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Redox cycling compounds generate H₂O₂ in HTS buffers containing strong reducing reagents – real hits or promiscuous artifacts?

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Abstract

Redox cycling compounds (RCCs) generate μM concentrations of hydrogen peroxide (H₂O₂) in the presence of strong reducing agents, common buffer components used to maintain the catalytic activity and/or folding of target proteins for high throughput screening (HTS) assays. H₂O₂ generated by RCCs can indirectly inhibit the catalytic activity of proteins by oxidizing accessible cysteine, tryptophan, methionine, histidine or selenocysteine residues, and indeed several important classes of protein targets are susceptible to H₂O₂-mediated inactivation; protein tyrosine phosphatases, cysteine proteases, and metalloenzymes. The main sources of H₂O₂ in cells are the Nox enzyme/SOD systems, peroxisome metabolism, and the autoxidation of reactive chemicals by enzyme mediated redox cycling at both the microsomal and mitochondrial sites of electron transport. Given the role of H₂O₂ as a second messenger involved in the regulation of many signaling pathways it is hardly surprising that compounds which can generate intracellular H₂O₂ by enzyme mediated redox cycling would have pleiotropic effects. RCCs can therefore have serious negative consequences for the probe and/or lead generation process: primary HTS assay hit rates may be inflated by RCC false positives; critical resources will be diverted to develop and implement follow up assays to distinguish RCCs from real hits; and screening databases will become annotated with the promiscuous activity of RCCs. In an attempt to mitigate the serious impact of RCCs on probe and lead generation, two groups have independently developed assays to identify RCCs.

Introduction

In the pursuit of small molecules that modulate the activity of isolated protein targets or cellular phenotypes the selection of the screening assay format is arguably one of the most critical factors that influences whether a chemical biology endeavor will be successful [1,2]. The pairing of a particular target or cellular phenotype with an assay technology not only requires the development, optimization and validation of an assay that is biologically appropriate, but also one which provides a robust and reproducible signal window with sufficient throughput and capacity to screen the compound diversity of interest [1–4]. The

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potential for compound interference with an assay format must also be considered, especially since it typically requires the integration of orthogonal counter screens into the follow up paradigm to identify and eliminate false positives [1,2,5,6]. Assay interference can be attributed to both chemical and physical compound properties [1,5–10]. Compounds that are colored, fluorescent, or that form aggregates have the potential to promiscuously interfere with a wide variety of biochemical and cell based assay formats [1,2,5,6,11]. Alternatively, some inhibitors of the firefly luciferase (FLuc) enzyme that stabilize intracellular FLuc can produce a gain in signal in cell based luciferase reporter assays that may be misconstrued as transcriptional activation, and represent a more restricted example of assay format interference [6,7,12]. Redox cycling compounds (RCCs) generate hydrogen peroxide (H_2O_2) in the presence of strong reducing agents like dithiothreitol (DTT) (Figure 1A) or tris(2-carboxyethyl)phosphine (TCEP), common buffer components used to maintain the catalytic activity and/or folding of target proteins for high throughput screening (HTS) assays [8–10,13,14]. H_2O_2 generated by RCCs in HTS assay buffers containing DTT/TCEP can indirectly inhibit the catalytic activity of proteins by oxidizing accessible cysteine, tryptophan, methionine, histidine or selenocysteine residues, and indeed several important classes of protein targets are susceptible to H_2O_2 -mediated inactivation; protein tyrosine phosphatases (PTPs), cysteine proteases (cathepsins and caspases), and metalloenzymes (Figure 1A) [8–10,13,14]. The current review focuses on RCCs as nuisance/pan assay interference compounds with the potential to annotate screening databases with promiscuous bioactivity profiles (Figure 1B) [6,8–10].

