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Redox Platforms in Cancer Drug Discovery and Development

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Abstract

Redox homeostasis is frequently dysregulated in human disease, particularly cancer. Recent and ongoing efforts seek to validate and extend this platform for the discovery/development of anticancer drugs. As the primary source of cellular redox buffer, thiols (in particular glutathione) have been therapeutically targeted in cancer treatment, myeloproliferation, hematopoietic progenitor cell mobilization and immune response. A number of “redox modulating” drugs have been, or are, under development and the pipeline seems viable. Moreover, S-glutathionylation is a protein post-translational modification that influences a number of critical cell pathways and in the medium term, defining the “glutathionome” has the possibility to provide opportunities for target identification for therapeutic intervention perhaps with a relevance that parallels ongoing efforts with the kinome.

Introduction

As a consequence to the improvement of human health, cancer has become a leading disease for which academic institutions and pharmaceutical industries have allocated much effort in the discovery and development of new drugs. Redox homeostasis is critical in regulating many cellular processes pertinent to cell survival [1,2] and there is growing evidence that it is dysregulated in cancer cells. Moreover, redox balance, particularly involving thiols such as glutathione (GSH) influences aspects of myeloproliferation, hematopoietic progenitor cell mobilization and immune response. Since myelosuppression is a dose limiting toxicity of many cancer drugs, redox chemotherapeutics that enhance hematologic and immune recovery could be useful.

In a biological setting, sulfur is one of the more flexible elements and has been used liberally in organism evolution. The flexible valence state of sulfur can yield a range of biological oxidation states that range from +6 in sulfates to −2 in hydrogen sulfide (H₂S). Glutathione (GSH) is a tripeptide of glutamic acid, cysteine and glycine and is the most prevalent redox buffer and predominant non-protein thiol in biological systems. GSH exists in reduced, oxidized or mixed disulfide forms, where the GSH:GSSG ratio is a critical determinant of

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redox homeostasis. GSH is a cofactor for a number of enzymes including, glutathione S-transferases (GST) and glutathione peroxidases (GPx), but also plays critical roles in metabolism, signal transduction, proliferation and apoptosis [3]. Glutathione-dependent redox signaling may also be mediated through post-translational modification involving covalent binding of GSH to protein cysteine residues (S-glutathionylation). Human diseases of GSH deficiency are associated with immune disorders, accelerated pathogenesis of viral disease and increased incidence of malignancies. Many tumors show elevated levels of GSH emphasizing the link between the dysregulation of GSH homeostasis in the disease.

Since the turn of the century, a renaissance in the importance of soluble gases in regulating cellular functions has occurred. H₂S adds a further variable to the redox platform. It is a weak acid of high aqueous solubility and readily ionized at physiological pH. The latter property impedes membrane transport, but empiric evidence supports a model of passive diffusion across cellular and organelle lipid membranes [4]. Concentrations of H₂S are much lower than those of low molecular weight thiols, (particularly GSH) and its redox potential (+0.17V) is comparatively high [5]. As a consequence, H₂S is unlikely to have a significant antioxidant role in cells. Nevertheless, genetic abnormalities in the enzyme systems that regulate H₂S (cystathionine β-synthase, γ-cystathionase, mercaptopyruvate sulfur transferase) do produce human clinical pathologies, primarily cardiovascular, inflammatory and CNS in nature. Secondary disease associations include liver cancer and neuroblastoma [6]. Interesting associations of H₂S with mammalian hibernation have been linked with reversible inhibition of the enzyme cytochrome c oxidase and the authors have speculated that H₂S may also act as a source of electrons during hibernation when energy from food is low [5]. Clearly the general importance of H₂S is an evolving field, the challenges of which are well described in recent reviews [5,7]*. Targeting H₂S and the pathways associated with its metabolism may prove enterprising.

