Cyclic 5-Membered Disulfides Are Not Selective Substrates of Thioredoxin Reductase, but Are Opened Nonspecifically by Thiols

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The cyclic five-membered disulfide 1,2-dithiolane has been used as the key element in numerous chemical biology probes. Contradictory views of this disulfide motif populate the literature: some reports describe it as being nonspecifically reduced, others as a highly specific substrate for thioredoxin reductase (TrxR). We show that 1,2-dithiolanes are nonspecifically reduced by a broad range of thiol reductants and redox-active proteins, and that their cellular performance is barely affected by TrxR inhibition or knockout. We conclude that inhibitor screenings and probe designs treating 1,2-dithiolanes as TrxR-selective substrates should be treated with caution and previous interpretations may need careful re-evaluation. Considering ring-opening polymerisation, and stringently interpreting assays involving the thiophilic gold-based inhibitor auranofin, are critical to assess 1,2-dithiolane’s true behaviour. We present an approach to control against assay misinterpretation with reducible probes, to ensure that future TrxR-targeted designs are robustly evaluated for selectivity, and to better orient redox probe research in the future.
Cyclic 5-membered disulfides are not selective substrates of thioredoxin reductase, but are opened nonspecifically by thiols

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\textbf{ABSTRACT:} The cyclic five-membered disulfide 1,2-dithiolane has been used as the key element in numerous chemical biology probes. Contradictory views of this disulfide motif populate the literature: some reports describe it as being nonspecifically reduced, others as a highly specific substrate for thioredoxin reductase (TrxR). We show that 1,2-dithiolanes are nonspecifically reduced by a broad range of thiol reductants and redox-active proteins, and that their cellular performance is barely affected by TrxR inhibition or knockout. We conclude that inhibitor screenings and probe designs treating 1,2-dithiolanes as TrxR-selective substrates should be treated with caution and previous interpretations may need careful re-evaluation. Considering ring-opening polymerisation, and stringently interpreting assays involving the thiophilic gold-based inhibitor auranofin, are critical to assess 1,2-dithiolane’s true behaviour. We present an approach to control against assay misinterpretation with reducible probes, to ensure that future TrxR-targeted designs are robustly evaluated for selectivity, and to better orient future research.

INTRODUCTION

Specific dithiol/disulfide-exchange reactions underlie a great number of crucial pathways in biology. Often, these are coordinated through conserved, highly specialised networks of oxidoreductases.\textsuperscript{1} The thioredoxin reductase - thioredoxin (TrxR/Trx) system, and the glutathione reductase – glutathione - glutaredoxin (GR/GSH/Grx) system, are central "nodes" in these networks. TrxR (nM cellular concentration) passes reducing equivalents from NADPH to the effector protein Trx (µM). Similarly, GR (nM) passes reducing equivalents from NADPH to the redox-active peptide GSH (mM), that can directly function as a cellular reductant or be further shuttled to the effector Grx proteins (µM). These systems drive hundreds of redox reactions vital to cellular metabolism, and also regulate protein activity, protein–protein interactions, and protein localisation by reversible dithiol/disulfide-type reactions.\textsuperscript{2} Their complex homeostasis is dysregulated in many diseases, particularly in autoimmune disorders and cancer;\textsuperscript{3} making Trx and TrxR promising therapeutic targets.\textsuperscript{4} Designing selective probes or substrates that report on or target these redox nodes, would enable a broad range of applications in both basic biological and applied biomedical research, and is therefore a subject of intense development both through genetic engineering and chemical biology approaches.\textsuperscript{5,6}

Disulfides are the native substrates of these redox manifolds, and both linear and cyclic disulfides have been exploited as artificial substrates in biophysics, materials chemistry and chemical biology.\textsuperscript{5}

Driven by the high intracellular concentration of thiols (ca. 50 mM total, ca. 5 mM GSH) compared to low concentrations in plasma or in the extracellular space, linear disulfides undergo irreversible and nonspecific transthioleation and reduction in cells (Fig 1a).\textsuperscript{7} Linear disulfides are thus used for nonspecific intracellular release and/or activation of appended cargos, exploiting the cellular thiol pool.

