Selective cellular probes for mammalian thioredoxin reductase TrxR1: rational design of RX1, a modular 1,2-thiaselenane redox probe

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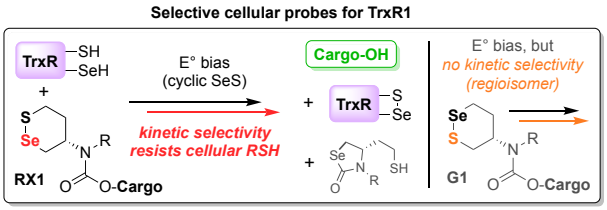
ABSTRACT: Dynamically driven cellular redox networks power a broad range of physiological cellular processes, and additionally are often dysregulated in various pathologies including cancer and inflammatory diseases. Therefore it is vital to be able to image and to respond to the turnover of the key players in redox homeostasis, to understand their physiological dynamics and to target pathological conditions. However, selective modular probes for assessing specific redox enzyme activities in cells are lacking. Here we report the development of cargo-releasing chemical probes that target the mammalian selenoprotein thioredoxin reductase (TrxR) while being fully resistant to thiol reductants in cells, such as the monothiol glutathione (GSH). We used a rationally oriented cyclic selenenylsulfide as a thermodynamically stable and kinetically reversible trigger that matches the chemistry of the unique TrxR active site, and integrated this reducible trigger into modular probes that release arbitrary cargos upon reduction. The probes' redox biochemistry was evaluated over a panel of thiol-type oxidoreductases, particularly showing remarkable, selenocysteine-dependent sensitivity of the "**RX1**" probe design to cytosolic TrxR1, with little response to mitochondrial TrxR2. The probe was cross-validated in cells by TrxR1 knockout, selenium starvation, TrxR1 knock-in, and use of TrxR-selective chemical inhibitors, showing excellent TrxR1-dependent cellular performance. The **RX1** design is therefore a robust, cellularly-validated, modular probe system for mammalian TrxR1. This sets the stage for *in vivo* imaging of TrxR1 activity in health and disease; and the thermodynamic and kinetic considerations behind its selectivity mechanism represent a significant advance towards rationally-designed probes for other key players in redox biology.



1. INTRODUCTION

The thioredoxin reductase (TrxR) - thioredoxin (Trx) system and the glutathione reductase (GR) - glutathione (GSH) - glutaredoxin (Grx) system are the two highly evolutionarily conserved "central nodes" of redox biology, and are of fundamental importance across all eukaryotes (**Fig 1a**).[1,2] They drive and buffer a range of vital biological redox reactions that are crucial to metabolism, protein folding, signaling, protein regulation, and to many aspects of cellular homeostasis and stress responses.[3,4] These systems are driven by reducing equivalents from NADPH that are harvested through the enzymes TrxR and GR, and distributed by downstream effector proteins, mainly isoenzymes of Trx and Grx, into various manifolds of dithiol/disulfide-type reactions. The two major forms of TrxR in mammals are the cytosolic TrxR1 and mitochondrial TrxR2. For all these redox enzymes, both chemocompatibility and protein-substrate binding determine their substrate scopes; these combine with subcellular compartmentalisation of the isoforms and substrates in each cascade to allow sophisticated regulation and spatial organisation of redox reactions in cells.[5]

Due to the fundamental importance of redox networks across biology, developing techniques to monitor and to respond to their dynamics is critical for understanding cellular physiology. Biological approaches to monitor redox biochemistry include genetically engineered, redox-responsive ratiometric fluorescent protein fusions. These are well-established tools for imaging redox *poise* (the balance between reduced and oxidised fusion protein), and include sensors for Grxs and Trxs.[6–8] However, they do not reveal the redox reaction rates these species undergo, which are integral to a network understanding of redox homeostasis. Additionally, since TrxR and GR are NADPH-driven enzymes, they are not suited to ratiometric monitoring of their redox poise. Measuring mRNA or protein expression levels is also insufficient to understand cellular redox systems, since reaction rates through the networks are dynamically controlled on multiple levels e.g. by protein binding partners, post-translational modifications, subcellular localisation, and throttling flow from upstream reductants (or to downstream electron acceptors). Instead, molecular probes that could selectively report on the activity of individual redox-active proteins within the TrxR/Trx and GR/GSH/Grx networks, would be ideal tools for studying redox biology. However, no such probes currently exist.

Here, we aimed to develop TrxR-selective molecular probes, to reveal the activity of this key reducing enzyme *in the cellular setting*. We focused on chemical designs to monitor enzymatic turnover by irreversible accumulation of signal, in the form of an activated probe. We additionally required the probes to be *modular*, i.e. the same approach should be applicable to activating arbitrary cargos, including drugs. This would be of particular interest since redox dysregulation, including upregulation of TrxR activity and expression, is correlated to disease progression, severity, and resistance to conventional therapeutics, in a range of pathologies. These include nearly all solid tumors (hypoxia-induced gene expression shifts), as well as auto-immune and inflammatory conditions (redox signaling and microenvironment effects).[9] Developing modular strategies to activate both imaging agents (for diverse diagnostic modalities) as well as drugs (for redox-targeted treatment of pathologies) could therefore be a powerful approach not just to understand, but also to actively respond to cellular redox activity.

The challenge for selective small-molecule redox probes is to distinguish between similar chemistries of the dithiol/disulfide-type proteins. The TrxR and GR systems are the major mammalian dithiol/disulfide reductants (**Fig 1a**) and have been well reviewed.[5] In brief: (i) TrxRs and GRs are rather low-expression enzymes (ca. 20 nM cellular concentration) that harvest electrons from NADPH to dithiol/disulfide active sites (CVNVGC motif).[10] GR has high specificity for glutathione disulfide (GSSG) and little activity with other substrates. Mammalian TrxR has a broader substrate scope than GR. This is due to its additional C-terminal selenolthiol/selenenylsulfide active site (CU motif) located on an exposed flexible tail, that relays electrons from the NADPH-driven dithiol site to its substrates. Its native substrates include Trxs and TRP14; several small molecules can also be reduced (**Fig 1b**).[11,12] The rare selenolthiol of TrxR endows it with distinctive redox properties compared to dithiols, including enhanced reaction kinetics, lowered reduction potential, and a resistance to permanent oxidation, i.e. loss of function.[13,14] (ii) Trxs and dithiol Grxs are proteins with exposed dithiol/disulfide redox-active sites (CxxC motif) which reduce a broad scope of protein substrates, and they have relatively high expression levels (up to ca. 10 μM). Trxs have lower reduction potentials and are reduced by TrxR, while Grxs have higher reduction potentials and use monothiol GSH (ca. 2-5 mM cytosolic concentration) for recovery via a net trimolecular reaction.[15]

Note: the fascinating (bio)chemistries of selenium, selenenylsulfides, and TrxR have inspired much devotion,[16] with e.g. the computational studies of Bachrach,[17,18] practical investigations of Iwaoka,[19,20] and biochemical and mechanistic analyses of Holmgren,[21] Hondal,[22] and more. The interested reader is referred to these works for much useful information that cannot be given here.

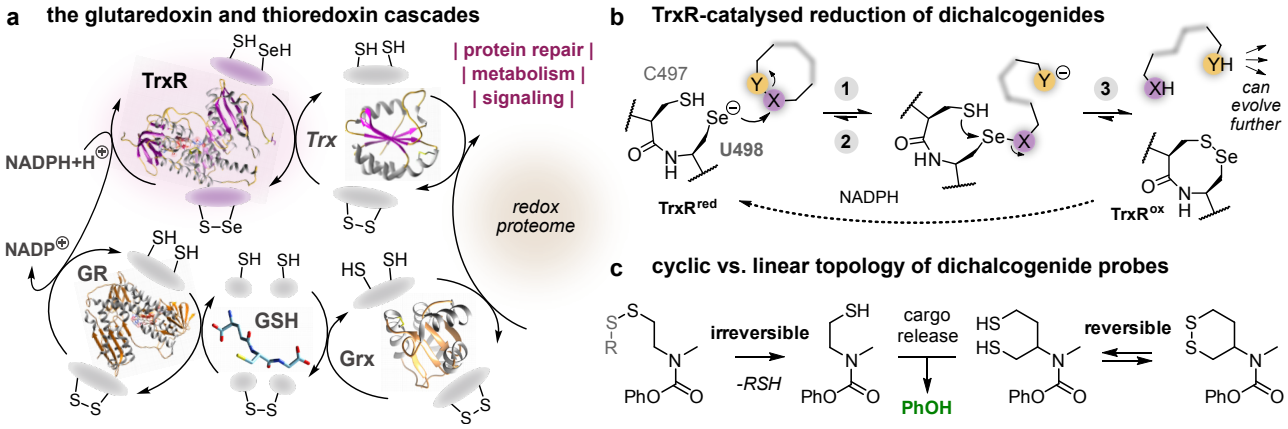


Figure 1 General mechanistic considerations for TrxR-selective probes: (a) The central dithiol/disulfide-type redox cascades of cell biology. **(b)** Reaction mechanism for TrxR reducing a generalised dichalcogenide XY, using its C-terminal Sec-Cys active site. Initial *exchange* (1) gives an intermediate that can either evolve backwards by reforming the dichalcogenide (2) or else evolve forwards by full *reduction* (3) to the dichalcogenol. Recovery of TrxR by NADPH makes this a non-equilibrium process. **(c)** *Topology considerations:* linear topology dichalcogenides (shown: disulfides) are irreversibly committed to cargo release after monothiol exchange, whereas for cyclic topology dichalcogenides, the exchange / reduction pathways is reversible: for full mechanism, see **Fig S3**.

