment with a second inducer of oxidative stress might overcome the observed insensitivity. To test this hypothesis, we co-treated cells with nontoxic ROS-elevating compounds and a nontoxic dose of L-buthionine sulfoximine (BSO), an inhibitor of glutathione biosynthesis. Strikingly, for more than 20 nontoxic ROS-enhancing compounds, co-treatment with 5 μM BSO in EJ cells led to potent cell death (Figure 3C−E, Supporting Figure 3). Co-treatment of many nontoxic ROS-enhancing compounds with BSO did not lead to cell death, highlighting the mechanism-dependent nature of the observed effect. The loss of viability caused by the combination of ROS-enhancing compounds and BSO could be prevented by the chemically unrelated antioxidants NAC and vitamin E (Figure 3C−E, Supporting Figure 4), implicating ROS elevation in the observed cell death. Enhanced potency for depletion of total cellular glutathione was also observed for several ROS-enhancing compounds when paired with BSO (Figure 3F). The ability to synthesize glutathione may become a dependency of cells treated with some chemical agents that give rise to a more oxidizing cellular environment.

In two additional cancer cell lines (U2OS and H1703), distinct sets of ROS-enhancing compounds showed highly potent sensitization when paired with nontoxic doses of BSO (Supporting Figure 5A,B). BRD5459 decreased viability in both EJ and H1703 cells when paired with BSO, while PL-DHN, a piperlongumine analogue previously shown to elevate ROS with little effect on cell viability,[25] was more potent in the presence of BSO in all three cell lines (Supporting Figure 5C). The genetic and physiological responses underlying the observed cell line-dependence remain to be elucidated. Cellular metabolism, in particular pathways that generate NADPH, a key cofactor in many ROS-quenching processes, may play a role in shaping cellular redox state and responses to our probe compounds in this and other assays.

Finally, ROS-enhancing nontoxic compounds were also able to enhance the potency of two chemotherapeutic agents previously shown to enhance ROS levels, vinblastine and etoposide (Supporting Figure 6).[26,27] Combining chemical probes that induce oxidative stress may be a useful strategy to enhance ROS-mediated cell death, even when each agent lacks toxicity individually.

Further Evaluation of Toxic Screening Hits. In addition to the many compounds that elevate ROS levels without affecting cancer cell growth and viability, we also identified 90 small molecules that did lower ATP levels in either EJ or U2OS cells. Many small molecules previously shown to elevate ROS levels and cause cancer cell death contain electrophilic centers, including PEITC, parthenolide, and piperlongumine. Screening hits bearing electrophilic centers (defined as α,β-unsaturated carbonyl or sulfonyl, α-halo-carbonyl, thiophenyl ester, or 2-chloro-pyridine and related heteroaromatic groups) were substantially more likely to cause diminished growth and viability in our cell line panel than compounds lacking these functional groups (17 of 41 electrophilic compounds; 73 of 527 nonelectrophilic compounds).

To assess the contribution of elevated ROS levels to the cell death observed for our screening hits, we determined whether the cell death caused by these compounds could be rescued

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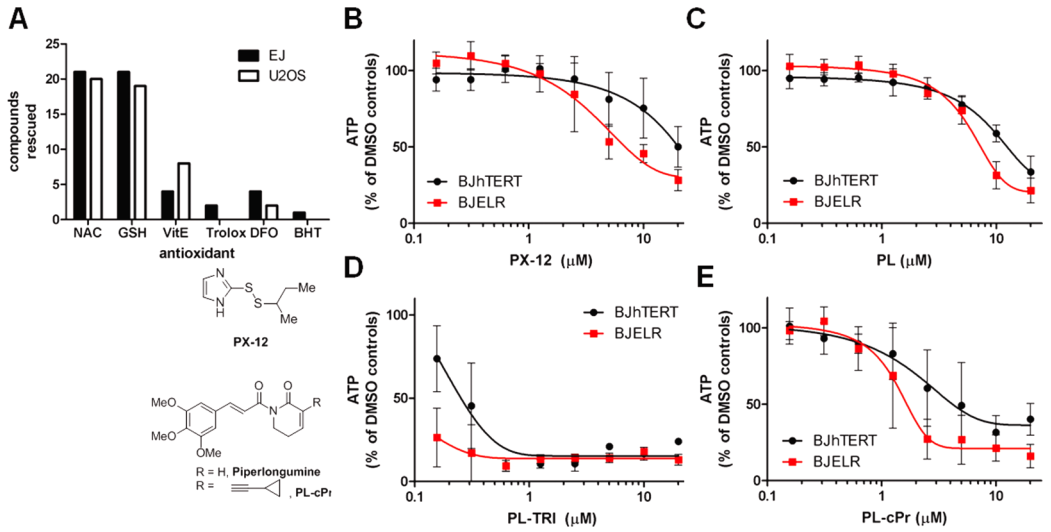

