Piperlongumine is a naturally occurring small molecule recently identified to be toxic selectively to cancer cells in vitro and in vivo. This compound was found to elevate cellular levels of reactive oxygen species (ROS) selectively in cancer cell lines. The synthesis of 80 piperlongumine analogs has revealed structural modifications that retain, enhance, and ablate key piperlongumine-associated effects on cells, including elevation of ROS, cancer cell death, and selectivity for cancer cells over nontransformed cell types. Structure/activity relationships suggest that the electrophilicity of the C2-C3 olefin is critical for the observed effects on cells. Furthermore, we show that analogs lacking a reactive C7-C8 olefin can elevate ROS to levels observed with piperlongumine but show markedly reduced cell death, suggesting that ROS-independent mechanisms, including cellular cross-linking events, may also contribute to piperlongumine’s induction of apoptosis. In particular, we have identified irreversible protein glutathionylation as a process associated with cellular toxicity. We propose a mechanism of action for piperlongumine that may be relevant to other small molecules having two sites of reactivity, one with greater and the other with lesser electrophilicity.

Reactive oxygen species (ROS) are natural byproducts of oxidative respiration and function in signal transduction and clearance of pathogens during innate immune responses. Cancer cells have been reported to harbor higher levels of ROS than nontransformed cells, and in some cases activation of a specific oncogene (for example, HRAS) is sufficient to elevate levels of ROS (1, 2). Because ROS are capable of damaging crucial cellular macromolecules, including DNA, some cancer cells may be faced with chronic “oxidative stress” that requires active enzymatic ROS detoxification to prevent induction of cell death. As such, one consequence of some of the genomic alterations leading to tumorigenesis may be a dependency on pathways facilitating the detoxification of ROS for survival, a form of “non-oncogene addiction” or “non-oncogene co-dependency” (3–5). Importantly, this dependency might not be shared by many nontransformed cells, whose lower basal ROS levels and/or elevated antioxidant capacity could provide resistance to treatments that impair ROS metabolism. In keeping with this hypothesis, various small molecules, including ones with disulfide, α,β-unsaturated carbonyl, sulfonate, or other electrophilic functional groups, have previously been shown to elevate ROS levels and induce cancer cell death (6). A subset of such compounds has also demonstrated a degree of selective toxicity toward cancer cells in vitro and in vivo models (7–12).

A cell-based, high-throughput screening approach was used to identify piperlongumine (PL), a naturally occurring, electrophilic small molecule capable of selectively killing a variety of transformed cell types while sparing primary normal cells (5). PLs in vivo antitumor efficacy was illustrated in mouse models of cancer, small molecule capable of selectively killing a variety of transformed cell types while sparing primary normal cells (5). PLs in vivo antitumor efficacy was illustrated in mouse models of cancer, including xenograft and spontaneous mammary tumor formation models. Mechanistic investigations correlated the observed selective toxicity with a cancer-selective increase in ROS and other markers of oxidative stress following treatment with PL, as well as increases in DNA damage and apoptotic cell death (Fig. 1A).

The small-molecule nucleophile and antioxidant N-acetyl-Lcysteine prevents PL-mediated cell death, and several proteins known to bind glutathione and to detoxify ROS were identified as potential cellular interaction partners of PL through affinity purification and quantitative proteomics.

To explore the biology and chemistry of this promising lead compound further, we have synthesized 80 structural analogs of PL that together enable us to examine the roles of modifications at every carbon atom. Extensive investigation of the actions of these analogs on cells has identified modifications that retain, enhance, or ablate the potency of PL. A parallel chemical analysis suggests that the electrophilicity of the C2-C3 olefin is required for these actions. Several analogs, including those with markedly enhanced potency, retain the selective toxicity for cancer cells observed for PL in in vitro models of cancer. Other analogs retain PL’s ability to elevate cellular ROS while displaying notably lower cellular toxicity, suggesting that mechanisms independent of ROS elevation, including covalent protein modifications like glutathionylation, may play a role in inducing cancer cell death.

Results

Synthesis of PL Analogs. PL analogs were in general synthesized by a convergent strategy that entailed coupling commercially available or synthetically accessible lactams and carboxylic acid chlorides (Fig. 1B). Additional analogs bearing substituents at C2 were generated by selective iodination of PL at C2 and palladium-catalyzed cross-coupling (for complete synthesis details, see SI Appendix, Fig. S1).