As disulfides are their native substrates, chemocompatible disulfide-based probes have long been explored for their ability to report on these redox-active enzyme systems. Disulfide trigger-cargo constructs[23] are a conceptually simple, modular turn-on design. Ideally, the cargo is chosen and attached to mask a key structural element, such that (i) the intact probe is fully deactivated, but (ii) trigger reduction causes a cascade that irreversibly restores activity by unmasking, often by simply liberating the cargo (**Fig S1c**). We have reviewed the state of the art in disulfide trigger-cargo probes elsewhere.[23] The most important outcome for probe design is that both disulfide topology (linear or cyclic), as well as geometry (strained or stabilised), determine the probe's sensitivity to monothiols.[23] Monothiols are highly concentrated in cells, with 1-8 mM GSH, and an even larger pool of protein thiols (PrSH).[24,25] To report selectively on redox-active dithiol/disulfide proteins, probes must resist attack by this monothiol background. However, any thiol/disulfide exchange reaction upon linear-topology disulfides irreversibly commits a probe to cargo release (**Fig 1c**, **Fig S3a**). This makes linear topology probes nonspecifically labile to cellular monothiols,[26–28] and therefore prevents them from being enzyme-selective reporters in the cellular context.[29] Thus, although some reports have used linear disulfide probes (**Fig S1a**), we believed that selective reporters would require a different design.[23]

Cyclic-topology disulfide probes can, however, resist triggering by monothiols in two ways. They can reform the disulfide after the initial thiol-disulfide exchange by expelling the attacking monothiol, or after reduction to the dithiol they can be re-oxidised by other

disulfides in their environment: both of which prevent them from committing to cargo release (**Fig S3b**; full discussion in ref. [29]). The geometry of the cyclic disulfide is critical for its tendency to perform these reactions, and therefore determines the cellular performance of cyclic-disulfide-based probes. Strained cyclic disulfides were well characterised by Whitesides in the 1990s.[30–32] They are kinetically labile and are rapidly and nonspecifically opened by monothiols, and opening is irreversible since the disulfide strain disfavours reclosure. This means that in the cellular context, they cannot be selective for dithiol proteins. This rules out cyclic 7-membered,[23] ETP-type-6-membered,[33] and cyclic 5-membered disulfides[29,34] as substrates that can be cellularly selective for distinct dithiol-based enzymes. Although probes based on cyclic 5-membered disulfides have been published and commercialised as TrxR-selective (**Fig S1a**),[35–37] the unselectivity and kinetic problems of such disulfides have been shown historically as well as recently (**Fig S1b,c**).[22,29,34,38–40] Alicyclic 6-membered disulfides (1,2-dithianes), however, are stable against monothiols. We recently characterised monocyclic and annelated bicyclic 1,2-dithianes for their performance as reductive triggers, showing that they resist GSH[28] as well as TrxR[41], and can be harnessed in trigger-cargo constructs that are selective for Trx (**Fig S1c**) (see **Supporting Note 1**).[23]

Here, we wished to leverage these design principles to now develop robust, modular probes that could resist the cellular monothiol background and would instead monitor TrxR1 activity in live cells. For achieving TrxR1 selectivity, we analysed probe activation pathways in detail. A combination of thermodynamic, kinetic and mechanistic considerations oriented us to target novel cyclic 6-membered selenenylsulfide reduction triggers. We developed scalable syntheses of a panel of cyclic dichalcogenides and mechanistic controls, and using a modular design, we applied them to create a palette of cumulative-release fluorogenic probes. We evaluated these across several redox-active enzymes in cell-free assays, highlighting their remarkable TrxR1-sensitivity and -selectivity. Finally, a range of cellular assays including genetic knockout, knock-in, chemical inhibition, and selenium depletion studies, confirmed the high cellular specificity of the selenenylsulfide probes for reporting on TrxR1. One trigger structure in particular offered consistently excellent rapidity and homogeneity of TrxR1-dependent response in the cellular context. We name this probe **RX1**, for TrxR1-redox-probe 1. This work highlights **RX1** as a flexible basis for further development of probes and prodrugs responding to TrxR1. Furthermore, the mechanistic analyses behind its development can light a path to other key redox-active enzymes and proteins in urgent need of probes and prodrugs.

2. RESULTS

2.1 Design of a TrxR-selective dichalcogenide trigger. We wished to design non-disulfide dichalcogenide triggers which would react selectively with the unique selenolthiol motif found in TrxR, and which would give rapid cargo release even with nM TrxR concentrations, without releasing cargo due to the action of other cellular reductants: whether μM vicinal dithiols or mM monothiols.

Since we were designing irreversible-release probes, we first considered kinetic aspects on the path to cargo release. We consider that the same topology restrictions apply as in the disulfide series,[23] i.e. that linear dichalcogenides are likely to be nonselective due to irreversibility of triggering under monothiol challenge. We note that two linear (non-disulfide) dichalcogenide-trigger probes have been published as selective (**Fig S1a**);[42,43] but according to our analysis, selectivity in the cellular context was not shown (see **Supporting Note 2**). We instead proceeded with cyclic-topology designs. We also considered that the same geometric factors would apply to dichalcogenides as for disulfides, thus ruling out strained designs from being cellularly selective. In support of this view, Matile has shown the lability of cyclic 5-membered diselenide probes[39] to nonspecific thiol exchange, mirroring that of the corresponding disulfides (**Fig S1b**).

We therefore expected that only cyclic 6-membered dichalcogenides would be stable enough to avoid irreversible triggering in cells. Selecting them is only a partial solution, as this only addresses the overall challenge of ensuring that monothiol attack has the potential to be reversible (discussion in[23]). As highly reducing dithiol proteins Trx and Grx are present at ca. 10 μM concentrations, and GSH at up to 8 mM, the next challenge is to ensure that only the ca. 20 nM vicinal selenolthiol enzyme TrxR can efficiently catalyse cargo release, despite the presence of these and other redox-active species. This challenge cannot be met with thermodynamics alone (e.g., Trx-reducible 1,2-dithianes are reduced only slowly by even 10 μM Trx, while kinetically resisting the more powerful reductant TrxR[23]) but requires specificity either in binding or in the reductive mechanism.

The following analysis of reductive release pathways then convinced us that probes based on a desymmetrised cyclic selenenylsulfide trigger **A** (**Fig 2a**) should represent a unique TrxR-selective solution to all these problems (see also **Fig S4** and **Supporting Note 3**); and it also suggested that the thermodynamically identical regioisomer **G** (**Fig 2b**) ought not be TrxR-selective, so suggesting a highly stringent control of this postulated specificity mechanism:

(1): Selenenylsulfides should be rapidly attacked at Se by TrxR selenolate: Literature indicates that rates of attack upon the Se of a selenenylsulfide should be orders of magnitude faster for selenolates, than for thiolates (k_{R1} vs k_{T1}; though, there are no data for cyclic 6-membered systems); and also emphasises that attack at Se will be fast in an absolute sense. This could indicate good initial reaction rates between TrxR and a cyclic selenenylsulfide, compared to the TrxR-inert 1,2-dithianes: (i) In the linear series for which data are reported, selenenylsulfides are ca. 10^4 times as electrophilic to selenolate attack at Se as disulfides are at S.[44] (ii) In general, selenolates are ca. 100 times as nucleophilic as thiolates.[44] (iii) Selenols are also 2-3 pKa units more acidic than thiols,[39] and the acidity of RSeH acidity in TrxR is still greater[16], which enhances the general reactivity of the TrxR selenolate as compared to thiol species, which are more likely to be protonated. Importantly too, (iv) nucleophilic attack on the selenenylsulfide at Se is kinetically (and thermodynamically, see below) much more favoured than at S,[18] which should fix the site of initial attack by any chalcogenide at the selenium.