Figure 4. ROS-enhancing compounds show varying selectivity in isogenic models of tumorigenesis. (A) Prevention of compound-induced toxicity using antioxidants. We defined "rescue" as >30% increase in ATP levels at any compound dose following antioxidant co-treatment. No prevention of toxicity was observed using ascorbic acid, uric acid, or β-carotene (not shown). (B–E) Measurement of ATP levels in BJhTERT and BJELR after 48-h treatment with PX-12 (B), piperlongumine (C), and two synthetic piperlongumine analogues including a piperlongumine trimer (D, E). All data are expressed as mean ± SD, n = 3.

using a panel of 6 chemically diverse antioxidants. Although the precise reactive species quenched by these antioxidants (and their associated rates) are not well-defined, a causal role for elevated ROS levels in cancer-cell death has previously been inferred based on the ability of antioxidant molecules to prevent toxicity when co-treated with ROS-enhancing stimuli.[20−22] Although these antioxidants reduced basal ROS levels as measured by CM-H_2DCF-DA by up to 45% (Supporting Figure 7A), the viability of only a minority of toxic screening hits was successfully rescued by our antioxidant panel, with rescue defined as >30% increase in ATP levels at any dose following antioxidant co-treatment (Figure 4A, Supporting Table 1). Glutathione and *N*-acetyl cysteine were most effective at preventing loss of viability. These two thiol-based antioxidants rescued cell death caused by a largely overlapping set of small molecules, most of which contain electrophilic centers. As these antioxidants can react with and inactivate electrophilic compounds prior to entry into cells,[28] and as other antioxidants were generally unable to prevent cell death mediated by electrophilic small molecules such as piperlongumine (Supporting Table 1), substantial caution is warranted in interpreting rescue of electrophilic compounds by thiol antioxidants. Vitamin E rescued a smaller, orthogonal set of compounds. A subset of compounds for which NAC or vitamin E co-treatment rescued viability was also assessed for antioxidant-mediated rescue of ROS levels. In most cases, co-treatment with the antioxidant that prevented cell death also mitigated compound-induced ROS increases (Supporting Figure 7B). However, the general inability of antioxidants to prevent cell death for most toxic screening hits suggests that compound-induced ROS elevation may frequently be a symptom of, or mechanistically unrelated to, cell death.

Evaluation in Isogenic Models of Tumorigenesis. A desirable feature of some ROS-enhancing compounds is selective induction of cell death in cancer cells but not nontransformed cells.[1,12,13] We next assessed our collection of toxic ROS-enhancing screening hits for differential effects on growth and viability in engineered, isogenic models of tumorigenesis. Such models rely on the serial transfection of human primary cells with defined genetic factors that promote immortalization, and ultimately full transformation, to cell types capable of initiating cancers in animal models. These engineered cell lines provide a controlled setting for high-throughput comparisons of immortalized versus fully transformed cells. We began by comparing human foreskin fibroblasts immortalized by addition of the protein subunit of telomerase (BJhTERT) with a derivative transformed by the addition of SV40 early region and activated HRas (BJELR).[29] Several ROS-enhancing small molecules with electrophilic functionalities, including the putative thioredoxin inhibitor PX-12 and piperlongumine and its synthetic derivatives,[25] showed modest selectivity in this viability assay (Figure 4B–E, Supporting Figure 8A–C). Although previously we have identified distinct cellular effects for electrophilic small molecules containing one or more electrophilic centers,[25] in this assay small molecules bearing one (PX-12), two (PL, PL-cPr), or more (PL-TRI) electrophilic centers showed similar magnitudes of selectivity. In contrast, one small molecule generated by diversity-oriented synthesis showed notable selectivity for immortalized BJhTERT cells over the transformed BJELR derivative (Supporting Figure 8D,E).

Two additional isogenic models were analyzed that derive from distinct human primary cells (small airway epithelial cells, mammary epithelial cells) but use the same genetic factors to create immortalized (SALE, HMEL) and ultimately transformed (SALER, HMELR) cell lines.[30,31] Unlike the BJhTERT/BJELR model, no ROS-elevating screening hits showed significant selective cell death in these cell line pairs (data not shown). Together, these studies suggest that cancer-selective killing is an uncommon feature of ROS-enhancing small molecules and is most likely to be observed for those that contain electrophilic centers.