By contrast, cyclic disulfides can exhibit quite different kinetics and thermodynamics for disulfide transthioleation or reduction, and may therefore display different specificity. Cyclic systems are found in nature, perhaps most remarkably in the epidithiodiketo-piperazine class of natural products (ETP).\textsuperscript{6,10} ETPs such as gliotoxin (Fig 1b) and chaetocin feature a near-planar diketopiperazine that is 1,4-bridged by a disulfide with an unfavourable dihedral angle of 0° as compared to the more favourable dihedral angle found in linear disulfides (90°) or 6-membered cyclic disulfides (60°). ETPs were reported to inhibit a range of enzymes and cause a variety of toxic cellular effects, but these often poorly reproducible bioactivities are now understood as relatively nonspecific chemical reactivity of their highly strained disulfide.\textsuperscript{11}

A particularly important cyclic disulfide is the 5-membered 1,2-dithiolane (in this work also termed SS-50). This motif underlies the critical cellular redox cofactor lipic acid, as well as being found in several natural products (Fig 1c),\textsuperscript{12,13} and it has emerged as a motif of general interest within the last decade.\textsuperscript{14-16}

The strained 1,2-dithiolane is kinetically labile to transthioleation,\textsuperscript{18} which is likely the reason for its biological role as a redox cofactor. The disulfide's opening/reduction kinetics have made it the focus of numerous chemical biology approaches, although these have been predicated on two mutually contradictory views of its cellular behaviour, which in this paper we will examine and resolve.

Following one view, 1,2-dithiolane has been cast as an easily, nonspecifically, and irreversibly transthioleated and/or reduced motif. Whitesides' systematic disulfide investigations highlighted that its unfavourable CSSC dihedral angle of ca. 30° destabilises it by more than 8 kJ/mol relative to linear disulfides (90°), and that its reduction potential (ca. -240 to -270 mV) is not significantly below that of linear disulfides (ca. -230 mV). In the 1950s, Fava observed that alkyl thiolates transthioleate 1,2-dithiolane ca. 5000 times faster.
than linear aliphatic disulfides. Creighton reported that its transsthiolation by the vicinal diol dithiothreitol (DTT) is over 100 times faster still, and Whitesides showed that in DMSO, this rate is only 100-fold slower than the diffusion limit. With favourable thermodynamics and high kinetic lability, 1,2-dithiolanes readily polymerise at low concentrations by nucleophile-catalysed ring-opening polymerisation, in particular in the presence of thiols. Matile showed that the sterically less shielded 1,2-dithiolane in asparagusic acid derivatives (two primary thiols) polymerise even more easily than do lipoic acid derivatives, giving them valuable applications exploiting proximity-induced polymerisation.

The intrinsic lability of 1,2-dithiolanes has also been extensively applied for thiol-mediated cellular uptake systems. Promoted by strain, 1,2-dithiolanes are transsthiolated at the cell surface, then are internalised through a series of dynamic covalent reactions with membrane and intracellular thiols. Attaching the strained disulfide to a cargo can therefore dramatically enhance its cellular uptake rate. It will be important to note that this process may be affected by thiophilic inhibitors: uptake rates could be decreased 5-fold by treating cells with thiol-alkylating or thiol-oxidising reagents, or instead enhanced by treatment with reducing agents. Other recent studies further demonstrate that 1,2-dithiolanes undergo fast strain-driven cross-linking or polymerisation initiated by thiols or other nucleophiles. In contrast however, 1,2-dithiolanes have also been reported as reduction-sensing units with a remarkable selectivity for TrxR.

**Figure 1: 1,2-dithiolanes in chemical biology.** (a) Principle of disulfide-based cellular delivery: irreversible cleavage of linear disulfides after cell entry leads to intracellular cargo release (Pr-SH: intracellular protein thiol). (b) Increasing ring strain from linear, to 6-membered and S-membered disulfides, to ETPs. (c) Strained 1,2-dithiolanes in natural products. (d) Fast, irreversible and non-specific transsthiolation of 1,2-dithiolanes with exofacial protein thiols permits rapid dynamic exchange cascades enhancing cellular cargo uptake. (e) 1,2-dithiolane-based probes that have been reported as selective cellular substrates of thioredoxin reductase.

Fluorogenic TRFS probes and prodrugs have been commercialised and extensively reviewed (Fig 1e; overview in Fig S1b). These probes have since been used to study the role of TrxR in Parkinson’s disease and stroke, and have been employed for mechanistic validation of putative TrxR inhibitors during screening approaches.