(2): A selenenylsulfide's exchange intermediate should have the attacking nucleophile bound to Se, not to S: The thermodynamic preference for exchange intermediates to be Se-bound rather than S-bound is strong, regardless of what the attacking nucleophile is. For attack by thiolates, a linear selenenylsulfide intermediate will have ca. 70 mV lower E° than the alternative disulfide (ca. -280 mV vs -210 mV),[45] favouring e.g. **D** rather than **F** in **Fig S4**. For attack by selenolates, linear diselenides are typically reported to have ca. -480 mV, although proximal *N*-acylation as in our design lowers this to -550 mV[16], therefore being ca. -270 mV lower than the alternaitve selenenylsulfide (e.g. **B** rather than **E** in **Fig S4**). These values can also be compared to the ca. -364 mV expected for the intact cyclic selenenylsulfide triggers on the basis of Iwaoka's elegant study of cyclic dichalcogenides (**Fig S1d**), which is predictably more stable

than the corresponding cyclic disulfides (ca. -317 mV).[19] Crucially, it is also known for symmetrical cyclic dichalcogenides that initial kinetic products can rearrange intramolecularly within milliseconds to form the thermodynamic products.[39] Taken together, this implies that: (i) the kinetically-favoured attack at Se gives exchange intermediates that are also the strongly favoured thermodynamic intermediates; and that the Se preference is particularly strong for TrxR selenolate attack. Thus, the only intermediates relevant to probe evolution are **B**, **D**, **H**, and **K** - which simplifies the pathways to cargo release (compare **Fig 3a-b** to **Fig S4**). (ii) The much greater stability of linear diselenide intermediates **B** and **H** (from TrxR selenol attack; -550 mV) might much better offset the energetic penalty of opening the cyclic selenenylsulfide (-364 mV), than linear selenenylsulfide intermediates **D** and **K** can (ca. -280 mV); this could greatly favour the fast generation and evolution of the TrxR intermediates **B**/**H**, rather than undesired thiol intermediates **D**/**K**.

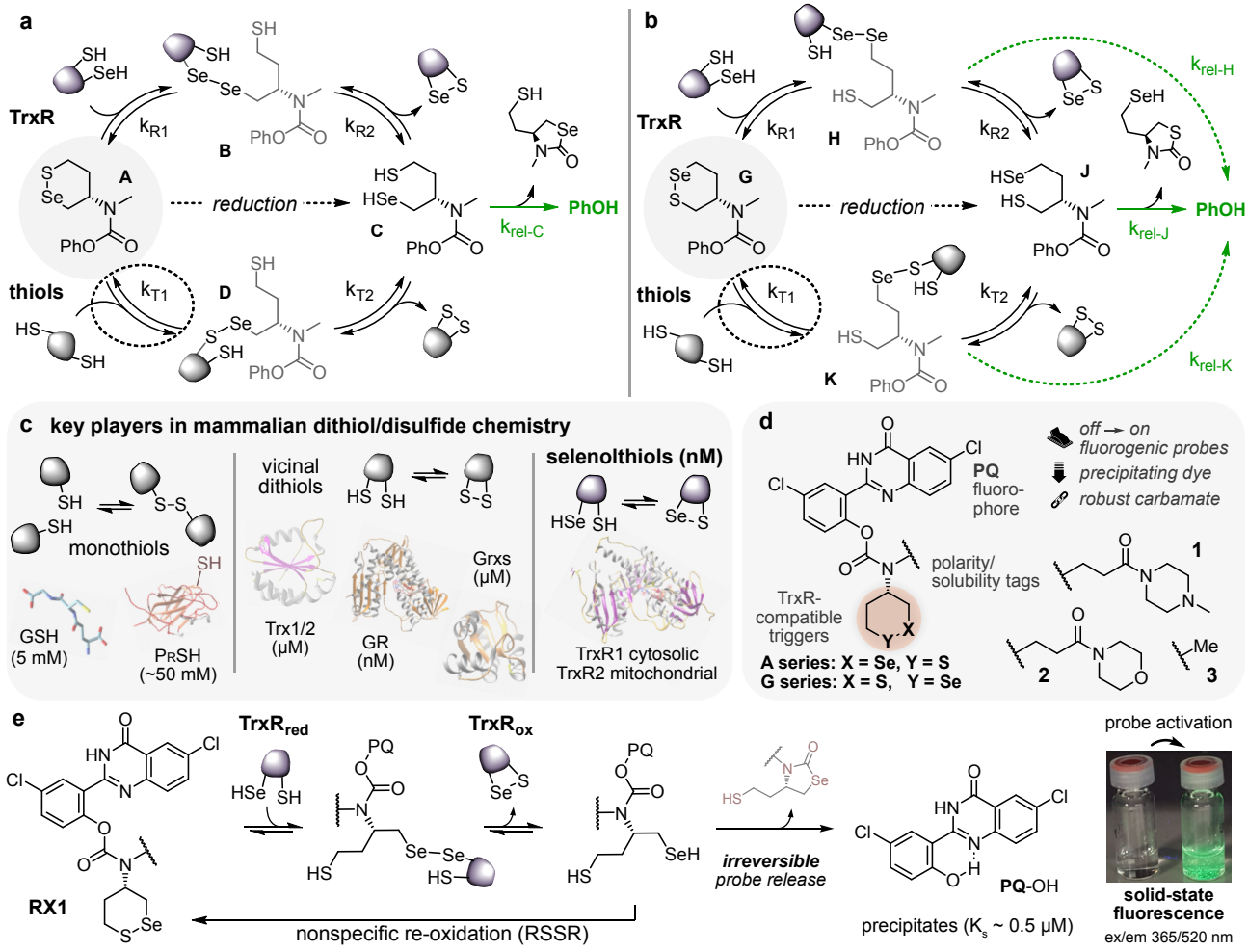


Figure 2 Despite identical thermodynamics, a specific 6-membered cyclic selenenylsulfide is proposed for TrxR-selectivity: (a-b) Probes of type **A** (includes **RX1**) are predicted to avoid thiol-based signal generation, whereas regioisomeric control probes of type **G** are predicted to be thiol-labile (full mechanism in **Fig S4**). **(c)** Overview of chemocompatible cellular reductants that a TrxR-selective probe must resist. **(d)** Modular trigger-cargo design for fluorogenic probes with flexibly-chosen polarity. **(e)** Signal activation pathway for **RX1**.

*(3): **A** should avoid cargo release before reduction, and so have monothiol resistance*: We anticipate that initial thiol-exchange of **A** to **D** is unavoidable due to the high cellular concentration of monothiols (**Fig 2c**), but also that the exchange reaction to **D** (or **B**) does not contribute directly to cargo release because the 6-*exo*-trig cyclisation of the thiolate is a relatively slow process: so **A** will only release its cargo if reduced to **C**. In contrast, thiol-exchange of **G** to **K** (or **H**) allows the thiolate to cyclise by rapid 5-*exo*-trig, without requiring full reduction to **J**. We call this "on-reductant release" ($\mathbf{k_{rel\text{-}K}}$, $\mathbf{k_{rel\text{-}H}}$). Therefore, *although the thermodynamics and kinetics of overall reduction of the trigger motifs of **A** and **G** are likely to be almost indistinguishable*, we expect that probes of type **G** will suffer significantly more unwanted cargo release from unavoidable initial exchange reactions with the monothiol background. (Though **H** can also give on-protein release $k_{rel\text{-}H}$, TrxR is more likely to fully reduce the probe than monothiols are, so we would expect the $k_{rel\text{-}H}$ pathway to play little role in TrxR-based cargo release: this is revisited in section 2.4).

(4): Cargo release after reduction: Reduced **C** or **J** are, after all, small flexible mimics of the reduced TrxR active site[19], and they can reduce any accessible disulfide in their environment (k_{T2}) instead of cyclising to release cargo. However, as **C** is likely to cyclise faster than **J** (see 1: its selenolate should be a superior nucleophile), we anticipated that **A** would also be the more competent design for signal generation after full reduction.

Taken together, these four aspects informed our hypotheses that probes of type **A** (which includes the **RX1** probe) will be: (1-2) rapidly attacked by TrxR, (2) much more efficiently reduced through to

C by TrxR than by thiols, (3) resistant to on-reductant signal generation by (mono)thiols, and (4) rapidly cyclise after reduction to **C**. In contrast, though probes of type **G** will share features (1-2), they will be (3) labile to thiols, and (4) slower to report on TrxR than the **A**-type. We then set out to test whether these considerations would indeed enable us to reach TrxR-selectivity using **A**-type probes.

2.2 Probe design. We designed selenenylsulfide-based probes that use carbamate cyclisation to release phenolic cargos (**Fig 2d**). This is a modular system that can be applied to any phenol-type cargos, including drugs. It offers a range of performance features that we have reviewed elsewhere.[23,46] Important aspects of this phenolic carbamate system are: (i) the phenolate is a good leaving-group, resulting in fast cargo release that is orders of magnitude faster[23] than the aniline carbamates that are more often[41] used. (ii) Though primary amine phenolic carbamates are so unstable due to E_{1cB} elimination that they are usually discarded[41] (halflife ~seconds[47]), secondary amine carbamates avoid this mechanism, so such probes are entirely robust to spontaneous hydrolysis (only ca. 1% in 3 weeks).[46] (iii) Masking the hydroxyl of many phenolic systems completely blocks their activities; so this system allows true off-to-on performance (zero activity before cargo release) with a range of cargos. For example, phenol *O*-unmasking is required for activation of masked fluoresceins,[23] indigo-type chromophores,[48] luciferins,[49] and bioactivity in many series of drugs (irinotecan[50], duocarmycin[51]). Thus, it should be possible to extend this work's modular design to probes with diverse imaging modalities, and to many prodrug types.