By assembling a large, apparently unbiased collection of small molecules that increase ROS levels in cancer cells, we have been able to assess systematically the effects of chemically induced ROS elevation on cell viability and other cellular processes. We provide evidence that most ROS-enhancing compounds are unable to induce cancer-cell death as single

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agents. However, such compounds frequently cause additional markers of oxidative stress, and more than 20 caused potent cell death when co-treated with a nontoxic dose of the glutathione biosynthesis inhibitor BSO (Figure 5). Three such probe

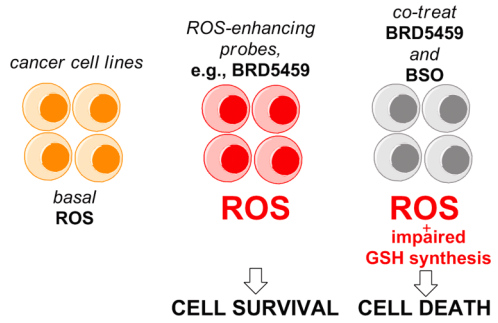

Figure 5. ROS-enhancing chemical probes frequently create a more oxidizing cell state without toxicity to cancer cell lines. Glutathione synthesis can be a dependency of cells treated with such probes, as co-treatment with the glutathione synthesis inhibitor BSO often leads to potent cell death.

compounds (BRD5459, BRD56491, and BRD9092) are highlighted that strongly and generally elevate oxidative stress without impacting cell viability until co-treated with BSO. Together with PL-H_2 and PL-DHN, piperlongumine analogues previously noted to elevate ROS with minimal loss of cell viability,[25] these probes form a novel class of nontoxic ROS-enhancing agents that may be of use in a variety of settings in which creating a more oxidizing cell state is desirable. Additionally, analysis of toxic ROS-enhancing screening hits in isogenic, engineered "models" of tumorigenesis revealed several electrophilic compounds with modest selectivity for fully transformed cells over isogenic immortalized cells. These observations stress the need for caution in interpreting correlations between ROS-elevating manipulations and cell death but also suggest novel combination strategies and a deeper investigation of electrophilic small molecules as potential cancer-selective agents.

METHODS

Cell Culture. U2OS, EJ (T24), H1703, and HeLa were acquired from ATCC and cultured in recommended media. HEC108 were obtained from the Broad Institute/Novartis Cancer Cell Line Encyclopedia and cultured in EMEM + 15% FBS. HMEL, SALE, and SALER were a gift of Dr. Jesse Boehm, Broad Institute Cancer Program. HMELR cells were generated (by addition of activated H-RAS) and donated by Dr. Yashaswi Shrestha (Broad Institute Cancer Program). BJhTERT and BJeLR were a gift of Prof. Brent Stockwell, Columbia University, and were cultured in 4:1 DMEM/M199 + 15% FBS. The isogenicity of these three models of tumorigenesis was confirmed using STR profiling (Molecular Diagnostics Laboratory, Dana Farber Cancer Institute).

ROS Assays. CM-H_2DCF-DA. Cells were plated at 5,000 per well of 384-well black plates (Corning 3712) and allowed to recover overnight. The next day (ca. 90% confluence), dilutions of compounds in DMSO were added by pin transfer (CyBio Vario, 100 nL per well). Cells were incubated for 1 h. (For experiments measuring ROS at 8 h, 4,000 cells were plated; for 24 h measurements, 3,000 cells were plated.) Media was then removed and replaced using a Combi liquid handler with colorless DMEM (no supplements) containing CM-H_2DCF-DA and Hoechst 33342 as described previously.[23] During high-throughput screening, light fixation using 0.5% paraformaldehyde was performed for 5 min prior to two additional washes with PBS and a FLiPR plate reader was used. Intensity values were normalized on a per-plate basis using the Genedata software package. During subsequent studies, images were obtained using an IX_Micro automated fluorescence microscope (Molecular Devices). Quantitation of pixel intensity was performed using MetaXpress software and signal intensity was calculated relative to wells in the same plate treated with DMSO. **Dihydroethidium:** The assay was performed as above except for use of DHE at 10 μM instead of CM-H_2DCF-DA.