**Which is the real situation?** 1,2-dithiolane cannot simultaneously be TrxR-selective and yet highly and nonspecifically reactive. To develop a systematic understanding of redox biology, it is necessary to clarify such fundamental disagreements, and reveal why such contradictory results could arise.

Towards this goal, we have investigated the novel 1,2-dithiolane-based reduction-sensing probe SS-50-PQ. We show that (a) its 5-membered cyclic disulfide is nonspecifically reduced by a broad range of monothiols, diols, proteins and enzymes, and (b) its cellular processing does not exclusively require TrxR.

Taken together, we conclude that 1,2-dithiolane-based probes are not selective for cellular TrxR, so the previous work interpreting the performance of TRFS probes as TrxR reporters will benefit from re-evaluation. We speculate that strained disulfides are best understood as probes to monitor thiol-mediated uptake rates, and can be legitimately applied for enhanced delivery and nonspecific activation of trigger-cargo-systems, which opens up promising avenues for chemical biology. We also outline a strategy to control against assay misinterpretations with reducible probes, to promote progress towards a robust and useful toolset of probes for redox biology.
RESULTS AND DISCUSSION

Probe design: We aimed to explore the properties of 1,2-dithiolanes using a reduction-activated probe. Reduction-activated probes and prodrugs are typically trigger-cargo designs, where a reduction-sensitive trigger is connected to the cargo while masking a key functional group. Trigger reduction then results in a fragmentation reaction that restores activity by unmasking that key functional group. This concept has been used for a range of imaging agent and drug cargos.

The TRFS probes are also designed as trigger-cargo constructs with 1,2-dithiolane as the redox sensor, attached to aniline fluorophores through a carbamate (TRFS-red, TRFS-green; Fig 1e) or urea (Fast-TRFS; Fig 1e). For the carbamate probes, disulphide reduction and thiol cyclisation releases the active aniline fluorophore; though the urea probe operates without aniline unmasking, and the fluorescence turn-on mechanism is not fully understood. Generally, most trigger-cargo disulphide probes employ aniline rather than phenol cargos (Fig S1b). While aniline carbamates have high hydrolytic stability,\(^{14}\) phenol-releasing designs would in many ways be more attractive targets, due to a large scope of cargos and to their improved release kinetics. Therefore, to test the reduction selectivity of 1,2-dithiolane with a rapidly-responding probe - and at the same time to establish a modular design that allows delivering a wide range of agents in the future - we created the novel phenol-releasing probe SS-50-PQ (Fig 2a).

The SS-50-PQ design has several advantages. As a tertiary carbamate, this probe cannot decompose by S\(_{1,2}\) elimination,\(^{5,46}\) avoiding the instability\(^{14}\) that has blocked previous phenol-releasing 1,2-dithiolane probes. The choice of 2-(2-hydroxyphenylethyl)-4-(3H)-quinazolinone (PQ-OH) as the cargo, ensures a complete-off-to-on signal readout for carbamate cleavage, making it a sensitive and easily interpreted sensor of disulphide cleavage.

Following disulphide transthiolelation and/or reduction and thiolate cyclisation, expulsion of the electron-poor phenolate (Fig 2a) results in PQ-OH precipitating upon reaching its low aqueous solubility limit (ca. 0.5 \(\mu\)M).\(^{67,46}\) Only in the solid-state precipitate does PQ-OH exhibit its large-Stokes-shift ESPIT-based fluorescence, that involves intramolecular transfer of the phenolic hydrogen (ex/em 360/530 nm). Therefore, fluorescence is unambiguously due to cyclisation-mediated cargo release, and combined with the large Stokes shift, the system gives excellent signal-to-background ratios of typically \(>100\) without needing background subtraction.

Probe synthesis: 1,2-dithiolane 6 was prepared using an approach initially reported by Raines\(^{67}\) followed by N-methylation and Boc-deprotection (Fig 2b). All intermediates containing the S-membered cyclic disulphide showed degradation upon standing, that we presume to be linear polysulphide oligomers. This occurred even without clear stimuli (e.g. stirring in dichloromethane). The cyclic monomer could typically be recovered by stirring in dilute methanolic solution, although isolating the monomer from this solution while avoiding re-polymerisation was not straightforward. The N-methylation step suffered particular tendency towards polymerisation, until we found that the monomeric product could be extracted from methanol by hexane washes (see Supporting Information). The final fluorogenic probe SS-50-PQ was assembled by carbamate coupling with PQ-OH.