We selected Haugland's precipitating fluorophore **PQ-OH** (**Fig 2d-e**)[52] as our proof-of-concept fluorogenic phenolic cargo. Acylating its phenol completely mechanistically quenches its high-quantum-yield, environment-independent, large-Stokes-shift fluorescence (ex/em 360/520 nm), since this depends on excited state intramolecular proton transfer (ESIPT) of the phenolic hydrogen. Therefore **PQ**-releasing probes can operate as true turn-on systems, with zero self-background and excellent signal-to-noise ratios, typically above 300 even without background signal subtraction. There are many further advantages[53] and few drawbacks of **PQ-OH** for cell-free and cellular proof-of-concept studies (see **Supporting Note 4**).

Finally, while we were investigating the simplest *N*-methylated trigger designs (**Fig 2d**), we noted that the resulting **PQ** probes had borderline solubility for cell culture work, with insufficiently reproducible results above 25 μM in aqueous buffer with 1% DMSO. To address the solubility challenge presented by the flat hydrophobic **PQ** cargo without switching cargos, we used two other *N*-alkyl sidechains as cell-compatible solubilisers, to minimally crowd the redox-active site while being tracelessly removed during cyclisation. We chose a basic *N*-methylpiperazinamide, and a neutral morpholinamide (**Fig 2d**). The reliability of the probes mirrored their solubility, so we numbered the sidechains as 1 (piperazinamide, preferred; reliable to >250 μM), 2 (morpholinamide), or 3 (methyl).

The modularity of this design ensures that any trigger can be fitted with any solubiliser sidechain and any phenolic cargo. We freely combined triggers and sidechains to create **PQ**-releasing-probes, which we name by the letter of the trigger (e.g. **A**) combined with the number of the sidechain (e.g. 2), thus e.g. **A2**.

As well as the **A**-type selenenylsulfides and the corresponding **G**-type design controls, we added extra hydrolytic, kinetic/thermodynamic, and mechanistic controls to test specific aspects of our design: eventually reaching a panel of eleven cyclic probes and one linear probe (**Fig S5**). We used an isosteric cyclohexane "trigger" as a non-reducible control for any spontaneous or enzymatic hydrolysis, or aminolysis/thiolysis, of the carbamate (**C**-type); this is particularly important when applying mM concentrations of thiolate reductants (that might intermolecularly attack carbamates, circumventing the mechanistically desired intramolecular cyclisation). We used a cyclic disulfide (**S**-type) and a cyclic diselenide (**Se3**) as reducible controls with different thermodynamic and kinetic sensitivity profiles that we anticipated would make them *not* TrxR-selective. Linear disulfide **SS00**[29] (**Fig S5**) was used as our mechanistic control, since according to our hypotheses it ought to access the irreversible pathway and so should be thiol-labile. (see **Supporting Note 2**).

2.3 Synthesis: We prepared the key selenenylsulfide building blocks in a straightforward, divergent manner from bis-mesylate **1** (**Fig 3a**), which can easily be accessed from aspartic acid.[54] Reaction with one equivalent of potassium thioacetate afforded a mixture of mono-substitution products **2** and **3**, with a small preference for **2**. These regioisomers were separated by chromatography. Treatment with potassium selenocyanate followed by thioester cleavage directly resulted in the formation of the cyclic dichalcogenides **5** and **8** by expulsion of cyanide. Boc-deprotection then gave the amine hydrochlorides **6** and **9**. In parallel to our work, Iwaoka elegantly reported the synthesis of compounds **6** and **9** via a similar approach, but using non-commercial PMBSeH as the selenium source.[19] In comparison, our synthesis offers more atom economy, reduced step count, fewer chromatographic separations and more rapid access to the final compounds, although these differences are not crucial. To obtain the control **Se** and **S** series dichalcogenides (**Fig 3a**), bis-mesylate **1** was either treated with excess potassium thioacetate[54] and KOH/air, or excess KSeCN and KOH, followed by Boc-deprotection to form disulfide **10** or diselenide **11**, respectively. The symmetrical linear disulfide **SS00** was accessed from commercial cystamine. We diversified these trigger building blocks by installing the sidechains, giving secondary amines. Finally, deprotection and coupling with chloroformate **PQ-OCOCl**[46] (**Fig 3b**) yielded a panel of twelve **PQ** probes (**Fig 3c** and **Fig S5**): the **A**-type and **G**-type cyclic selenenylsulfides **RX1**, **A2-A3** and **G1-G3** as candidates for TrxR-selective probes; the cyclic diselenide **Se3** and cyclic disulfides **S1-S3** as well as the linear disulfide **SS00** as reduction-responsive controls; and the non-reducible cyclohexyl probe **C1** as a control for non-reductive activation. All probes were nonfluorescent as solids and in solution (see **Supporting Information**).

2.4 Cargo release mechanistic aspects. To test the 5-*exo*-trig cyclisation release mechanism of **Fig 2a-b**, we performed an HPLC-MS study of TCEP-mediated probe activation (see **Supporting Information** section 5). This is of interest since alternative post-reductive release mechanisms could feature different parameters of kinetics and selectivity. For example, 3-*exo*-tet cyclisation releasing an *N*-methyl probe carbamate via thiirane or selenirane formation (similarly to a mechanism reported by Melnyk[55] with halflife ca. 4.5 h), followed by fast $E1_{cb}$, could also be postulated as a mechanism. We also wished to test whether the triggers resist parasitic reactions such as TCEP deselenisation[56] which would otherwise complicate cell-free benchmarking.

In the TCEP challenge, **RX1** reacted fully to selenocarbamate and **PQ-OH**. **G1** reacted similarly although more slowly to give the thiocarbamate and **PQ-OH**, potentially reflecting the desired

difference of **C**/**J** cyclisation kinetics (fourth design consideration in section 2.1). The 5- rather than 6-*exo*-trig cyclisation is supported in two ways: (i) The **RX1** cyclisation byproduct was found as the monomer, while the **G1** byproduct was a monooxidised dimer matching interpretation as an easily-formed and highly stable linear diselenide. (ii) Equimolar selenolate alkylators almost entirely blocked **PQ-OH** release from **RX1**, but only slightly reduced **PQ-OH** release from **G1**. This latter observation crucially suggests that on-reductant cyclisation (which also proceeds from an intermediate in which the selenolate is engaged) may be significant for **G1** but not **RX1**: so matching the third design consideration in section 2.1. The controls ran as expected: **SS00** reacted fully to thiocarbamate and **PQ-OH**, while **C1** remained intact (for full discussion see **Supporting Information** section 5).

In summary, the HPLC studies showed that reductive release is fast and operates by 5-*exo*-trig cyclisation according to the proposed mechanism; that deselenisation is not a complicating factor; and indicated that on-reductant cyclisation may be speedy (**G1**) or absent (**RX1**). As on-reductant cyclisation can be key for probes to resist (**RX1**), or succumb to (**G1**), monothiol-exchange-based triggering, we expected this to determine their reduction selectivity in cells.