False Positive Hits due to Redox Cycling Compounds

A HTS of > 700,000 compounds to identify inhibitors of caspase-8 produced a high hit rate (~1%), and it was found that 85% of the inhibitors from the initial set of 20,000 compounds were RCCs that inhibited the enzyme through the generation of H_2O_2 in the presence of DTT [14]. 97% of the actives in a HTS campaign to identify activators of glucokinase (GK) activity were found to be nuisance RCCs that interfered with the resazurin coupled assay format independently of the GK enzyme [9]. A retrospective analysis of screening data revealed that the pyrimidotriazinedione hits identified in a Cathepsin L cysteine protease inhibitor screen (structurally related to the RCC compound presented in Figure 1A) had been promiscuously active in many historical HTS campaigns, predominantly those conducted against cysteine proteases, metalloenzymes, and other drug targets with an active site cysteine [9]. Furthermore 2.3% of a 10,000 compound library that was used to validate the performance of HTS assays prior to conducting a full diversity screen were found to be redox active at 10 μ M [9]. In a HTS to identify inhibitors of the dual-specificity phosphatase mitogen activated protein kinase phosphatase-1 (MKP-1), 10.4% of the concentration dependent MKP-1 inhibitors were found to be RCCs [8,15], and in another HTS to identify inhibitors of the dual-specificity phosphatase cell division cycle 25B (Cdc25B), 55% of the concentration dependent Cdc25B inhibitors were shown to be RCCs [16]. The thirty seven (0.02%) RCCs that were identified in the profiling of ~200,000 compounds from the of the NIH's Molecular Library Screening Center Network (MLSCN) compound library exhibited a significantly higher number of active flags and "confirmed" active flags in the PubChem database compared to non-RCCs (p-value < 0.01) [10]. Interestingly, the majority (91.9%) of the RCC's had not been screened against any of the well known oxidation-sensitive targets in the database, suggesting that more targets may be susceptible to oxidation than has previously been recognized [10]. RCCs can therefore have serious negative consequences for the probe and/or lead generation process: primary HTS assay hit rates may be inflated by RCC false positives; critical resources will be diverted to develop and implement follow up assays to distinguish RCCs from real hits; and screening databases will become annotated with the promiscuous activity of RCCs [8–10].

Functional Characteristics of Redox Cycling Compounds

Several hallmark characteristics have been utilized to distinguish the behavior of RCCs from hit compounds that modulate target activities directly [8,13,14,16–18]. The degree of inhibition of the target protein activity by the RCC increases over time, consistent with the time dependent redox cycling generation of H_2O_2 . The inhibition of the target protein activity by the RCC can be abolished by the addition of catalase (CAT) to the assay to degrade any H_2O_2 produced. RCCs can inhibit target protein activity in buffers containing strong reducing agents like DTT or TCEP, but not in buffers containing weaker reducing agents such as glutathione (GSH), cysteine (Cys) or β -mercaptoethanol (BME). The inhibition of the target protein activity by the RCC depends on the concentrations of both the compound and the reducing reagent. For example, the potency and efficacy of sixteen RCC Cdc25B inhibitors are significantly reduced when the conditions of the Cdc25B assay are modified: if the strong non-physiological reducing agent DTT is replaced with the weaker BME or by the physiologically relevant GSH; if CAT is added to the assay; if the DTT concentration is increased from 1 to 25 mM; or in reactions conducted in the presence of both CAT and high levels of DTT [16].

Non-enzymatic Redox Cycling Generation of H_2O_2

A reaction scheme for the non-enzymatic generation of H_2O_2 by redox cycling between a quinone RCC and DTT in aqueous solutions containing oxygen has been proposed (Figure 2) [8,10,13]. (1) DTT reacts with the quinone RCC to form dihydroxydithiane (ox-DTT) and a hydroquinone. (2) When the hydroquinone and quinone RCC are present together they undergo a synproportionation to form a transient semiquinone radical anion species ($\text{RCC}^{\cdot-}$) and 2H^+ . (3) The semiquinone radical anion reacts with O_2 to form superoxide anion ($\text{O}_2^{\cdot-}$) and regenerate the quinone RCC. (4) In neutral to acidic pH DTT may also react with O_2 in aqueous buffers to generate $\text{O}_2^{\cdot-}$ and ox-DTT [19]. (5) $\text{O}_2^{\cdot-}$ produced in reactions 3 and 4 can oxidize a hydroquinone resulting in the production of H_2O_2 and the regeneration of the $\text{RCC}^{\cdot-}$ semiquinone radical anion. In aqueous buffers containing a strong reducing agent and molecular oxygen, quinone RCCs cycle between the quinone, hydroquinone and semiquinone radical anion species to generate the reactive oxygen species $\text{O}_2^{\cdot-}$ and H_2O_2 .