Chemical Reactivity of PL and its Analogs. As PL contains multiple potentially electrophilic sites that may influence its actions on cells, we assessed the chemical reactivity of PL and several of our analogs using methyl thioglycolate as a representative achiral thiol nucleophile. Treatment of PL with three equivalents of methyl thioglycolate in DMSO provided the product of conjugate addition at C3 in 62% yield (Fig. 1C). No addition was observed at C8 by LC-MS or 1H NMR, and 2,3-dihydropiperlongumine (Table 1, entry 2) was unreactive under these conditions. We next assessed whether PL analogs with modifications proximal to the highly reactive C2-C3 olefin yield similar patterns of reactivity. Substitution of methyl at C2 (Table 1, entry 3) ablated hetero-conjugate addition, indicating that C2 alkyl substituents can impede reaction at C3. A ring-expanded cycloheptenimide analog (PL-7; Table 1, entry 4) also provided the expected hetero-conjugate addition.


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Contributed by Stuart L. Schreiber, August 1, 2012 (sent for review June 16, 2012)
Piperlongumine (1; BRD2293) DNA damage, apoptotic cell death (cancer cells only)

Cellular Actions. We next sought to determine the performance of the synthetic analogs relative to PL in cells. Changes in ROS levels were assessed 90 min after compound treatment by automated microscopy using the redox-sensitive dye CM-H$_2$DCF-DA, which shows greatest sensitivity to hydroxyl radical and other highly reactive species. As a second assay, effects of compound treatments on ATP levels, a surrogate for cell viability, were measured after 48 h. Monitoring cellular reducing equivalents as a surrogate for viability gave closely correlated results (SI Appendix). Both assays were performed in 384-well plates on two human cancer cell lines, H1703 (lung) and HeLa (cervix).

As PL contains multiple electrophilic sites and can undergo hetero-conjugate addition with small-molecule thiols, we hypothesized that the electrophilicity of PL might be central to its bioactivity. Our first analogs sequentially eliminated the two reactive α,β-unsaturated olefins (Table 1, entries 1, 2, and 6. Table 1 includes viability data for representative analogs in two cell lines. For viability and ROS dose-response data for all 80 analogs, see SI Appendix). Notably, 2,3-dihydropiperlongumine (PL-2,3H$_2$; Table 1, entry 2), lacking the C2-C3 olefin, neither elevated ROS levels nor decreased viability of the two cell lines tested, demonstrating that this functionality is essential for PL's biological activity (Fig. 2). By contrast, removal of the C7-C8 olefin (PL-H$_2$) led to substantial reductions in toxicity (Fig. 2A), but did not diminish ROS elevation (Table 1, entry 6; Fig. 2B and C). These results indicate that while the presence of the C2-C3 olefin is sufficient to elevate ROS, both olefins are necessary to recapitulate the level of cellular toxicity observed for PL.

The study of additional analogs confirmed that modifications diminishing the reactivity of the C2-C3 olefin yielded compounds with minimal activity in these assays (Table 1, entries 7 and 8). Piperlongumine-thiol adduct PL-MTG, a potential PL pro-drug, was found to be substantially less potent than PL at inducing cancer cell death and elevating ROS (Table 1, entry 7).

Additional analogs disrupting the electrophilicity of the C7-C8 olefin by steric blockade or cyclization to an aromatic heterocycle showed substantially diminished toxicity in cells (Table 1, entries 9–11). Together with 7,8-dihydropiperlongumine PL-H$_2$ discussed above, these analogs highlight the need for both Michael acceptors to observe potent cell death.

Although the C2-C3 and C7-C8 olefins appear critical for PL's actions on cells, many modifications can be made at positions distal from these olefins without greatly affecting performance in our ROS and ATP assays. Modification of the aromatic

![Fig. 1.](image)

(A) PL and its cellular phenotypes. (B) Convergent strategy for the synthesis of PL analogs. (C) PL reacts with small-molecule thiols at C3 under neutral conditions. Reaction time, 72 h.