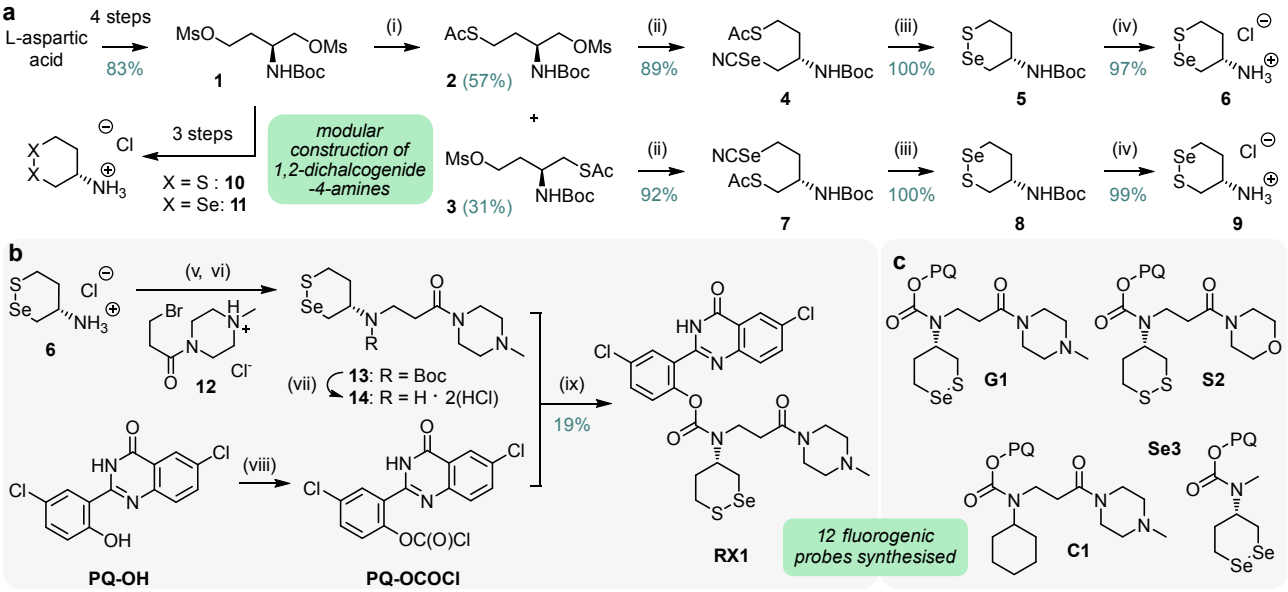


Figure 3: Trigger and probe synthesis. **(a)** Divergent synthesis of amino-1,2-thiaselenane triggers: (i) KSAc, 18-crown-6, DMF, 15 h, r.t.; (ii) NaI, 18-crown-6, KSeCN, THF, 10 h, 50°C; (iii) KOH, MeOH/THF, 1 min, r.t.; (iv) HCl, dioxane/DCM, 11 h, r.t.. **(c)** Representative probe synthesis, shown for **RX1**: (v) NEt_3, MeOH, 1 h, MW 120°C; (vi) Boc_2O, NEt_3, DCM, 15 h, r.t.; (vii) HCl, dioxane/DCM, 11 h, r.t.; (viii) triphosgene, DIPEA, DCM, 2 h, 0°C → r.t.; (ix) DIPEA, DCM, 4 h, 0°C → r.t. **(c)** Representative **PQ** probes (full list in **Fig S5**).

2.5 Cell-free reductant profiling. To report on a protein selectively in cellular settings, a trigger must firstly be completely resistant to reduction by the cellular monothiol background (ca. 50 mM, of which up to 8 mM GSH).[24] Resistance or sensitivity to a given reductant is not properly revealed by "single-concentration bar chart" data display, but rather is best understood by determining dose-response functions. Therefore, to meaningfully characterise the response to reductant challenges, we titrated reductants up to supraphysiological concentrations, and acquired full timecourse rather than endpoint data for more rigorous interpretation.[23,29]

We began with GSH challenge tests. These were highly encouraging: over 6 h, **A**-type probes showed zero fluorescence response up to 10 mM GSH, indicating outstanding monothiol resistance (**Fig 4a**, **Fig S9-S10**). The thermodynamically identical **G**-type pathway controls were GSH-labile (3 mM GSH activated to ca. 25%, **Fig S9-S10**), supporting our pathway-based design (**Fig 2b**). Dose-response plots of GSH resistance using the 6 h timepoint data (**Fig 4b**, **Fig S10**) likewise highlight the GSH-robustness of all **A**-type probes and the partial GSH-lability of **G**-type pathway controls. The comparison probe types behaved as expected. We had hoped that cyclic disulfides **S1**-**S2**, which are analogues of our previously reported vicinal-dithiol-selective probe **S3**,[23] would likewise be monothiol resistant (i.e. no destabilisation by the solubiliser sidechains); they indeed resisted GSH up to 10 mM (**Fig S9-S10**). In comparison to these, linear disulfide **SS00** is a mechanistic control for the need of a cyclic dichalcogenide topology (**Fig 1c**), and indeed we confirmed its sensitivity to monothiol-based release (nearly full activation by 1 mM GSH; **Fig 4a**, **Fig S9**). We noted in passing that the cyclic diselenide **Se3** was entirely nonreactive (**Fig S9**), suggesting that selenium is tolerated at both positions of the reduction-based probes without Se-oxidation-mediated release mechanisms (see **Supporting Note 2**). The zero-level fluorescence seen with **RX1** (and **Se3**) under a thousandfold challenge by GSH is significant, qualifying the secondary amine phenolic carbamate design as exceptionally stable to monothiolysis, as well as to spontaneous hydrolysis and aminolysis.

We next tested the probes against a range of purified recombinant reducing enzymes in cell-free assays, including different TrxRs as well as non-targeted Trxs, Grxs, GR, TRP14, and GPx1. The source and production methods are relevant for TrxRs, since the key selenocysteine (U498) residue in their active sites that is predominant in native enzymes,[57] is typically not incorporated using standard recombinant expression systems.[58] Therefore, we used human cytosolic TrxR1 and human mitochondrial TrxR2 produced with recent

recombinant production methodologies that approach 100% UC active site content.[59] To stringently test the validity of our design for selenolthiol-dependent activation, we also used the U498C point mutant of rat TrxR1 (rTrxR1^{U498C}).[60]

The selenenylsulfide probes showed excellent response to TrxR1. **RX1** at 10 μM was strongly activated by 20 nM TrxR1 when supplied with NADPH, with a halftime to maximal signal generation of ca. 30 min (**Fig 4c**). This contrasts exceptionally with its total stability to a 1 million times higher concentration of monothiol GSH over a period of 6 hours. **RX1** responded well to TrxR1 down to the sub-physiological concentration of 5 nM (**Fig 4d**). As this cell-free assay even underestimates probe sensitivity to low concentrations of TrxR (see **Supporting Note 5**), this was very encouraging for cellular use. The TrxR1-turn-on kinetics and response profiles were similarly good for all other cyclic selenenylsulfide probes (**A2-A3**, **G1-G3**) (**Fig S13**). This unity of TrxR1-selective performance despite the structural diversity of the probes matched our hypothesis that the cyclic selenenylsulfide itself is responsible for reactivity, and that the solubilising motif can be exchanged flexibly without altering the triggers' redox activity. Also, since the rates of probe activation by TrxR1 were essentially identical between the **A**-type and **G**-type triggers (**Fig S13**), this indicates an important result for enzymatic selectivity which completes the $k_{rel\text{-}H}$ discussion (section 2.1). If on-TrxR cyclisation (**Fig 2a-b**) were significant for probe release, the **G**-type probes would be significantly faster than the **A**-type in releasing cargo. However, the data rather support that TrxR1 indeed fully reduces the cyclic selenenylsulfide probes to **C**/**J**, before they cyclise.

Disulfide controls **S1-S3** were nearly inert to TrxR1 (**Fig S13**); we noted however that the piperazinamide sidechain 1 generally gave faster reaction speeds compared to the less soluble methyl and morpholinamide sidechains, which may be associated to hydrophobic adsorptive loss[29] of the intact probes as well as to local pH modulation. This effect was often reproduced in later assays including with **A**- and **G**-type probes, and supported the choice of piperazinamides as the standard sidechain for the probes. Diselenide control **Se3** was completely nonresponsive to TrxR1, as it later proved also to be to all other reductants except to DTT (**Fig S9, Fig S12**).

Of the selenenylsulfides, only **RX1** and **G1** were reduced by the mitochondrial isoenzyme TrxR2, matching the trend of greater reactivity for the piperazinamides, and this only at supraphysiological TrxR2 concentrations (50 - 100 nM; **Fig 4c**, **Fig S13**). Some TrxR2 response was expected, since TrxR2 features the same Sec-Cys motif as TrxR1, but this result mirrors ample literature precedents of TrxR2 being slower to process small molecule substrates than TrxR1.[61] Since cytosolic TrxR1 has many-fold higher total expression levels in most cell types, we therefore expected the faster-reactive TrxR1 to be the dominant cellular reductant for our selenenylsulfide probes.