1,2-dithiolane is unstable in probes: Polymerisation was also observed for probe stock solutions in DMSO. Their maximal fluorescence, determined by applying a standardised reducibility test (aq. buffer, pH 7.4, 10 eq of the quantitative disulphide reductant tris(carboxymethyl) phosphine, TCEP) decreased over time. We understood this as a consequence of polymerisation, since the hydrophobic polymeric degradation products would have decreased accessibility to solvated reductants. Fresh probe stocks were therefore prepared immediately for each assay from powdered solid, then assayed for quality with comparison to calibration curves established by precipitating the theoretical amount of PQ-OH. Only stocks yielding TCEP-related signals within 10% of the calibration intensity were used in assays. In fact, we re-prepared SS-50-PQ five times during this research to maintain high-quality stocks.

Figure 2: 1,2-dithiolane probe design and synthesis. (a) After reduction of the 1,2-dithiolane in SS-50-PQ, thiolate cyclisation releases the precipitating phenol PQ-OH that gives ESPIT-based fluorescence in the solid state. (b) Synthesis of SS-50-PQ: (i) Boc,O, NEt\(_3\), dioxygen/H\(_2\)O\(_2\), r.t., 15 h (96%) then either MsCl, py, DC, followed by KSAc, acetone, 60°C, 2 h (89%), or HSac, PPh\(_3\), DIAD, THF, 0°C to r.t., 15 h (88%). (ii) KOH, MeOH, open to air, r.t., 15 h (98%). (iii) Me, NaH, DMF, 0°C to r.t., 0.5 h (70%). (iv) PQ-OH, triphosphene, NEt\(_3\), DC, 0°C to r.t., 1 h; then 6, NEt\(_3\), DC, 0°C to r.t., 1 h (49%).

1,2-dithiolane is nonspecifically reduced by various thiols: A trigger can only be enzyme-selective in the cellular context, if it resists transthiolelation or reduction by the cellular monothiol background (ca. 50 mM, of which ca. 5 mM GSH\(^{49-52}\)). Hence, we began testing the potential for selectivity by performing cell-free incubations of SS-50-PQ (10 \(\mu\)M) with GSH. Probe “sensitivity or resistance” to challenge by a species, is often cited according to a measurement at a single challenge concentration at a single timepoint. However, this allows inconsistent or selectively chosen results to oversimplify or misrepresent a more complex situation. To provide a useful characterisation of probe resistance to monothiols, we titrated GSH...
over a wide concentration range (0.01 to 10 mM) and collected time-course fluorescence data (Fig 3a). For more meaningful representations, we normalised the signals at each timepoint to the maximum fluorescence time-course value at that timepoint (TCEP), and also calculated dose-response curves at various endpoints (Fig 3b; see Fig S2). This normalisation is important and can be generally recommended: it helps to separate kinetics of fragmentation from kinetics of reduction (otherwise, slow fragmentation can hide reductive sensitivity); and it allows direct comparison of experiments relative to their theoretical maxima. We observed strong, fast probe response to even subphysiological GSH levels. The GSH concentrations causing half-maximal fluorescence ("EC_{50}^{0.5}\text{mM}") were ≤ 1 mM (Fig 3b; Fig S2). This indicates that 1,2-dithioleane probes can be rapidly and fully reduced by cellular GSH concentrations, even without enzyme catalysis involved.

We also screened other monothiol reductants, e.g. cysteine (Cys), N-acetylcysteine (NAC), N,N-dimethyl-cysteamine (MEDA), and cysteamine (CA) and found fast probe activation (Fig 3c-d) with similar concentrations and kinetics compared to GSH. This suggests that 1,2-dithioleane is generally instable to monothiols, so that probes derived from it might be rapidly activated by the intracellular thiol background. Matching expectations from Creighton and Whitesides, the probe was quantitatively and rapidly triggered by equimolar vicinal dithiol DTT. We controlled for release by mechanisms other than transthiolation/reduction-triggered cyclisation, using serine (Ser) and glutathione disulphide (GSSG). The probe was entirely stable to non-reductive degradation as e.g. aminolysis, highlighting the stability of the tertiary phenolic carbamate, and supporting that transthiolation/reduction is its pathway for signal generation (Fig 3c).