Table 1. Effects on cellular ATP levels for selected PL analogs in two cell lines

<table>
<thead>
<tr>
<th>EC$_{50}$ H1703, μM</th>
<th>EC$_{50}$ HeLa, μM</th>
<th>EC$_{50}$ H1703, μM</th>
<th>EC$_{50}$ HeLa, μM</th>
<th>EC$_{50}$ H1703, μM</th>
<th>EC$_{50}$ HeLa, μM</th>
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<tbody>
<tr>
<td>1</td>
<td>2.8</td>
<td>7.1</td>
<td>7</td>
<td>&gt;20</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>n.t.</td>
<td>n.t.</td>
<td>8</td>
<td>n.t.</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>n.t.</td>
<td>n.t.</td>
<td>9</td>
<td>&gt;20</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>5.1</td>
<td>10</td>
<td>18</td>
<td>&gt;20</td>
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<tr>
<td>5</td>
<td>0.7</td>
<td>1.3</td>
<td>11</td>
<td>14</td>
<td>n.t.</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>n.t.</td>
<td>12</td>
<td>3.5</td>
<td>7.1</td>
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n.t. indicates no loss of viability at 20 μM; >20 μM indicates less than 50% decrease in viability observed at 20 μM.
substituents of PL was largely without effect: all three aryl methoxyl groups could be removed and replaced with a variety of substituents at the ortho, meta, or para positions without substantially altering elevation of ROS or cellular toxicity (Table 1, entries 12 and 13). Likewise, substitution at C5 or C4 with aromatic, heteroaromatic, and some alkyl substituents provided analogs of comparable potency to PL (Table 1, entries 14 and 15). One-carbon ring expansion of the dihydropyridone, replacement of the C6 carbonyl with a sulfonyl moiety, and several other modifications also were largely neutral (Table 1, entries 4 and 16).

Analogs with substituents at C2 provided a wide range of activities. A variety of alkynyl substituents at C2 induced cell death more potently than PL, with EC\textsubscript{50} values as low as 0.4 μM (Table 1, entries 5 and 17). However, other substituents at C2 were uniformly less potent than PL, with C2 alkyl or aryl groups lacking activity in both assays (Table 1, entries 3 and 18). The diminished chemical reactivity of analogs with C2 alkyl substituents may be one factor contributing to the observed lack of cellular activity (SI Appendix, Fig. S2).

Because both the C2-C3 and C7-C8 olefins are necessary to observe the levels of toxicity seen for PL, we speculated that multivalency—the ability to interact with multiple cellular targets or a single cellular target at more than one location—might alter toxicity in this system (13–16). Thus, we synthesized a structurally analogous PL “monomer,” “dimer,” and “trimer” using a Mitsunobu approach (Fig. 3A and SI Appendix, Fig. S1). Remarkably, the PL dimer (PL-DI) showed roughly 10-fold greater potency in the ATP and ROS assays relative to a closely related monomeric analog (PL-MON) (Fig. 3B and C). Moreover, a PL trimer (PL-TRI) was found to be approximately 2-fold more potent than the dimeric analog.

**Additional Cellular Actions.** Beyond elevation of ROS, PL affects other cellular markers of oxidative stress, including depletion of glutathione (5). We next characterized a subset of analogs in additional oxidative stress assays to identify phenotypes that might correlate with cellular toxicity more closely than ROS elevation.

Separation of ROS Elevation From Cellular Toxicity. For PL and many analogs, doses at which ROS and cellular toxicity are elevated are closely correlated. However, two series of analogs appear to decouple the elevation of ROS and cell death. In both cell lines tested, 7,8-dihydropiperlongumine (PL-H\textsubscript{2}) and a dihydronaphthalene analog (PL-DHN) led to robust enhancement of ROS levels but to diminished cell death relative to PL (Figs. 2 and 4). Conversely, various analogs bearing alkynes at C2 showed greatly enhanced toxicity (Fig. 4B) without altering potency for the elevation of ROS (Fig. 4A). Although this enhanced cell death could be explained by C2-alkynyl analogs having additional toxic mechanisms of action, we were surprised to find analogs like PL-H\textsubscript{2} and PL-DHN that showed elevation of ROS comparable to PL but greatly diminished cellular toxicity. A similar pattern was observed in two additional cancer cell lines (U2OS, osteosarcoma; HEC108, endometrial), indicating that the observed decoupling of ROS and cell death may be general (SI Appendix, Fig. S3). Although elevation of cellular ROS likely places cancer cell lines under enhanced oxidative stress, this stress appears insufficient in some cases to induce cell death.