A review of some redox active agents has recently appeared [8]*, where therapeutic potential is discussed in the context of toxicities and off-target effects. In most instances, improvements in therapeutic index have been the driving force in the selection of redox targets in cancer drug discovery and there are many ongoing efforts in this preclinical arena. One of the earliest approaches used for predictive small molecule drug discovery was affinity fingerprinting, directly leading to a technology designated TRAP (Target-Related Affinity Profiling; [9]**). TRAP took advantage of the principle that all pharmaceutically active molecules work by interacting with proteins, but that certain amino acid motifs possess enabling characteristics for ligand binding [10]. The platform measures binding of a small molecule to a proprietary reference panel of proteins to create a profile for each lead. Subsequently, computational tools were used to search created databases to identify drug candidates. Early efforts with this platform yielded glutathione S-transferase inhibitors. These efforts subsequently morphed into two novel candidate drugs, *Telcyta* and *Telintra*. Since each has progressed into clinical trials, a brief perspective on their development will serve to exemplify the process of using redox approaches in drug discovery [11].

Telintra (Ezatiostat HCl) began as TER199 and is a small molecule peptidomimetic inhibitor of GST P1-1. Although initial development of the agent focused on sensitizing tumors that over-express GSTP to standard anticancer drugs, serendipitous findings made *Telintra* a product candidate as a myelostimulant [12]*. Mechanistically, GSTP1-1 is a key protein in signaling pathways that control c-jun N-terminal kinase (JNK) and *Telintra* can act by interfering with the complex formation between GSTP and JNK [13]**. Myelodysplastic syndrome (MDS) is a form of pre-leukemia in which the bone marrow produces insufficient levels of one or more of the three major blood elements (white blood cells, red blood cells and platelets). Either pharmacological or genetic ablation of GSTP increases white blood cell production in normal animals as well as in animals treated with

cancer drugs. Thus, clinical trials with *Telintra* have focused on Phase I/II studies in MDS patients. *Telintra* treatment results in improvement in all three types of blood cells in patients with all types of MDS, including those in intermediate and high-risk groups [14]. An oral formulation of the drug has been developed and pre-NDA trials continue.

Telcyta (Canfosfamide HCl) started as TER286 and was designed to exploit the high levels of glutathione S-transferase P1-1 (GST P1-1) in many human tumors frequently associated with poor prognosis and resistance to certain drugs [15]. Preclinical studies have shown that GST P1-1 splits the drug into an active tetrakis (chloroethyl) phosphorodiamidate alkylating species and a vinyl sulfone derivative of the glutathione backbone. *Telcyta* has been through a number of Phase II and Phase III clinical trials in advanced cancers. While it showed clinical activity in advanced ovarian, non-small cell lung, colon and breast cancers, a pivotal Phase III trial in platinum resistant ovarian cancer gave negative results. Nevertheless, additional clinical testing is ongoing and will determine the ultimate registration status [16,17]*. It is perhaps worth reflecting that the clinical trials did not incorporate any pharmacogenetic components in their analysis. There is ample evidence that GST isozymes generally, and GSTP in particular, have human polymorphic variants. Although inclusion of correlative biomarkers in clinical trials can be time consuming, expensive and delay NDA submission, it is becoming generally more appreciated that their absence can sometimes adversely influence the outcomes analysis of end points. In the initial *Telcyta* trial there was no prior knowledge of patient GST characteristics. As such, an individual's response (or lack thereof) to drug treatment may have been influenced by an individual's GSTP polymorphic variant expression. For example, the *GSTP1* gene is on chromosome 11, spans ~3kb and encodes 210 amino acids in seven exons. Expression of GSTP1 has been identified in all tissues and cells, except red blood cells. The allele frequencies for the polymorphic variants *GSTP1* *A, *B, and *C in Caucasian populations are 0.685, 0.262 and 0.068, respectively [18]. Homozygosity for *GSTP1**B is favorable in the treatment of patients with cancer because such patients have a diminished capacity to detoxify platinum-based anticancer agents [19].