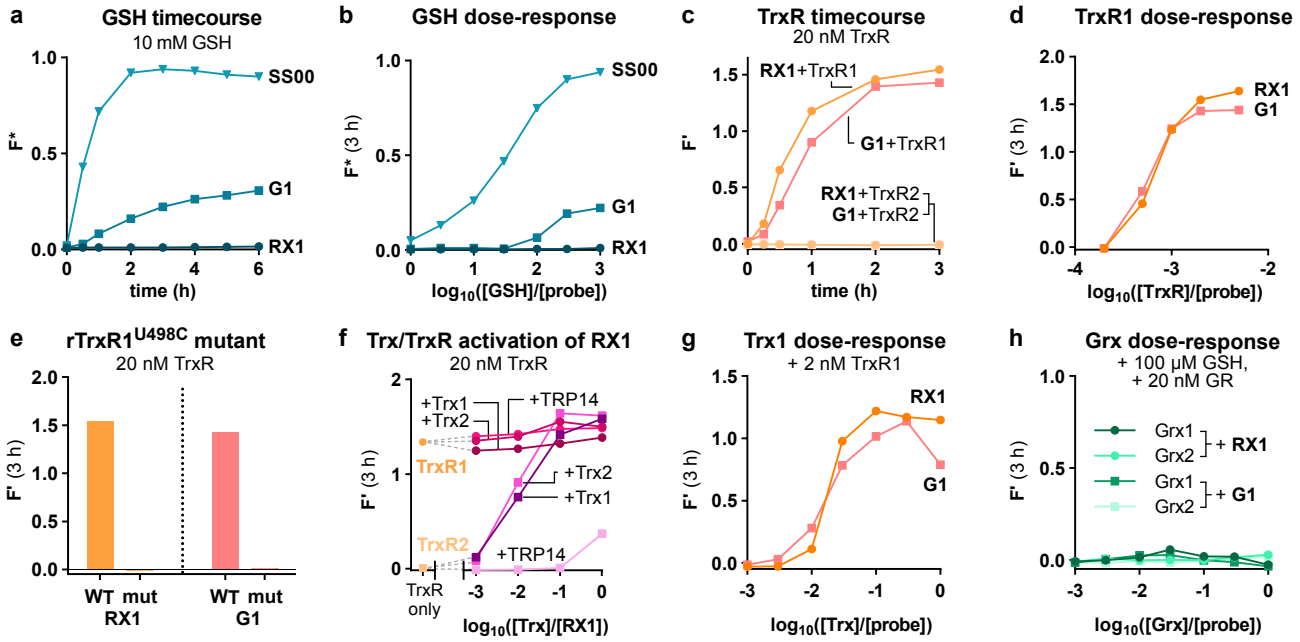


FIG 4 *In vitro* characterisation of TrxR-selective **RX1** and comparison probes. All probes used at 10 μM in TE-buffer. TCEP (10 equiv., 100 μM) was used as a reference for fast, quantitative probe reduction and activation; **F*** in enzyme-free assays is the relative fluorescence signal compared to full activation (**F*** = F/F^{TCEP}). NADPH (200 μM) was applied as native upstream component for enzymatic assays; **F'** in enzymatic assays is F* additionally corrected for NADPH autofluorescence (see Supporting Information). **(a-b)** Selected GSH-resistance timecourses, and GSH-dose-response (0.01-10 mM GSH). **(c)** Selected TrxR1-activation timecourses (20 nM TrxR1 and TrxR2). **(d)** TrxR1-dose-response. **(e)** Selenenylsulfide probe activation by wildtype TrxR (Sec-Cys) compared to Cys-Cys mutant rTrxR1^{U498C}. **(f)** Dose-response titrations with vicinal dithiols Trx1, Trx2, and TRP14 (0.01-10 μM) in presence of 20 nM TrxR. **(g)** Trx1 dose-response (0.01-10 μM) titrations in the presence of 2 nM TrxR. **(h)** Dose-response (0.01-10 μM) profiles to the GR/GSH/Grx cascade (20 nM GR, 100 μM GSH).

Next, we wished to test our hypothesis that the selenenylsulfides should interact selectively with mammalian TrxR on the basis of the TrxR selenolthiol. TrxR has two redox-active sites, with the NADPH-driven N-terminal Cys-Cys shuttling electrons to the C-terminal Sec-Cys. As Hondal *et al* showed by studies of a mitochondrial TrxR2 in a paper aptly titled "No Selenium Required",[22] many compounds that had been assumed to require a selenol for reactivity not only turned out to be similarly processed by the C-terminal Cys-

Cys mutant TrxR^{U498C}, but indeed were better or exclusively processed by the TrxR N-terminal site or by other dithiols. We therefore compared probe activation by wildtype TrxR (Sec-Cys) with its Cys-Cys mutant rTrxR1^{U498C}. This Cys-Cys mutant no longer reduced either the **A**- or **G**-type probes, while its reduction of standard substrates DTNB and juglone was unimpeded (**Fig 4e, Fig S14**). This supported that both **A**- and **G**- selenenylsulfide probes *do* indeed require the native mammalian TrxR's C-terminal selenolthiol for efficient reduction, matching our designs.

With excellent monothiol resistance, and strong processing by selenolthiol TrxR1 rather than its dithiol mutant, it remained an open question whether physiological concentrations of other selenol-bearing proteins would also be capable of probe triggering, which is important for enzymatic selectivity. Fortunately, there are only 25 selenoproteins in the human proteome; only a few of these have ubiquitous expression at substantial concentrations; and nearly all of those have monoselenol active sites (rather than selenolthiols). The highest-concentration ubiquitous selenoproteins are monoselenol glutathione peroxidases GPx1 and GPx4.[9] We challenged **RX1** and **G1** with cytosolic GPx1 up to supraphysiological 1 μM concentration, and were pleased to see no detectable probe activation over 6 hours (**Fig S15**). This was an encouraging step towards the possibility of cyclic selenenylsulfides acting as TrxR-selective probes (see **Supporting Note 6**).

Having tested the major biological monothiol, monoselenol, and selenolthiol reductants, we finally examined the effect of vicinal dithiols on probe activation. We began by screening the probes' dose-response to dithiothreitol (DTT), a nonphysiological vicinal dithiol that is ca. 50 mV more reducing than Trx1, but which reproduces some aspects of its behaviour and so can be helpful[23] to study dithiol reactivity. Excess DTT triggered all **A**- and **G**-type selenenylsulfide probes. Although selenenylsulfides are more thermodynamically resistant to reduction than disulfides, their reaction with DTT was more rapid and more sensitive than that of the corresponding **S1-S3** series (**Fig S11**). This is a reminder of the importance of kinetics to probe release, as the greater electrophilicity of Se in selenenylsulfides than S in disulfides determined the assay outcomes.

We then tested Trxs, titrating them up to physiological 10 μM in the presence of 10 μM probe. These assays require TrxR and NADPH to reduce Trx from the non-reactive, oxidised, stored state, and to re-reduce it after each oxidation by probe or by molecular O_2 in the non-degassed assay buffer. **RX1** is already fully and rapidly activated by 20 nM TrxR1 alone (**Fig 4c**), so, unsurprisingly, titrating in Trx1/2 or other Trx-fold proteins such as TRP14 did not reveal extra processing by those species (cf. TrxR1 data in **Fig 4f**). To study direct probe reduction by Trx only, we instead used concentrations of TrxR1 and TrxR2 that were ineffective at directly activating **RX1**, observing half-maximal probe activation with ca. 0.1-0.2 μM of Trx1 or Trx2 regardless of which TrxR was chosen (TrxR2 data in **Fig 4f**, **Fig S17** and **Fig S19**; TrxR1 data in **Fig 4g** and **Fig S22**). This represents a ca. 50-fold lower sensitivity to Trx than to TrxR1, under continually re-reduced conditions. The TrxR-resistant disulfide comparison probes **S1-S3** were activated by Trx as expected,[23] with half-maximal activation around 0.3 μM Trx (**Fig S16-20**).

We finally tested alternative redox-active dithiol proteins. We first profiled dose-response to Grx1 and Grx2, the other major vicinal dithiol protein redox effectors, applied as part of the full GR-GSH-Grx cascades (**Fig 4h**, **Fig S23-S24**). Both **RX1** and **G1** fully resisted up to 10 μM Grx1/Grx2 over 3 hours (including 20 nM GR, 200 μM NADPH, and 100 μM GSH for Grx reduction). This is a significant result. Although the Grxs are vicinal dithiol redox enzymes similar to the Trxs, they are more RSSG-selective. Therefore the cyclic probes should resist Grx processing inasmuch as they resist monothiol exchange (note too that although GR is NADPH-driven, it has very high specificity for GSSG so is not expected to directly reduce the probes). We also examined the TrxR-reduced redox-active vicinal dithiol TRP14, finding that the probes resisted it up to 3-10 μM (**Fig 4f**, **Fig S18**, **Fig S21**). These results show that the probes are not sensitive to all vicinal dithiol proteins *per se*, but have a specific sensitivity to the highly reducing Trxs at least in cell-free conditions. While it would have been best for our TrxR-reporter goal if Trxs were incapable of probe reduction, sensitivity can be expected in a cell-free assay where excess NADPH and an absence of other oxidants and binding partners offers no alternative pathway except probe reduction. In cells however, Trx as an effector protein has many binding partners that recognise and oxidise its thioredoxin fold, while NADPH-powered TrxR binds fewer substrates (mainly Trx, also TRP14, and potentially Grx2).[12,62,63] This gave hope that if Trx in cells would be substantially engaged with preferred protein substrates, then **RX1**'s extraordinary kinetics of turnover by TrxR1, combined with its total stability to GSH, might give it functional TrxR1-selectivity in cells. In line with this understanding, previous research on Trx has identified cyclic dichalcogenide probe chemotypes that were highly sensitive to purified Trx (but not to TrxR) in cell-free settings, yet were unexpectedly *inactive* in cellular settings despite the high cellular concentrations of this universal reductant.[23]

2.5 Cellular assays: By this stage we had shown that, in line with our pathway hypothesis, **RX1** was monothiol resistant while its regioisomer **G1** had some lability to physiological GSH concentrations; **RX1**'s GSH resistance could render it a protein-selective redox probe. Under cell-free conditions, both **RX1** and **G1** were rapidly activated by TrxR1, dependent upon its selenolthiol active site, and had minor sensitivity to TrxR2; both were sensitive to fully reduced Trx but almost entirely nonresponsive to the alternative vicinal dithiol thioredoxin-related protein TRP14; and were inert to GR/Grx. We now entered cell-based assays, aiming to test the probes' TrxR-dependency indicated in the cell-free assays as well as their potential for *functional* TrxR1-selectivity in the cellular context, by elucidating the cellular mechanism of activation of these compounds (see **Supporting Information** for assay conditions, processing methods, full data for all probes and accompanying discussions; and **Supporting Note 5** for discussion of fluorescence normalisation).