![Fig. 2. Contribution of PL’s electrophilic functionalities to cellular phenotypes. (A) Measurement of cellular ATP as a surrogate of viability (CellTiter-Glo) and (B) cellular ROS levels (CM-H\textsubscript{2}DCF-DA) in two cell lines. Data are expressed as mean ± SD for four (CTG) or three (ROS) independent experiments. (C) Representative fluorescence microscopy images of HeLa cells treated for 1 h with 20 μM PL, PL-H\textsubscript{2}, or PL-2,3H\textsubscript{2}.](https://www.pnas.org/content/109/38/15117)

![Fig. 3. Oligomerization of PL leads to greatly elevated potency for elevation of ROS and cell death in two cell lines. (A) Oligomers of PL: monomer (PL-MON), dimer (PL-DI), and trimer (PL-TRI). (B) ATP levels and (C) ROS levels in HeLa and H1703 cells after 48 h (ATP) or 1.5 h (ROS) treatment with the indicated concentrations of oligomer. Data are expressed as mean ± SD for four (CTG) or three (ROS) independent experiments.](https://www.pnas.org/content/109/38/15117)
A three compounds in HeLa cells, with PL most effective (Fig. 5).

Using a luminescence-based assay for cellular glutathione (GSH/GSSG-Glo) in the EJ bladder carcinoma line (5), a similar decrease in total cellular glutathione (ca. 60%) was observed for PL and two analogs with diminished toxicity (PL-H$_2$, PL-DHN; Fig. S4). Decreases in total glutathione were also observed for all three compounds in HeLa cells, with PL most effective (Fig. S4).

We also examined an additional oxidative stress phenotype, protein glutathionylation, using an immunofluorescence approach that relies on a monoclonal antibody recognizing glutathione. In HeLa cells, we observed large and rapid elevations in protein glutathionylation for PL and its potently toxic cyclopropyl alkynyl analog PL-cPr (Fig. 5B and SI Appendix, Fig. S4). However, no elevation in protein glutathionylation was observed for PL-H$_2$, and minimal elevation was observed for PL-DHN. Examination of our 80-analog set in both HeLa and EJ cells suggests that an unhindered, chemically reactive C7-C8 olefin is necessary for elevation of protein glutathionylation. Analogs with an unreactive or absent C7-C8 olefin, modifications that also diminish toxicity, show minimal elevation of protein glutathionylation (see SI Appendix). Similarly, several small molecules unrelated to PL bearing two Michael acceptor functionalities elevated glutathionylation, while nine other small molecules with a single electrophilic site did not (SI Appendix, Fig. S4). Elevation of protein glutathionylation also correlated with toxicity, as toxic PL analogs bearing multiple Michael acceptor functionalities showed robust protein glutathionylation.

Additionally, although proteins are commonly glutathionylated during periods of oxidative stress via readily reversible disulfide bond linkages (17, 18), the protein glutathionylation observed following PL treatment could not be reversed by treatment with 0.1 M dithiothreitol, indicating a role or roles for non-disulfide covalent attachments (Fig. 5C). By contrast, the elevation of protein glutathionylation observed following treatment with glutathione disulfide (GSSG) was strikingly reversed by treatment with 0.1 M dithiothreitol.

Selectivity for Cancer Cells Over Nontransformed Cells. We evaluated whether our analogs, like PL, could selectively target cancer cells over nontransformed cells using an established isogenic model of tumorigenesis (5). Such models rely on serial transduction of primary human cell types with defined genetic factors to create an engineered cancer cell line. We compared BJ human fibroblasts with the BJ-ELR line, which is fully transformed by the addition of hTERT, large-T antigen, and an oncogenic HRAS-V12 allele (19). Eight representative analogs were evaluated by crystal violet staining for cell numbers. Phenethylisothiocyanate and parthenolide, two electrophilic small molecules previously shown to be selectively toxic to cancer cells by a mechanism involving ROS elevation (8, 12), also showed selectivity in this assay. Initial screening of the eight analogs established that most retained a degree of selective toxicity in this isogenic cell line pair, although some were inferior to PL (SI Appendix, Fig. S5). Further testing confirmed that four analogs showed selectivity comparable to PL (PL-7, PL-DI, PL-TRI, and sulfonimide derivative PL-SO$_2$), with PL-7 the most selective (Fig. 6 and SI Appendix, Fig. S6).