H_2O_2 and Signal Transduction

Of all the reactive oxygen species produced by cells, H_2O_2 best meets the criteria of an intracellular second messenger involved in the transduction of external signals [20–23]. H_2O_2 is a small molecule that diffuses rapidly, can cross membranes, and is rapidly synthesized and destroyed in cells in response to external stimuli [20–24]. Many cell types generate low levels of H_2O_2 in response to a variety of extracellular stimuli including; cytokines (TNF- α , IL-1, GM-CSF, IL-3, IFN- γ , & TGF- β 1), growth factors (PDGF, EGF, bFGF, & insulin) and GPCR agonists (angiotensin II, thrombin, thyrotrophin, parathyroid hormone, lysophosphatidic acid, sphingosine-1-phosphate, serotonin, acetyl choline, platelet activating factor, and bradykinin) [20,22,23].

The NADPH oxidase (Nox) family of proteins are membrane associated multi-unit enzymes that catalyze the reduction of oxygen using NADPH to form the superoxide anion radical ($\text{O}_2^{\cdot-}$) that is in turn dismutated to H_2O_2 by cellular SOD enzymes [20–23]. Five Nox family members (Nox 1-5) and two dual oxidase enzymes (Duox 1-2) are expressed in many cell types and tissues [20]. Stimuli that activate H_2O_2 production in cells may promote Nox-complex formation with regulatory subunits by altering the activity or expression of Nox enzymes and their regulatory subunits, or by increasing intracellular Ca^{2+} concentrations [20]. Nox proteins are targeted to different subcellular localizations, and signaling specificity may be achieved by the coupling of an external stimulus to H_2O_2 production by a

specific Nox homologue in a distinct cellular location [20]. H_2O_2 derived from Nox enzymes can react with cellular proteins to alter their activity, localization and half-life [20–23].

The addition of exogenous H_2O_2 or its intracellular production in response to external stimuli modulates (activates or inhibits) the activity of a variety of proteins including protein kinases, protein phosphatases, transcription factors, phospholipases, ion channels and G proteins [20,22,23]. The removal of H_2O_2 in cells is mediated predominantly by CAT, glutathione peroxidase (GPx) and peroxiredoxins (Prxs) [20,22,23]. CAT is localized exclusively in peroxisomes, the major isoform of GPx is located in the cytosol and mitochondria, while the six Prx isoforms are distributed throughout the cytosol, mitochondria and endoplasmic reticulum [22,23]. H_2O_2 is a relatively mild oxidant that can oxidize the cysteine residues of proteins to cysteine sulfenic acid (Cys-SOH) or to form disulfide (-S-S-) bonds that can be reduced back to cysteine by cellular thiol donors such as GSH, glutaredoxin (Grx) and thioredoxin (Trx) [21–23]. The pK_a of sulfhydryl groups of most cysteine residues (Cys-SH) in proteins is ~ 8.5 and they do not react at physiologically significant rates with H_2O_2 unless the reaction is catalyzed [21–23]. The cysteine thiolate anion (Cys-S⁻) is much more readily oxidized by H_2O_2 and for some signaling proteins, most notably the PTP's, the active site cysteine is preferentially in a thiolate form [21–23]. The 107 PTPs found in the human genome are defined by the active site sequence C(X)₅R(S/T), with X being any amino acid, and they are critical regulators of mammalian cell proliferation, differentiation, and apoptosis [21–23,25]. The active site cysteine of PTPs is required for catalytic activity and performs a nucleophilic attack on the phosphotyrosine residues of the substrate to form a covalent thiol-phosphate intermediate followed by hydrolysis and the release of the phosphate [21–23,26–31]. Due to the unique environment and the invariant arginine, PTP active site cysteines have an unusually low pK_a of 4.7–5.4 and exist as thiolate anions at neutral pH, which enhances catalytic function as a nucleophile but also increases susceptibility to inactivation by H_2O_2 [21–23,26–31]. The PTP cysteine thiolate reacts with H_2O_2 to yield the sulfenic acid of cysteine which renders the PTP enzyme inactive against phosphorylated substrates, but can be reduced back to the active thiolate species by cellular thiol donors such as GSH, Grx, and Trx [25]. Sulfenic acids are highly reactive and readily undergo further oxidation to sulfinic and sulfonic forms, which are irreversibly oxidized and inactive [26–31]. The specific and reversible oxidation and inactivation of PTPs by H_2O_2 has now been demonstrated for numerous PTP targets; PTP1B, PTP α , LAR, VHR, PTEN, SHP-2, Cdc25B, MKP-1, and MKP-3 [8,13,16,17,30–32]. Other proteins with critical cysteine residues in the thiolate form that are susceptible to oxidation by H_2O_2 include AP1 and NF κ B transcription factors, caspases and some protein kinase C isoforms [23,27,29,33,34]. The protein serine-threonine phosphatases are metalloenzymes regulated by specific regulatory subunits, but some family members (PP2B, PP1 & PP2A) may be inactivated by the H_2O_2 mediated formation of disulfide bonds between the redox-sensitive cysteine residues of these proteins [29]. A number of protein serine-threonine kinases and protein tyrosine kinases have been also shown to be activated by H_2O_2 ; EGF-receptor, MAPK, c-Ret, Abl, Src and LCK [23,27,29,33,34].