Signal generation by the selenenylsulfide probes was strong in different cell lines (**Fig 5a-b**), was dose-dependent (**Fig 5b**), was stable over long timecourses, and was not due to direct carbamate cleavage (**C1**, **Fig 5a**). The potentially TrxR-selective **RX1** was, as expected, slower to generate signal than the nonselective linear disulfide control **SS00** or the GSH-sensitive **G1**. Fluorescence imaging showed near-ubiquitous turn-on of **RX1** with **PQ-OH** fluorophore being intracellularly retained over hours (**Fig 5d**). The solid-state nature of the **PQ-OH** fluorophore tends to saturate pixel intensities at the detector, so while microscopy images are excellent as a readout of which cells have activated the probe, platereader assays are better suited to quantify fluorophore release.

We then performed several orthogonal tests of whether cellular activation of the **RX1** probe is indeed TrxR-selective.

Cells cultured without selenium supplementation do not fully incorporate Sec in TrxR but instead incorporate Cys, so lowering the cellular concentration of the selenolthiol form of TrxR.[64] **RX1** signal was four times higher in cells supplemented with selenium than without (**Fig 5c**), whereas **SS00** and **G1** were essentially unaffected. This is consistent with an interpretation that **RX1** activation in cells depends on native TrxR, while the mechanistic control **G1** (just like nonselective **SS00**) is not selective in the cellular context for TrxR (presumably, the residual GSH lability of **G1** allows it to be activated by the monothiol background; **Fig 4b**).

We next explored how chemical inhibition of TrxR affects signal generation. We used the metal-free compounds TRi-1 and TRi-3 which are inhibitors with good cellular TrxR-selectivity,[65] and avoided the often-used thiophilic gold complex auranofin which has potential pitfalls and known liabilities[29] (see **Supporting Note 1** and **Fig S2**). Cellular **RX1** signal was strongly inhibited by acute TRi treatment, with just 2 µM TRi-1 reducing signal by 66%; whereas **G1** signal was only reduced by 15% and **SS00** signal was unaffected by this treatment (**Fig 5e, Fig S26**). TRi-3 dose-dependently reproduced the same inhibitory effects. Taken together, this supports the interpretation that cellular **RX1** activation is highly dependent on the activity of the TRi-1/3 target TrxR, whereas activation of its regioisomer **G1** is largely TrxR-independent.

Finally, we stringently evaluated **RX1**'s TrxR1-dependency using TrxR1-knockout (ko) cells (MEF: mouse embryonic fibroblasts)[66], which we compared against their corresponding wildtype control (fl/fl). This knockout upregulates compensatory thiol-based reductive pathways to survive (e.g. the GSH system), with Trx being reduced instead by Grx2.[67,68] Therefore, we expected that the only circumstances under which we would see zero signal for a hitherto cellularly-active probe with strong dependency on uninhibited, Se-containing TrxR1 for processing, would be if that probe was *exclusively* activated by TrxR1 in the cellular context. We were pleased when this indeed proved to be the case for **RX1**. The TrxR1^{ko} MEFs showed zero **RX1** activation, while the corresponding wildtype gave strong signal (**Fig 5f**). As the mitochondrial TrxR2 is still functional in the knockout, this strongly suggests that cellular **RX1** signal reports essentially on cytosolic TrxR1 at least in this cell line. Signal from mechanistic control **G1** was suppressed by ca. 60% though not abolished in the knockout cells (**Fig S27a**), again matching expectations that **G1** is not fully TrxR1-selective in cells. To obtain still stronger proof of mechanism, we tested TrxR1-knock-in (ki) to the MEF TrxR1-knockout background[67]. This fully restored the strong, dose-dependent **RX1** signal generation (**Fig 5g, Fig S27b**).

Taken together, the data strongly support that **RX1** is exclusively activated in cells by native TrxR1. **RX1** is therefore the first validated cumulative-release probe design that can selectively and meaningfully report on the cellular activity of mammalian TrxR1.

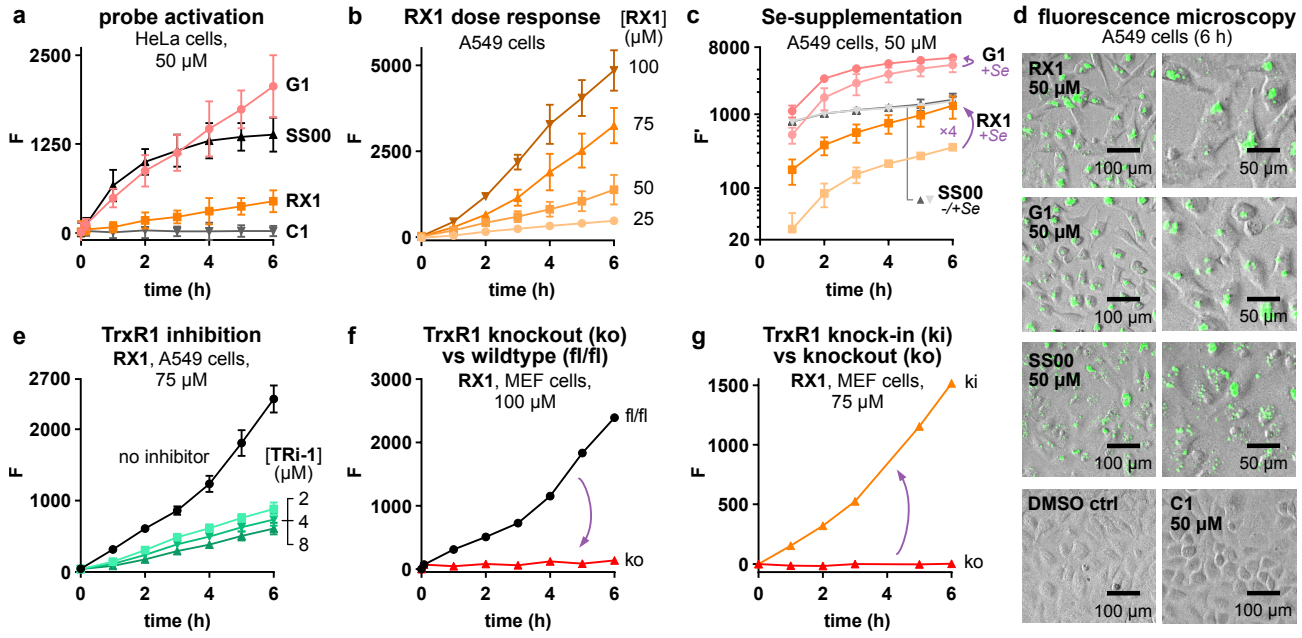


FIG 5 Cellular probe evaluations. **F** is the raw fluorescence signal minus the signal of DMSO-only controls (platereader assay; average over ca. 20,000 cells per well, three wells per datapoint). **(a-b)** fluorescence activation in HeLa and A549 cell lines (three independent experiments, mean with SD). **(c)** Cellular **RX1** signal depends on selenium starvation/supplementation, while comparison probes **SS00** and **G1** do not (**F'** is the raw signal minus its time-zero value, i.e. $\mathbf{F'}(t_0) \equiv 0$ by definition (hence a time-zero point is not shown), which gives a more sensitive comparison of conditions; see also Supporting Information). **(d)** Fluorescence microscopy images showing cell-marking performance of the **PQ** probes. Overlay of **PQ-OH** fluorescence (green) on brightfield images. **(e)** Cellular **RX1** signal is efficiently suppressed by the TrxR inhibitor, TRi-1. **(f-g)** Genetic knockout of TrxR1 entirely suppresses signal generation by **RX1**, whereas reconstitution of TrxR1 expression restores **RX1** activation (MEF [TrxR1^{ko}/TrxR1^{ki}] cell lines).