Discussion

By synthesizing and testing an array of PL analogs, we have identified the C2-C3 olefin as a key pharmacophore, with the C7-C8 olefin also playing a significant role in determining toxicity (Fig. 7A). Although a wide range of modifications at positions distal to these olefins is largely neutral, modifications expected to impair the reactivity of these olefins diminish analogs’ effects on cells. The C2-C3 olefin reacts with a small-molecule thiol under neutral conditions in DMSO, but exposure of the resulting PL-thiol adduct to cells was largely without effect. Addition of a C2 methyl group ablates both chemical reactivity in vitro and all observed cellular phenotypes, further supporting the necessity of reactivity at C3 for actions in cells. Although addition of thiols at C8 was not observed using our neutral in vitro conditions, the presence of cellular thiolate nucleophiles or the enhanced effective molarity following addition of a cellular nucleophile at C3 may greatly enhance the rate of thiol addition at C8.

As both olefins appeared necessary for PLs toxicity to cells, we evaluated several oligomers of PL to explore further the role of multivalent electrophilicity in determining toxicity. Notably, a PL
Fig. 6. PL analogs show selective toxicity toward transformed human fibroblasts (BJ-ELR). Viability was measured by Crystal Violet staining after 48-h treatment with (A) PL-7, (B) PL-SO$_2$, (C) PL-DIM, or (D) PL-TRI. Data are expressed as mean ± SD for three independent experiments.

dimer resulted in a nearly 10-fold increase in toxicity. The ability to cross-link additional cellular nucleophiles may limit the reversibility of compound binding or cause more extensive disruption of the structure and function of targeted proteins relative to PL itself. A PL trimer was only 2-fold more potent than the analogous dimer, suggesting diminishing returns for addition of further PL units. These highly potent oligomers, as well as several other analogs, also retained similar selectivity for transformed cells as observed for PL in an isogenic model of tumorigenesis.

As noted above, PL analogs with only a single electrophilic moiety showed diminished cell death but often gave rise to substantial increases in ROS, suggesting that ROS elevation may not be sufficient or even necessary for cell death in some cellular contexts. Although the specific ROS measured and their subcellular localization may vary between analogs, PL and its active analogs are likely capable of reaction with a variety of cellular protein thiolis, including some that may contribute to cell death independent of elevation of ROS levels.

We also assessed the performance of our analogs in additional oxidative stress assays. Several analogs with varying degrees of toxicity showed a similar ability to deplete cellular glutathione. As previous reports have established that substantial reductions in glutathione (for example, as induced by the glutathione biosynthesis inhibitor BSO) need not result in cell death, the contribution of glutathione depletion to PLs cellular toxicity remains unclear (20–22). Additionally, we note that reported estimates of HeLa cell volume (2.600 μm$^3$) (23) and typical concentrations of reduced cellular glutathione (ca. 5 mM) (24) suggest that PL is present at quantities greatly in excess of cellular glutathione under our assay conditions (1,000 cells per well, 50 μL per well). As such, direct conjugation of PL with glutathione at C3 is a plausible explanation for the observed decrease in total glutathione.

Treatment with PL or analogs with two reactive electrophilic sites also gives rise to enhanced glutathionylation of cellular proteins, while analogs with a single Michael acceptor did not. We propose that formation of a covalent complex linking glutathione, PL, and a glutathione-binding protein via both PL's electrophilic Michael acceptor functionalities can account for these observations (Fig. 7B). We imagine a sequence involving first a Michael addition of glutathione to PL's more electrophilic C2-C3 olefin followed by the formation of a non-covalent complex between the PL-glutathione adduct and a glutathione-binding protein, and finally a Michael addition of a nucleophilic residue of the glutathione-binding protein to the less electrophilic C7-C8 olefin that is accelerated by the formation of the complex. Mechanistically analogous protein glutathionylation under conditions of electrophilic stress has been observed recently for the metabolic byproduct 4-oxo-nonenal (25) and the chemotherapeutic busulfan (26), both of which are bivalent electrophiles. Such a model also provides a chemical rationale for the results of an unbiased quantitative proteomics analysis of proteins binding to PL, which identified numerous glutathione-binding proteins among the highest confidence interactions (5). We note that there exist other naturally occurring, biologically active small molecules with two sites of reactivity—one with greater and the other with lesser electrophilicity. Thus, it is possible that the proposed mechanism, where a small organic molecule is inserted between glutathione and a protein in the protein glutathionylation process, has generality beyond PL.