The S-Glutathionylation Cycle and The Glutathionome

In following the theme of this review section, post-translational modifications of cysteine residues localized in a basic environment (vicinal to lys, arg or his) can have low pKa values [20], making them targets for the addition of glutathione, i.e. can be S-glutathionylated. There is no one method for quantitatively measuring the various redox states of cellular thiols including protein cysteines and mixed disulfides, but a recent review summarizes the best approaches [21]. S-glutathionylation adds 305Da and introduces a net negative charge (as a consequence of the addition of glu) and occurs through a dynamic, reversible cycle. The forward reaction can be catalyzed by GSTP [22]* and the resultant mixed disulfide can protect the protein from oxidative damage, or can effect a change in conformation (and/or charge) that may alter protein function and/or cellular localization. Since many cellular processes rely upon sequential formation and disassembly of protein complexes, critical protein:protein interactions can be significantly influenced by glutathionylation and deglutathionylation. One such example is the interaction of GSTP with JNK, where the complex is subject to disassociation following S-glutathionylation [22]*. As such, critical in ascribing any regulatory function to this process is the reversibility of S-glutathionylation by small molecule cysteine rich proteins such as glutaredoxin, thioredoxin and sulfiredoxin. Relative to the proteome, the actual number of S-glutathionylated proteins is probably not large, likely numbering in the hundreds, although new target proteins appear regularly in the literature. As a consequence, the growing body of modified proteins might best be described as a "Glutathionome." The most prevalent S-glutathionylated protein is actin where the modification alters the ratio of its soluble and polymerized forms. Changes in microfilament

structure/number, membrane ruffling, cellular adhesion, cell:cell interactions and intracellular trafficking are some of the consequences of this modification [23].

There appear to be six general clusters of proteins subject to S-glutathionylation: 1 cytoskeletal constituents; 2 energy metabolism and glycolysis controlling proteins in mitochondria; 3 signaling proteins, particularly kinases and phosphatases; 4 and 5 calcium and redox homeostasis regulating proteins; 6 protein folding and stability controlling proteins. A more thorough analysis of these proteins is included in a recent review [24]**. Since S-glutathionylation has a direct effect upon phosphatases and kinases, perhaps this is an evolutionarily conserved nexus between sulfur and phosphorus biochemistry. Like phosphorylation, cysteine modification is critical to cellular signaling and its deregulation impacts multiple human diseases. At least one direct connection between these signaling pathways is derived from our earlier results showing that Srx reversed S-glutathionylation of phosphatases impacting their catalytic activity [25]. As an admittedly biased question in a redox review, it may be interesting to determine if as potential drug targets, catalytic components of the S-glutathionylation cycle may prove to have value equivalent to kinases and phosphatases. Certainly the latter “kinome” constituents have engendered a high degree of intellectual and fiscal investment over the last decade.

Redox and myeloproliferation

The bone marrow is a relatively hypoxic tissue (1% to 2% oxygen) with both vascular and osteoblastic niches that have oxygen and calcium gradients. The hypoxic osteoblastic niche encourages HSC quiescence, while the more oxygenated vascular niche promotes differentiation into myeloid and lymphoid hematopoietic cells that eventually enter the peripheral blood supply [26]*. Because thiol active agents influence hematopoiesis, the possibility that they may impact a thiol/redox gradient within the marrow matrix (in concert with those of O₂ and Ca²⁺) and influence HSC migration and differentiation would seem to be a testable hypothesis. Moreover, the features of niche microenvironments and their influence on HSC sub-populations may present opportunities for the development of targeted therapeutics. For small molecule thiol/redox active myeloproliferative agents, at least one component of their mechanism of action may involve effects on the expression profiles of cytokines and growth factors. These effects, however, would have to be viewed in context with the pleiotropic impact of many cytokines when placed in a specialized environment [27,28].

Hematopoietic stem cell (HSC) self-renewal and differentiation is dependent upon exogenous cysteine and is influenced by niche microenvironments within the bone cavity [29]. Redox status and the equilibrium of free thiol:disulfide couples are important in modulating immune response and lymphocyte activation, proliferation and differentiation. T cells differentiate into T helper (Th)-1 and Th2, which are characterized by different cytokine production profiles. Thiol balance has been shown to modulate Th1/Th2 lymphokine production by T cells [30]. Antigen presenting cells (APC) are central to the initial Th1 or Th2 mediated immune response patterns. GSH depletion in APC's decreases IL-12 secretion and biases cytokine patterns towards Th2 cell production. N acetyl cysteine (NAC) treatment highlights the involvement of different APCs and cytokines in the outcome of a Th1 or Th2 immune response. Free thiol groups are critical in this redox-mediated lymphokine production [31]. Secreted thioredoxin (Trx) can also act as a potent cytokine that induces IL-12 production and a Th1 mediated immune response.