3. DISCUSSION AND CONCLUSION

TrxR's position as a central redox node in diverse physiological processes drives the hitherto-unmet need for precise, selective molecular probes to characterise and decrypt its activity in cell biology and in physiological responses to stress. Furthermore, the significant and potentially unique dysregulation of cellular redox systems in pathology, notably of the thioredoxin system, opens exciting vistas towards using TrxR-selective probes as tools both to study and

ultimately to diagnose pathologies featuring redox dysregulation, such as autoimmune diseases and cancer. Finally, if such molecular probes are also *modular* designs, they offer to meet the full spectrum of needs in translational chemical biology: from redox-triggered diagnostics of diverse modalities (that are predictive of arbitrary cargo release), to the redox-triggered prodrugs that may allow targeting bioactivity selectively to the identified pathological tissues.

The selenenylsulfide-based probe **RX1** exploits a "Selenium Required" mechanism-based design to perform as the first TrxR1-selective, modular, cargo-releasing cellular probe system. The cyclic topology of the stabilised selenenylsulfide substrates, and the mechanistic bias of the 5-*exo*-trig selenolate cyclisation, are both required for this performance. Its design is robust, yet its high selectivity for mammalian TrxR1 is accompanied by fast kinetics of processing and cargo release. Its true off-to-on optical performance (zero pre-activation background, to bright and photostable environment-independent fluorescence) and its cell-retained localisation permit cell-resolved imaging with excellent signal-to-noise ratios. **RX1** will thus be directly useful for redox biology studies quantifying TrxR1 activity in physiology and disease. Moreover, it can gain a crucial place in screening and development pipelines towards selective cellular inhibitors of TrxR1: a goal of significant pharmaceutical interest.

The modularity of **RX1** also suits it for flexible adaptation to TrxR1-triggered release of a range of cargos, far beyond the fluorogenic **RX1** probe for cell culture. The use of a traceless solubiliser sidechain to ensure reliable and cargo-independent solubility is explicitly intended to favour just such adaptation. The design is particularly suited for translation to any molecular cargo with unmaskable activity: of which the fluorogenic, indigogenic, or bioluminogenic probe possibilities given in section 2.2, and the accompanying drug-releasing prodrug possibilities, are but a small selection. Particularly given the strong pharmaceutical interest in TrxR-targeted and redox-activated drugs in the inflammation, immunity, and cancer therapy fields,[9] it will be exciting to test whether redox-responsive prodrug designs based around **RX1**-like selenenylsulfides can harness pathologically dysregulated TrxR1 activity to target drugs to diseased tissues. We also anticipate that improved **RX1** analogues can be rationally designed to retain its specificity while installing enhanced reaction kinetics, alternative cargo delivery scope, and maximising its still-untested resistance to *in vivo* metabolism-based cargo release mechanisms, which will further promote its utility in biological and therapeutic applications. We thus expect that the **RX1** system will impact both redox biology quantification/mapping studies, as well as translational research and therapy of locally-redox-dysregulated pathologies such as cancer, inflammation and auto-immune disorders.

The development and use of chemical probes for redox biology has been hampered by several systematic problems. Only very few redox-active trigger chemotypes have been explored, a lack of diversity which is exacerbated by the overwhelming use of linear disulfides in probes (**Fig S1**). The rational mechanism-based design of new redox-active motifs that are chemocompatible with the major biological redox systems, is urgently needed to drive innovation and research across the field. A second problem is the lack of clarity about what molecular information the known chemotypes do, or do not, provide. This occasionally verges on denying that predictive structure-activity relationships indeed apply to the reduction of probes. A third and related problem is that redox selectivity testing is rarely as systematic as it could be. Often too, selectivity has been stated despite it remaining obscure which families of cellular reductants have been checked, and which have not; or what proportion of a probe is said to be *cellularly* processed by the species for which selectivity is claimed. The combination of these problems has resulted in a large number of redox probe reports claiming selectivity for various species, albeit with the very same redox-active motifs being claimed as selective for a range of very different redox species (**Fig S1**); occasionally without or even going against supporting data or rationale, which has not fostered confidence or progress in the field.[22]

We also intended this probe research to test how such systematic problems may be overcome with chemistry. The two cyclic selenenylsulfide cores are new chemical species that open up broad possibilities for probe adaptations, which are easily accessible through their operationally simple, divergent, gram-scale syntheses. The exploration of the solubiliser sidechains, and the comparisons to related probe structures, confirms the rule of structure-activity relationships from cell-free to cellular applications. The battery of cell-free and cellular tests we have employed have stringently assayed all key aspects of redox probe performance, in particular to validate **RX1**'s TrxR1-selectivity. The comprehensive cell-free characterisations, using key redox-active species and proteins in their full interacting systems, tested several key features that cellular assays later confirmed by orthogonal measurements. **RX1**'s cell-free resistance to physiological levels of monothiols over hours was indeed shown to depend on mechanism, since its thermodynamically identical but mechanistically differentiated **G1** analogue is GSH-labile. The cell-free selenolthiol-selectivity of **RX1** was shown by its nonresponse to the TrxR(U498C) mutant, which until now was assumed to be similarly-reactive to most small molecule redox substrates including all that contain selenium[22]. The cellular counterpoint of these assays has been the validation of TrxR1 as the *only* relevant cellular target of **RX1**, by three independent biological and chemical methods (knockout/knock-in, chemical inhibition, and selenium starvation). Comparison to the performance of mixed-mode **G1** (processed by both TrxR and thiols, as revealed by all three methods) further substantiated **RX1**'s cellular selectivity, and provides a comprehensive and quantitative basis for reporting **RX1** as a TrxR1-selective probe.

The use of chemical diversity, i.e. regioisomeric as well as disulfide and diselenide probes, as mechanistic, thermodynamic, and hydrolytic controls, has thus been a key element in testing and rationalising **RX1**'s selenolthiol-selectivity. More broadly, the rationally-designed panel of selenium-containing motifs we present here complements the all-sulfur series we have reported elsewhere[23,29] to allow functional comparison and selectivity screening across a total of >40 dichalcogenide redox probes, with a level of intercomparability and rigour that to our knowledge has not yet been attempted. It was our aim to increase the breadth and depth of this body of data, and particularly also to provide explicit mechanistic descriptions (**Fig S3-S4**) of how these small molecule probes have been designed for selectivity despite lacking the protein recognition features upon which genetic approaches can rely. Our hope is that by doing so, this research will both inspire new chemical approaches in the still-untapped field of redox chemical biology, and will also open doors for a range of powerful biological studies: revealing the key cellular redox networks in action, and addressing them through therapeutics targeted by the physiology and the pathology of TrxR1.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the website. Synthesis, analysis, biochemical and biological evaluations (PDF)

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Author Contributions

L.Z. performed synthesis, chemical analysis, chemoreductant and enzymatic cell-free studies, coordinated data assembly and wrote the manuscript. J.G.F. performed synthesis, chemical analysis, chemoreductant and enzymatic cell-free studies. L.P. performed cellular studies. K.S. performed enzymatic specificity screenings and cellular studies. M.S.M. performed synthesis and analysis. Q.C. expressed and purified recombinant TrxR and Trx isoenzymes. E.S.J.A. supervised enzymatic specificity screenings and cellular studies and helped in interpretations of results and writing of the manuscript. J.T.-S. performed cellular studies and supervised cell biology. O.T.-S. designed the concept and experiments, supervised all other experiments, coordinated data assembly and wrote the manuscript.

Funding Statement

This research was supported by funds from the German Research Foundation (DFG: SFB 1032 project B09 number 201269156, SFB TRR 152 project P24 number 239283807, SPP 1926 project number 426018126, and Emmy Noether grant 400324123), LMUExcellent (Junior Researcher Fund), and the Munich Centre for NanoScience initiative (CeNS) to O.T.-S.; and from Karolinska Institutet, The Knut and Alice Wallenberg Foundations, The Swedish Cancer Society, The Swedish Research Council and the Hungarian Thematic Excellence Programme (TKP2020-NKA-26) to E.S.J.A..

Notes

L.Z., J.G.F., and O.T.-S. are inventors on a patent application filed by the LMU Munich in 2021 covering compound structures reported in this paper. The authors declare no competing financial interest.

ACKNOWLEDGMENT

L.Z. thanks the Fonds der chemischen Industrie for generous financial support through a Kekulé fellowship. J.G.F. thanks the Studienstiftung des deutschen Volkes for support through a PhD scholarship. L.P. thanks the GRK 2338 for support through a PhD scholarship. J.T.-S. thanks the Joachim Herz Foundation for fellowship support. We thank Marcus Conrad (Helmholtz Centre, Munich, DE) for MEF and knockout MEF cell lines; and Carina Schmitt (LMU) for synthetic assistance. We thank the attendees of the SPP 1710 conference Thiol-Based Redox Switches in 2019, and in particular Christopher Lillig (Greifswald, DE), for their supportive, collegial, and helpfully challenging discussions.

This paper is dedicated to Yasuteru Urano, whose rigorous and far-reaching developments towards translational applications of chemical biology probes are inspiring to the field.

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