HTS Assays to Identify Redox Cycling Compounds

Until recently, the process to identify and eliminate RCCs from HTS hit lists involved the development and implementation of multiple counter screens and secondary assays: (i) performing a detailed enzyme kinetic analysis to verify the time- and concentration-dependent inhibition of target activity by RCCs in the presence of DTT or TCEP; (ii) testing whether CAT abolishes the target inhibition by RCCs in DTT or TCEP; (iii) investigating RCC inhibition in the presence of weaker reducing agents such as GSH, BME or Cys; (iv) measuring the UV/Vis spectra of the RCC +/- the reducing agent to determine if the RCC is

reduced in a time-dependent manner; and (v) liquid chromatography and mass spectrometry analysis to confirm the oxidation of active site cysteines in target proteins [8,9,13,14,16,17]. Such detailed analyses consume critical resources and significantly lengthen the timelines for hit characterization and lead selection. In an attempt to mitigate the serious impact of RCCs on probe and lead generation, two groups have independently developed assays to identify RCCs (Figure 3) [8–10].

Lor et al. [9] describe a surrogate assay using the conversion of resazurin to resorufin to detect small molecule redox activity. In a coupled enzyme assay format to screen for glucokinase (GK) inhibitors, 97% of the primary screening actives generated the resorufin product in a process that was independent of GK activity and required only compound, resazurin and DTT [9]. These observations led to the development of a surrogate assay to identify redox active compounds; 100 nL of compounds are added to the wells of black low volume plates to which is added 10 μ L of a mixture containing 5 μ M resazurin and 50 μ M DTT in 50 mM HEPES, 50 mM NaCl at pH 7.5, and after a 60 minute incubation at ambient temperature the fluorescent intensity of resorufin (Ex 560 nm. Em 590 nm) is captured (Figure 3A). Although the homogeneous mix and read format and robust fluorescent assay signal window are attractive features of the resazurin surrogate assay, there are inconsistencies between the performance of the assay and the behavior of RCCs [8,13,14,16–18]. A pyrimidotriazinedione RCC (5 μ M) generated $O_2^{\cdot-}$ in the presence of DTT (10 μ M) that was readily detected in a cytochrome C assay and was prevented by the inclusion of SOD to scavenge the $O_2^{\cdot-}$ and convert it to H_2O_2 [9]. However the addition of SOD and/or CAT to the resazurin surrogate assay reaction does not prevent the generation of resorufin by RCCs in DTT, indicating that the reaction is not mediated by either $O_2^{\cdot-}$ or H_2O_2 [9]. Also, the amount of resorufin generated is limited by the amount of resazurin and DTT, but not the compound concentration. It was proposed that the compound is not a substrate but behaves as a catalyst facilitating the 2e⁻ reduction of resazurin by DTT [9]. There was also no indication whether weak reducing agents such as GSH or Cys can substitute for DTT in the surrogate assay. Despite these inconsistencies the surrogate assay successfully identified previously described RCCs and several other structural classes of RCCs in the hits from historical HTS campaigns conducted at GlaxoSmithKline (Figure 4) [9]. The reduction of resazurin to resorufin is a popular assay format used to measure cell viability (mammalian cells, bacteria and parasites), and is often used in coupled enzyme assays to measure the activity of target enzymes that reduce NAD to NADH [9,35,36]. During the initial planning and design phase of an HTS campaign it therefore might be advisable to consider the potential for RCCs to interfere with and greatly inflate the hit rates of screening assays that use a resazurin-resorufin format.