Transcription factors, such as NF-κB and AP-1, incorporate “redox switches” that are regulated by redox and contribute to control of immune response. Both NF-κB and AP-1 can control IL-2 transcription and T cell proliferation [32,33]. Tavocept and amifostine induce

expression of both NF- κ B and AP-1, indicating a cause/effect relationship with immunomodulation. S-glutathionylation of redox sensitive cysteines provides additional potential to modulate the activity of key transcription factors and signaling pathways. These data suggest the S-glutathionylation induced by agents such as NOV-002 or *Telintra* (Table 1) may be involved in modulating T lymphocyte behavior.

Redox active drug overview

Table 1 lists some of the agents that are in various stages of drug development. While this list is not designed to be complete, it does serve to exemplify some of the issues that currently impact drug discovery relative to a redox platform. Perhaps the simplest redox active agent is n-acetyl cysteine, a modified form of cysteine that crosses cell membranes. It is both a pharmaceutical and a dietary supplement with a primary clinical use as a mucolytic agent in the management of acetaminophen overdose. In addition, NAC has been used in HIV patients to restore GSH levels, prevent NF κ B activation and HIV viral replication and inhibit CD4 D2 mediated viral entry [34]. Some of the other examples listed encompass agents that emanate from the GSH/GST platform. These agents can be classified as cytotoxics – most with active electrophilic centers, small molecule myeloproliferatives and chemoprotectants.

Conclusion

The chemical flexibility of sulfur has imbued biology with a complex series of redundant pathways that are regulatory of many critical cell functions, amongst which signaling and proliferation are quite relevant to cancer. An existing body of work has used these thiol/redox platforms to discover and develop drugs. Some are cytotoxics, designed to kill cancer cells. Others piggyback on the principle that proliferation and differentiation of bone marrow stem/progenitor cells can be altered by pharmaceutical intervention with redox active small molecules. Since many cancer patients have direct or indirect problems with their hematopoiesis, there is a therapeutic opportunity to develop agents that act on bone marrow. The etymology of the term “-omics” stumbles from a Greek beginning through to the present day neologisms where biologists use the suffix to describe some (initially) arcane field of study. In keeping with this theme, post-translational modification of low pK cysteine residues can produce S-glutathionylated proteins. Sooner rather than later this field will be designated “glutathionomics” and the “glutathionome” will be defined as the group of proteins subject to this reversible modification. In light of the structural/functional consequences ascribed to S-glutathionylation and the biological importance of many of the protein clusters, it seems reasonable to suggest that there are, and will be, therapeutically druggable targets waiting for a discovery platform.

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Table 1

Drug or agent	Mechanism of action/target	Status/notes	References
Amifostine	Aminothiols protect against radiation toxicity.	FDA approved, initially for esophageal cancer	[35]*
MESNA/Tavocept	Tavocept is the disulfide of MESNA used with cancer drugs as a thiol protective agent.	Tavocept is in early clinical testing.	[36]
Disulfiram	Thiol disulfide exchange and metal (particularly Cu^{2+}) chelation.	FDA approved as alcohol deterrent, but Cu^{2+} chelation effects have antitumor effects.	[37]
Telcyta	GSTP activated prodrug in ovarian ca, NSCLC and others.	IND in Phase III trial	[38]
Telintra	GSTP inhibitor, small molecule myeloproliferative.	IND in Phase II trial	[14,39]*
PABA/NO	GSTP activated NO releasing cytotoxic prodrug.	Preclinical testing.	[40]
NOV-002	Mimetic of GSSG complex with non- therapeutic concentrations of cisplatin	IND in Phase II/III trial.	[41]
Arsenic trioxide (As_2O_3)	Covalent cross-linking of vicinal thiols.	FDA approved for promyelocytic leukemia.	[42]*
Menadione	Redox cycling center produces semiquinone radical	Similar activity to anthracyclines.	[43]
Artemisinin	Sesquiterpene with reactive electrophilic endoperoxide	Anti-malarial, but analogues have longer half-lives and anticancer activity.	[44]
Organosulfur isothiocyanates	Chemoprotective agents that includes sulforaphane, β -phenyl ethyl isothiocyanate.	Induce Phase II detoxification enzymes through the antioxidant response element.	[45]
PX-12	Irreversible inhibitor of thioredoxin-1.	Preclinical to clinical transition.	[46]
Ethacrynic acid	An example of Michael addition chemistry interface with GSH/GST pathways.	FDA approved as a diuretic, used in Phase I setting in cancer. Dose limiting diuresis terminated the trial.	[47]