In summary, these assays show that 1,2-dithioleanes do not resist uncatalysed reduction by monothiols, even at subphysiological concentrations. This provides initial evidence that probes using 5-membered cyclic disulphides may not be enzyme-selective in the biological context.

1,2-dithioleane is nonspecifically reduced by redox-active proteins and enzymes: We next tested probe reduction with redox proteins from the Trx/TrxR and Grx/GSH/GR systems. Each protein has multiple isoforms, as has been excellently reviewed.53 We employed recombinant human Trx1 and Trx2; the thioredoxin-related protein TRP14, which features a vicinal dithiol/disulphide redox-active site that is also reduced by TrxR; the oxidoreductases TrxR1, TrxR2 and Grx; and human vicinal dithiol glutaredoxins Grx1 and Grx2. Both Trxs and Grxs have orders of magnitude higher cellular concentrations (ca. 10 µM) than their upstream TrxR and Grx partners (ca. 20 nM), so we reflected these concentrations in our assays. SS-50-PQ was challenged with combinations of proteins from these redox systems. To study whether probes were reduced by the effectors Trx or Grx, and/or by direct reaction with the upstream reductants TrxR or Gr, we compared assays using both effectors and upstream reductants, against assays employing only upstream reductants or only effectors (TrxR/Grx assays included NADPH; GR+Grx assays included 10 µM GSH for Grx reduction; see Supporting Information).

The 1,2-dithioleane probe was nonspecifically reduced, with Trx1, Trx2, TRP14, Grx1, Grx2, TrxR1 and TrxR2 all rapidly reaching high conversion. Only the highly GSSG-specific enzyme Gr gave no signal (Fig 3e, Fig S3). Time-courses even showed that both enzymatic cascades (TrxR/Trx and GR/Grx; Fig 3f) have identical activation profiles.

Taken together, the 1,2-dithioleane probe is nonselectively and nonenzymatically triggered by GSH and monothiols at subphysiological concentrations, as well as by a broad range of dithiol/disulphide-type proteins and enzymes. The systematic variation and titration of chemo- and bioreductants, and the examination of both time-course and endpoint data, show that 1,2-dithioleane is not a TrxR-selective substrate in a cell-free setting.
The 1,2-dithiolane probe is cellularly activated: We tested the cellular activation of SS-50-PQ in four cell lines: HeLa cervical cancer, A549 lung cancer, Jurkat T-cell lymphoma cells, and mouse embryonic fibroblasts (MEF). All cell lines rapidly generated well-defined fluorescent precipitates of PQ-OH. Fluorescence plate reader quantification showed a nearly linear increase of signal in the first 6 h (Fig 4a) and the signal was concentration-dependent (Fig 4b), indicating that no saturation effects are operative. The solid precipitates of PQ-OH were intracellularly localized and visible in most cells (Fig 4c). Because plate reader data and microscopy images can misrepresent population-level response, we used flow cytometry to collect single-cell-resolved statistics of probe activation. Though this is unusual for small molecule probes, it is possible with SS-50-PQ because the solid PQ-OH precipitate is cellularly retained during cell fixation. These data showed a monomodal fluorescence intensity distribution with >60% of cells exhibiting strong PQ-OH fluorescence (Fig 4d, Fig S5).

The 1,2-dithiolane probe is activated in vivo: At this point, we considered it valuable to apply SS-50-PQ in a simple in vivo model, to test three design goals of our phenolic carbamate probe system: (a) zero signal background due to mechanistic quenching in the probe and high Stokes shift of the released fluorophore; (b) no spontaneous cargo release; due to the hydrolytic robustness of the tertiary carbamate; and (c) cellular retention of PQ-OH precipitates for high-spatial-resolution imaging in vivo. Therefore, we incubated zebrafish zygotes and embryos up to 3 days post fertilisation (dpf) in media containing SS-50-PQ and acquired time-courses by epifluorescence and live confocal microscopy (Fig 4e, Fig S7-S8). Probe activation began within two hours, with intriguing localization of the marked cells, although the interpretation of this pattern would need additional investigation and we do not believe that it is connected to TrxR activity. Most pleasingly however, all three probe-design goals were achieved: high-contrast images were obtained without background manipulation, with precise resolution that will be particularly important for future investigations.