Recently, a different group reported the development and optimization of a rapid, economical, 384-well colorimetric HTS compatible assay to measure H_2O_2 generated by RCCs incubated with strong reducing agents (Figure 3B) [8]. The assay is based on the H_2O_2 -dependent horseradish peroxidase (HRP) mediated oxidation of phenol red (PR) that produces a change in its absorbance at 610 nm in alkaline pH, and readily detects H_2O_2 in the 1–100 μ M range [8,10,37]. The assay is performed in 384-well flat bottomed clear polystyrene microtiter plates and involves three liquid transfer steps of 20 μ L each of compounds/controls, DTT, and the HRP-PR detection reagent to give a final assay volume of 60 μ L. Compounds (0.01 to 50 μ M, final), plate controls (100 μ M H_2O_2 or 1 % DMSO), DTT (0.5 to 1.0 mM, final), and the HRP-PR detection reagent (100 μ g/mL phenol red and 60 μ g/mL HRP, final) are all prepared in Hank's balanced salt solution (HBSS). Compounds and DTT are incubated together at ambient temperature for a minimum of 15 min prior to the addition of the HRP-PR detection reagent and after an additional incubation period at ambient temperature, minimally 5 min, the assay is terminated by the addition of 10 μ L of 1N NaOH to all wells and the absorbance of the phenol red is measured at 610 nm on a plate

reader (Figure 3B). For this assay, the redox cycling generation of H_2O_2 depends upon the concentrations of both the RCC and the reducing agent, is abolished by the addition of CAT, and whereas both DTT and TCEP support H_2O_2 generation by RCCs, weaker reducing agents (BME, GSH and Cys) do not [8,10]. The HRP-PR assay has been utilized to identify RCCs in the hits from two PTP inhibitor screens [8,16], to evaluate the redox regulation of Cdc25B by *para*-quinone-based inhibitors[18], and to profile ~ 200,000 compounds from the LOPAC and NIH MLSCN compound libraries for RCC activity [10]. Although ~ 50% of the RCCs that were identified in the RCC profiling screen are structurally similar to previously described pyrimidotriazinedione, ortho-quinone and para-quinone RCCs, several other RCC pharmacophores were identified (Figure 4).

Activities of Redox Cycling Compounds in Cells

The main sources of H_2O_2 in cells are the Nox enzyme/SOD systems described above, peroxisome metabolism, and the autoxidation of reactive chemicals by enzyme mediated redox cycling [20–23,38–40]. Within the cellular environment and in the presence of molecular oxygen and metal ions, phenols, catechols and hydroquinones are converted to quinones by intracellular monooxygenase and peroxidase enzymes [38]. In cells, quinones undergo enzymatic redox cycling at both the microsomal and mitochondrial sites of electron transport to generate $O_2^{\cdot-}$ that is dismutated to H_2O_2 by cellular SOD enzymes [38–40]. For example, quinones undergo an enzymatic one-electron reduction catalyzed by microsomal NADPH cytochrome P450 reductase to the semiquinone radical, and in the presence of molecular oxygen, the semiquinone radical can transfer an electron to generate $O_2^{\cdot-}$ and ultimately via SOD H_2O_2 [38,40]. Quinones cause toxicity *in vivo* through a variety of mechanisms including; GSH and/or ATP depletion, damage to DNA and/or mitochondria, and by the oxidation or alkylation of critical cellular proteins [18,38]. Quinones are also Michael acceptors that may covalently bind to cellular nucleophiles including GSH, Cys residues on proteins, and amino groups on proteins and DNA [18,38]. It has been proposed that the ability of quinone RCCs to undergo redox cycling and to generate H_2O_2 within cells may be the basis of their widespread cellular activities [13,17,38,40,41]. Given the role of H_2O_2 as a second messenger involved in the regulation of many signaling pathways it is hardly surprising that compounds which can generate intracellular H_2O_2 by enzyme mediated redox cycling would have pleiotropic effects.