Viability Assays. CellTiter-Glo. Cells were generally plated at 1,000 per well in white 384-well plates and allowed to attach overnight. BJhTERT and BJELR were plated at 500 per well, and HMEL and HMELR were plated at 750 per well, due to rapid growth kinetics. HEC108 cells were chosen for measurement of viability after 5 days of treatment on the basis of their slower growth kinetics and were plated at 500 cells/well. After addition of compounds by pin transfer, plates were incubated for 48 h (H1703, 72 h). At that time, media was removed and replaced with a solution of CellTiter-Glo reagent in PBS. Luminescence was read using an EnVision multimode plate reader, and signal intensity was calculated relative to in-plate DMSO control wells. For co-treatment with antioxidants and other compounds (e.g., BSO), after overnight recovery the culture media was removed and replaced with fresh media containing the desired antioxidant or other agent. After 1 h, test compounds were added by pin transfer and the assay proceeded as above.

GSH/GSSG Glo Assay. Cells were plated at 1,000 per well in white 384-well plates and allowed to attach overnight. After addition of compounds by pin transfer, plates were incubated for 6 h. At that time, media was removed and cells were washed with PBS. Total glutathione was then measured according to manufacturer's instructions (Promega) with measurement of luminescence performed using an EnVision multimode plate reader.

ARE-Luciferase Assays. IMR32 cells were plated at 10,000 per well in white 384-well plates and assayed using Bright-Glo (Promega) as previously described.[24]

Source of Chemicals. Screening hits were obtained from the Broad Institute Chemical Biology Platform and were assessed for purity by LC−MS analysis. BRD9092 and BRD56491 were additionally purchased from ChemDiv, and BRD5459 was purchased from Sigma. These repurchased supplies provided equivalent activity in all assays. Erastin, BSO, vitamin E (α-tocopherol), and *N*-acetyl cysteine were purchased from Sigma; PX12 was purchased from Tocris. BRD1378 was resynthesized and purified by HPLC and showed comparable activity to supplies provided by Broad CB Platform.

ASSOCIATED CONTENT

Supporting Information

Seven supporting figures, one supporting table. This information is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Trachootham, D., Alexandre, J., and Huang, P. (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat. Rev. Drug Discovery 8*, 579−591.

(2) Winterbourn, C. C., and Hampton, M. B. (2008) Thiol chemistry and specificity in redox signaling. *Free Radical Biol. Med. 45*, 549−561.

(3) Murphy, M. P., Holmgren, A., Larsson, N. G., Halliwell, B., Chang, C. J., Kalyanaraman, B., Rhee, S. G., Thornalley, P. J., Partridge, L., Gems, D., Nystrom, T., Belousov, V., Schumacker, P. T., and Winterbourn, C. C. (2011) Unraveling the biological roles of reactive oxygen species. *Cell Metab. 13*, 361−366.

(4) Baird, L., and Dinkova-Kostova, A. T. (2011) The cytoprotective role of the Keap1-Nrf2 pathway. *Arch. Toxicol. 85*, 241−272.

(5) Ma, Z. A. (2012) The role of peroxidation of mitochondrial membrane phospholipids in pancreatic beta-cell failure. *Curr. Diabetes Rev. 8*, 69−75.

(6) Victor, V. M., Rocha, M., Herance, R., and Hernandez-Mijares, A. (2011) Oxidative stress and mitochondrial dysfunction in type 2 diabetes. *Curr. Pharm. Des. 17*, 3947−3958.

(7) Chen, A. F., Chen, D. D., Daiber, A., Faraci, F. M., Li, H., Rembold, C. M., and Laher, I. (2012) Free radical biology of the cardiovascular system. *Clin. Sci. (London) 123*, 73−91.

(8) Andersen, J. K. (2004) Oxidative stress in neurodegeneration: cause or consequence? *Nat. Med. 10* (Suppl), S18−25.

(9) Li, Q., Spencer, N. Y., Pantazis, N. J., and Engelhardt, J. F. (2011) Alsin and SOD1(G93A) proteins regulate endosomal reactive oxygen species production by glial cells and proinflammatory pathways responsible for neurotoxicity. *J. Biol. Chem. 286*, 40151−40162.

(10) Haigis, M. C., and Yankner, B. A. (2010) The aging stress response. *Mol. Cell 40*, 333−344.

(11) Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. *J. Gerontol. 11*, 298−300.

(12) Schumacker, P. T. (2006) Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer Cell 10*, 175−176.

(13) Luo, J., Solimini, N. L., and Elledge, S. J. (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell 136*, 823−837.

(14) Watson, J. (2013) Oxidants, antioxidants and the current incurability of metastatic cancers. *Open Biol. 3*, 120144.

(15) Szatrowski, T. P., and Nathan, C. F. (1991) Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res. 51*, 794−798.