Although not measured in this study, PL may also covalently modify additional cellular proteins. Notably, proteins that do not bind glutathione were also high-signal outliers in the reported quantitative proteomic analysis, including four proteins previously shown to be modified following treatment with electrophiles (PRDX1, RPS5, VIM, and AHNAK) (5, 27–30). Taken together, our observations suggest that elevation of protein glutathionylation or other cellular cross-linking events may be a feature of cells treated with PL more closely associated with cellular toxicity than elevation of ROS or glutathione depletion. Further proteomic analyses will be required to identify specific protein glutathionylation events and proteins that interact with analogs of markedly enhanced (PL-DI, PL-TRI) or diminished (PL-H$_2$) electrophilicity and toxicity (31). These studies establish a central role for multivalent electrophilicity in the chemical biology of PL and related compounds, and indicate that both electrophilic and oxidative stress phenotypes can contribute to PLs promising cancer-selective toxicity.

Methods

Cell Culture. HeLa, U2OS, EJ, and H1703 were obtained from ATCC. HeLa and U2OS were cultured in DMEM+10% FBS in a 37°C incubator (5% CO$_2$); H1703

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was cultured in RPMI+10% FBS, and EJ in McCoy's 5A+10% FBS. HEC108 were obtained from the Broad Institute/Novartis Cancer Cell Line Encyclopedia and cultured in EMEM+15% FBS. BJ (human fibroblasts) were obtained from ATCC, while BJ-ELR (a fully transformed derivative containing HTERT, large-T antigen, and activated H-RAS) were a gift of Brent Stockwell, Columbia University, New York. Both lines were maintained in 1:1 DMEM:M199 supplemented with 15% FBS. BJ were maintained below 70% confluency and used below passage 8.

**ROS Assay.** Cells were plated at 5,000 cells/well in black 384-well plates (Corning) and allowed to attach overnight. The next day (ca. 90% confluency), dilutions of compounds in DMSO were added by pin transfer (CyBio Vario, 100 nl per well), and incubated for 90 min. Media was changed using a Thermo Multidrop Combi liquid handler to phenol red-free DMEM containing 10 μM CM-H2DCF-DA and 10 μg/ml Hoechst 33342. Following incubation for 15–30 min, cells were washed twice with PBS. 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.

**ATP 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 48 h. At that time, media was removed and replaced with a solution of CellTiter-Glo reagent (Promega) in PBS. After 10 min, luminescence was read using an EnVision multilabel plate reader (Perkin-Elmer), and signal intensity was calculated relative to in-plate DMSO control wells.

**Glutathione 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 3 h (EJ) or 6 h (HeLa). Cells were washed with a solution of CellTiter-Glo reagent (Promega) in PBS. After 10 min, luminescence was measured using an EnVision multilabel plate reader.


**Immunofluorescence Detection of Glutathionylated Proteins.** Cells were plated at 3,000 (HeLa) or 5,000 (EJ) per well and allowed to attach overnight. After addition of compounds by pin transfer, plates were incubated for between 10 min and 6 h. At the appropriate time, cells were fixed with 1% paraformaldehyde in PBS (20 min), permeabilized 30 min with PBS+0.1% TritonX-100 (PBST), and blocked 30 min with PBST+2%BSA. Primary antibody (Ms anti-glutathione, Abcam Ab19534) was added (1:1250 in PBST+2%BSA) and incubated at 4°C overnight. Following two washes with PBST, cells were incubated at RT 1 h in the dark with secondary antibody solution (Cy-2 or Cy-3-conjugated goat anti-mouse, Jackson Immunologicals, 1:500, plus Hoechst 33342, 10 μg/ml in PBST+2%BSA). Following two washes with PBST, images were collected using an IX Micro automated fluorescence microscope (Molecular Devices). Quantitation of pixel intensity was performed as above.

**Assessment of Cancer/Normal Selective Toxicity.** BJ vs. BJ-ELR: In 12-well dishes, BJ (33,000 cells/well) or BJ-ELR (25,000 cells/well) were seeded and allowed to grow to 40–50% confluency (24–36 h). Compound solutions in DMSO were added (0.2% DMSO final) and incubated 48 h. Cells were fixed with 2% paraformaldehyde (15 min), followed by staining with 0.01% aqueous crystal violet (30 min). Cells were washed twice with water and allowed to dry overnight. Once dry, the stain was resolubilized using ethylene glycol (2 ml per well, 2–16 h with shaking). When cells retained no stain, duplicate samples of 50 μl were then transferred to wells of a 384-well plate. Absorbance at 540 nm was measured with an EnVision plate reader, and relative viability was calculated relative to DMSO-treated control wells.

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