Conclusions

To illustrate the complexities and challenges that RCCs represent to the chemical biology approach, a recent case history is discussed [10,41–43]. Seven pyrimidotriazinedione hits, structurally related to the RCCs presented here (Figures 1A and 4) or that have been characterized previously [8–10,14], were identified in an HTS campaign to identify small molecules that disrupt the interaction between the C-terminal peptide of heat shock protein 90 (Hsp90) and the TPR2A domain of the Hsp organizing protein (HOP) (PubChem assay identifiers (AIDs) 595, 632 and 1400) [42,43]. The pyrimidotriazinedione hits all exhibited cytotoxicity against the BT474 human breast cancer cell line [42]. One of the pyrimidotriazinedione hits was shown to bind to TPR2A protein by isothermal titration calorimetry [42]. Although the hsp90 ATPase inhibitor 17-AAG exhibited selective killing for human breast cancer cell lines versus a normal mammary epithelial cell line, the pyrimidotriazinedione hit exhibited equivalent IC_{50} s (~ 1 μ M) against all three cell lines [42]. Unlike 17-AAG which induces hsp70 expression, the pyrimidotriazinedione hit had no apparent effect on hsp70 expression levels [42]. In contrast, treatment with both 17-AAG and the pyrimidotriazinedione hit reduced the levels of HER2 in the human breast cancer cell lines relative to untreated cells [42]. The authors concluded that the pyrimidotriazinedione hits represented a novel class of small molecule hsp90 inhibitors [42].

The TPR2A domain of HOP contains two Cys residues and binding to the C-terminal peptide of Hsp90 is better in the presence of DTT [43]. Both the primary HTS and secondary fluorescence polarization assays to measure Hsp90-TPR2A protein-protein interactions were conducted in the presence of DTT [42,43]. However the potential role of H₂O₂ generated by the pyrimidotriazinedione RCCs in the observed disruption of the Hsp90-TPR2A interaction assays was neither considered nor excluded [10,41]. Given the ability of pyrimidotriazinedione RCCs to generate μ M concentrations of H₂O₂ in buffers containing DTT, the apparent biological promiscuity of this class of compounds, and the role of H₂O₂ as an intracellular messenger [8–10,14–16,20–23], it would seem prudent to consider the redox cycling capabilities of these hits as they are further characterized and their mechanism of action explored.

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Figure 1. Redox Cycling Compounds Generate H_2O_2 in Reducing Environments and Exhibit Promiscuous Bioactivity Profiles in HTS Databases

A) Aqueous solutions containing dithiothreitol (DTT) and oxygen at neutral to acidic pH generate H_2O_2 via a chain reaction, and if compounds capable of redox cycling are added to this environment, micromolar (μM) concentrations of H_2O_2 can be produced. H_2O_2 generated by placing RCCs like 1-ethyl-6-methyl-3-phenyl-1H-pyrimido[5,4-e][1,2,4]triazine-5,7-dione, PubChem substance identifier (SID) 845167, in HTS assay buffers containing DTT can indirectly inhibit the catalytic activity of proteins by oxidizing accessible cysteine, tryptophan, methionine, histidine or selenocysteine residues. H_2O_2 generated by RCCs may also inhibit assays by having adverse effects on cofactor binding, or by disrupting disulfide bonds that contribute to the dimerization and/or folding of proteins.

