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

Drew J. Adams,*†‡, Zarko V. Boskovic,‡§, Jimmy R. Theriault,‡ Alex J. Wang,‡ Andrew M. Stern,‡ Bridget K. Wagner,† Alykhan F. Shamji,§ and Stuart L. Schreiber*†‡§,⊥

†Chemical Biology Program, ‡Chemical Biology Platform, and ⊥Howard Hughes Medical Institute, Broad Institute, 7 Cambridge Center, Cambridge, Massachusetts 02142, United States
§Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, United States

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. A minority of these compounds diminished the viability of cancer cell lines, indicating that ROS elevation by small molecules is 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, show selective toxicity for transformed cells over nontransformed cells in an engineered cell-line model of tumorigenesis. These studies suggest 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 aging10,11 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.13–14 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 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 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 is
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\(_2\)DCF-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\(_2\)DCF-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
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 (Figure 1D). Many compounds outperformed the positive control, including 38 compounds that elevated ROS to levels...
Although some nontoxic ROS-enhancing compounds had little effect 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). 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-H₂DCF-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 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.
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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.

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$_2$DCF-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,26 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 mechanically 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 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 an apparently unbiased collection of small molecules that increase ROS levels in cancer cells, we have been able to assess systematically the effects of small-molecule induced ROS elevation on cell viability and other cellular processes. We provide evidence that the majority of these ROS-enhancing compounds are unable to induce cancer-cell death as single 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 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-H₂ and PL-DHNF, 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). BjbTERT 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-H₂DCF-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% confluency), dilutions of compounds in DMEM 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-H₂DCF-DA and Hoechst 33342 as described previously.25 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-H₂DCF-DA.

**Viability Assays.** CellTiter-Glo. Cells were generally plated at 1,000 per well in white 384-well plates and allowed to attach overnight. BjbTERT 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.

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: dadams@broadinstitute.org; stuart_schreiber@harvard.edu.

**Notes**

The authors declare no competing financial interest.

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