Cellular probe activation is not blocked by TrxR suppression: We then tested the TrxR-specificity of cellular probe activation in several ways. Cells cultured without selenium supplementation may not fully incorporate SeCys in TrxR, lowering cellular TrxR activity. However, we did not observe significant dependency of SS-50-PQ signal upon NaSeO3 supplementation (Fig 5a). We more stringently evaluated TrxR-dependency using a TrxR1 knockout MEF cell line (TrxR1−/−) compared to its control (TrxR1+/+). Their signal time-courses were indistinguishable, i.e. probe activation is independent of TrxR1 expression (Fig 4b). We then explored whether chemical inhibition of TrxR affects signal generation, using the selective TrxR inhibitors TRi-1 and TRi-3. Cells pretreated with TRIs gave similar probe signals as the no-inhibitor controls (with partial inhibition observed for high-concentration experiments above 2 µM) and depending on the time of pre-incubation, regardless of whether this was evaluated as in time-course or endpoint (Fig 5c-d; and Fig S4) population-average assays, or with single-cell statistics (Fig 5e; and Fig S5). The inhibition effect was evaluated for different duration of the cells’ pre-treatment to elucidate, that the cells recover from TrxR inhibition and their biological consequences within a time window of 15 h. Therefore, neither TrxR inhibition nor TrxR knockout greatly alter the cellular activation of SS-50-PQ. Together with the cell-free results showing rapid and non-specific activation of 1,2-dithiolane by cellular thiol, we conclude that cellular activation of 1,2-dithiolane probes does not exclusively report on TrxR activity.
Why might 1,2-dithiolanes have been previously proposed as TrxR-selective substrates? Our cell-free and cellular assays had however shown that 1,2-dithiolane is not selective for TrxR. However, previous studies of 1,2-dithiolane probes have claimed excellent TrxR selectivity. This claim was based on assays where exposure of cells or lysates to auranofin (AF; Fig S5) dose-dependently reduced fluorescence signals as compared to untreated controls.\(^{24}\) We were intrigued to study AF more closely, to understand if/why it may have led to misinterpretation of selectivity, and to assess what information monomeric SS-50-type probes really provide in the biological context.

AF is a thiophilic Au(I) complex that is popularly used as an inhibitor of TrxR. AF binds TrxR in cell-free assays and in cells. However, AF is more generally a "potent thiol-reactive species"\(^{35}\) that is also reported to bind to at least 20 other thiol protein targets, and its therapeutic mechanisms of action are widely accepted to be unresolved,\(^{36}\) although it is clear that TrxR is one of its major targets.\(^{37}\) Notably, AF is a particularly strong binder of membrane thiols, possibly driven by its lipophilicity.\(^{38}\)

We confirmed that cells pretreated with AF (0.1 to 4 µM) had decreased probe processing, seen by both single-cell (Fig S5e, Fig SS-6) and population average (Fig 5d,f) measurements. However, this does not necessarily indicate that probe processing occurs through TrxR. Given AF's polypharmacology, the dithiolane's cell-free nonselectivity, and the minor effects of non-Au-based TrxR modulation on probe activation (Fig 5a-e), we were motivated to look more critically into this result. Conclusively, even in a fully cell- and enzyme-free setting, AF catalytically suppressed the fluorescence generated upon TCEP treatment, without a clear dose-dependence (Fig 5g). Therefore, signal suppression in the cellular assay cannot be used to argue that TrxR, or any other cellular target of auranofin, might be a selective reductant of 1,2-dithiolane.

[Figure 5: The activation of 1,2-dithiolane probes in cells does not require TrxR. (a) Cellular fluorescence time-courses in A549 cells cultured with/without NaN\(_3\)-supplemented medium (50 µM SS-50-PQ). (b) Cellular fluorescence time-courses of TrxR1-knockout (-/-) and -wildtype (+/+) MEF cells. (c) Cellular fluorescence time-courses of HeLa cells pretreated with the gold-free TrxR inhibitors TRI-1 and TRI-3 (25 µM SS-50-PQ, 15 h pre-treatment). (d) Cellular fluorescence dose-response plots for cells pre-treated with TRI-1, TRI-3 and AF (25 µM SS-50-PQ, 15 h pre-treatment). (e) Flow cytometry-based single-cell statistics of cellular fluorescence (25 µM SS-50-PQ) in HeLa cells optionally pretreated (15 h) with TRI-1, TRI-3 or the Au(I)-based inhibitor auranofin (AF; 4 µM), compared to reference fluorescence ("blank", no SS-50-PQ) and to uninhibited cells ("no inhibitor"). (f) Fluorescence time-courses of HeLa cells pretreated with AF (25 µM SS-50-PQ). (g) Cell-free, enzyme-free fluorescence time-course of TCEP (100 µM) reduction of SS-50-PQ (25 µM in TE-buffer) after pretreatment of the probe stock with AF (at rt for 2 h).