(16) Lee, A. C., Fenster, B. E., Ito, H., Takeda, K., Bae, N. S., Hirai, T., Yu, Z. X., Ferrans, V. J., Howard, B. H., and Finkel, T. (1999) Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *J. Biol. Chem. 274*, 7936−7940.

(17) Wondrak, G. T. (2009) Redox-directed cancer therapeutics: molecular mechanisms and opportunities. *Antioxid. Redox Signaling 11*, 3013−3069.

(18) Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., Chiao, P. J., Achanta, G., Arlinghaus, R. B., Liu, J., and Huang, P. (2006) Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell 10*, 241−252.

(19) Guzman, M. L., Rossi, R. M., Neelakantan, S., Li, X., Corbett, C. A., Hassane, D. C., Becker, M. W., Bennett, J. M., Sullivan, E., Lachowicz, J. L., Vaughan, A., Sweeney, C. J., Matthews, W., Carroll, M., Liesveld, J. L., Crooks, P. A., and Jordan, C. T. (2007) An orally bioavailable parthenolide analog selectively eradicates acute myelogenous leukemia stem and progenitor cells. *Blood 110*, 4427−4435.

(20) Raj, L., Ide, T., Gurkar, A. U., Foley, M., Schenone, M., Li, X., Tolliday, N. J., Golub, T. R., Carr, S. A., Shamji, A. F., Stern, A. M., Mandinova, A., Schreiber, S. L., and Lee, S. W. (2011) Selective killing of cancer cells by a small molecule targeting the stress response to ROS. *Nature 475*, 231−234.

(21) Dolma, S., Lessnick, S. L., Hahn, W. C., and Stockwell, B. R. (2003) Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells. *Cancer Cell 3*, 285−296.

(22) Shaw, A. T., Winslow, M. M., Magendantz, M., Ouyang, C., Dowdle, J., Subramanian, A., Lewis, T. A., Maglathin, R. L., Tolliday, N., and Jacks, T. (2011) Selective killing of K-ras mutant cancer cells by small molecule inducers of oxidative stress. *Proc. Natl. Acad. Sci. U.S.A. 108*, 8773−8778.

(23) Wagner, B. K., Kitami, T., Gilbert, T. J., Peck, D., Ramanathan, A., Schreiber, S. L., Golub, T. R., and Mootha, V. K. (2008) Large-scale chemical dissection of mitochondrial function. *Nat. Biotechnol. 26*, 343−351.

(24) Hur, W., Sun, Z., Jiang, T., Mason, D. E., Peters, E. C., Zhang, D. D., Luesch, H., Schultz, P. G., and Gray, N. S. (2010) A small-molecule inducer of the antioxidant response element. *Chem. Biol. 17*, 537−547.

(25) Adams, D. J., Dai, M., Pellegrino, G., Wagner, B. K., Stern, A. M., Shamji, A. F., and Schreiber, S. L. (2012) Synthesis, cellular evaluation, and mechanism of action of piperlongumine analogs. *Proc. Natl. Acad. Sci. U.S.A. 109*, 15115−15120.

(26) Fang, J., Nakamura, H., and Iyer, A. K. (2007) Tumor-targeted induction of oxystress for cancer therapy. *J. Drug Targeting 15*, 475−486.

(27) Hagenbuchner, J., Kuznetsov, A., Hermann, M., Hausott, B., Obexer, P., and Ausserlechner, M. J. (2012) FOXO3-induced reactive oxygen species are regulated by BCL2L11 (Bim) and SESN3. *J. Cell Sci. 125*, 1191−1203.

(28) Levy, E. J., Anderson, M. E., and Meister, A. (1993) Transport of glutathione diethyl ester into human cells. *Proc. Natl. Acad. Sci. U.S.A. 90*, 9171−9175.

(29) Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999) Creation of human tumour cells with defined genetic elements. *Nature 400*, 464−468.

(30) Lundberg, A. S., Randell, S. H., Stewart, S. A., Elenbaas, B., Hartwell, K. A., Brooks, M. W., Fleming, M. D., Olsen, J. C., Miller, S. W., Weinberg, R. A., and Hahn, W. C. (2002) Immortalization and transformation of primary human airway epithelial cells by gene transfer. *Oncogene 21*, 4577−4586.

(31) Elenbaas, B., Spirio, L., Koerner, F., Fleming, M. D., Zimonjic, D. B., Donaher, J. L., Popescu, N. C., Hahn, W. C., and Weinberg, R. A. (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev. 15*, 50−65.