B) The PubChem database (<http://pubchem.ncbi.nlm.nih.gov/>) was queried using the PubChem SID 845167 for the RCC 1-ethyl-6-methyl-3-phenyl-1H-pyrimido [5,4-e][1,2,4]triazine-5,7-dione. The bioassay activity information is captured in two PubChem data fields, the activity outcome and the activity score. A substance may be flagged active in a bioassay data uploaded to PubChem if it meets the active criterion for the assay that has been designated appropriate by the depositor. A substance may be flagged a “confirmed” active in bioassay data uploaded to PubChem if it exhibits a concentration response that has been designated appropriate by the depositor. RCCs represent nuisance/pan assay interference compounds with the potential to annotate screening databases with promiscuous bioactivity profiles. The RCC SID 845167 has been tested in 541 bioassays and was designated active in 173 (32%) of them. This RCC was confirmed active in 77 concentration response assays including a number of protein targets that are susceptible to H_2O_2 -mediated inactivation; protein tyrosine phosphatases (PTPs), and cysteine proteases (cathepsins and caspases).

Figure 2. Redox Cycling Reaction Scheme for a Quinone RCC in DTT

A reaction scheme for the generation of H₂O₂ by redox cycling between a quinone RCC and DTT in the presence of oxygen is presented. (1) DTT reacts with the quinone RCC to form dihydroxydithiane (ox-DTT) and a hydroquinone. (2) When the hydroquinone and quinone RCC are present together they undergo a synproportionation to form a transient semiquinone radical anion species (RCC^{•-}) and 2H⁺. (3) The semiquinone radical anion reacts with O₂ to form superoxide anion (O₂^{•-}) and regenerate the quinone RCC. (4) In neutral to acidic pH DTT may also react with O₂ in aqueous buffers to generate O₂^{•-} and ox-DTT. (5) O₂^{•-} produced in reactions 3 and 4 can oxidize a hydroquinone resulting in the production of H₂O₂ and the regeneration of the RCC^{•-} semiquinone radical anion. In aqueous buffers

containing a strong reducing agent and molecular oxygen, quinone RCCs cycle between the quinone, hydroquinone and semiquinone radical anion species to generate the reactive oxygen species $O_2^{\cdot-}$ and H_2O_2 .

Figure 3. HTS Assays to Identify Compounds that Redox Cycle in Reducing Environments

A) Surrogate assay using the conversion of resazurin to resorufin to detect small molecule redox activity [9]. 100 nL of compounds are added to the wells of black low volume plates to which is added 10 μ L of a mixture containing 5 μ M resazurin and 50 μ M DTT in 50 mM HEPES, 50 mM NaCl at pH 7.5, and after a 60 minute incubation at ambient temperature the fluorescent intensity of resorufin (Ex 560 nm. Em 590 nm) is captured. **B)** Colorimetric assay to measure H_2O_2 generated by RCCs incubated with strong reducing agents based on the H_2O_2 -dependent horseradish peroxidase (HRP) mediated oxidation of phenol red (PR) that produces a change in its absorbance at 610 nm in alkaline pH [8,10]. The assay is performed in 384-well flat bottomed clear polystyrene microtiter plates and involves three liquid transfer steps of 20 μ L each of compounds/controls, DTT, and the HRP-PR detection reagent to give a final assay volume of 60 μ L. Compounds (0.01 to 50 μ M, final), plate controls (100 μ M H_2O_2 or 1 % DMSO), DTT (0.5 to 1.0 mM, final), and the HRP-PR detection reagent (100 μ g/mL phenol red and 60 μ g/mL HRP, final) are all prepared in Hank's balanced salt solution (HBSS). Compounds and DTT are incubated together at ambient temperature for a minimum of 15 min prior to the addition of the HRP-PR detection reagent and after an additional incubation period at ambient temperature, minimally 5 min, the assay is terminated by the addition of 10 μ L of 1N NaOH to all wells and the absorbance of the phenol red is measured at 610 nm on a plate reader.

Figure 4. Representative Structural Classes of Redox Cycling Compounds

Representative RCCs reported in the literature [8–10,13,14,16–18] were subjected to structure based clustering and classification techniques based on recursive partitioning using the Leadscape Enterprise 2.4.6-1 software as described previously [8,10,15,16].