We present two hypotheses that plausibly explain why AF suppresses signal from SS-50-PQ, that are consistent with 1,2-dithiolane's being a nonspecific thiol probe, for which TrxR is not a significant cellular reductant. (1) The strained disulfide probe usually benefits from dynamic covalent exchange enhancement of cellular delivery, therefore AF's binding to membrane thiols\(^{39}\) suppresses cellular delivery and signal. Similar suppression of delivery of strained disulfide substrates has been achieved by thiophilic organic alkylators of exofacial thiols.\(^{40}\) This delivery inhibition is consistent with literature findings and our data showing that reaching the intracellular environment, 1,2-dithiolanes are nonspecifically opened by thiols (Fig 3), and it could be responsible for some or most of the cellular signal suppression by auranofin. (2) AF reacts with 1,2-dithiolane probes...
to suppress signal by both directly and catalytically diminishing their reducibility. Literature reports indeed show that Au(I)-(thiolate)(phosphine) complexes react with disulfides, initially forming triethylphosphine oxide and the thioglucose-gold-thiolate complex.22,26 Since Au(I)-bis(thiolate) complexes undergo facile net ligand exchange with disulfides, they mediate thiol-disulfide exchange reactions22, which in the case of 1,2-dithiolane would trigger ring-opening polymerisation.26 Polymerisation of the 1,2-dithiolane-based probe would generate hydrophobic polymeric products, which (as literature suggests22) are not reducible by even such strong aqueous reductants as TCEP, so suppressing fluorescent signal generation. This would explain auranofin’s cell-free (Fig 5g) signal suppression, and can even be sufficient to explain its cellular signal suppression, that we and others have observed.

Motivated by the known broad spectrum of auranofin’s targets and effects, it would be beneficial to at least take design precautions (e.g. cell-free controls testing AF-probe interactions, or using TRi inhibitors for competition assays) to identify likely confounding factors before advancing targets of strained disulfide probes based on AF experiments. We think it would be best to avoid Au-based reagents altogether, due to the high chance of any assay result being misinterpreted based on a populous literature of AF targets with no clear consensus. These may be valuable guides for analysis of cellular redox probe selectivities in the future.

CONCLUSIONS

Specific dithiol/disulfide-exchange reactions are central to biology, and engineered disulfides exploiting these reaction manifolds are finding applications from chemical biology probes to biophysics and materials chemistry. Linear disulfides have been known for decades as nonspecifically transthiolated/reduced substrates, and often used for intracellular release of cargos. 1,2-dithiolanes have emerged as substrates of interest in chemical biology13-15, although it has remained contentious whether its reduction is enzyme-selective, or nonspecific. We answered this question by studying the biochemical and biological performance of the novel 1,2-dithiolane-based redox probe SS-50-PQ. Here, the disulfide was integrated in a stable, modular design that can be adapted to release arbitrary phenols: in this case, a release-activated fluorescent cargo, that we could even use in flow cytometry studies and for cell-resolved imaging in live embryos.

A rigorous methodology of reductant titrations, enzyme panel screenings, and inhibitor/knockout cell experiments conclusively demonstrated the nonspecific reduction-based cleavage of the 1,2-dithiolane. While 1,2-dithiolane can indeed be rapidly opened by TrxR32 (Fig 3c), it is also rapidly opened by many other reducing thiol species. Therefore, in cells, it is highly unlikely to selectively report on TrxR, instead of on more abundantly present reductants (Fig 3). The cellular data show that neither TrxR knockout, nor TrxR inhibition by recently developed Au-free inhibitors, greatly modulate the cellular signal obtained from the 1,2-dithiolane probe (Fig 5). We pool literature references with our data to show that inhibition of cellular activation of probes based on 5-membered cyclic disulfides by treatment with auranofin can result from at least two plausible, precedent processes that do not involve TrxR: i.e., delivery inhibition by binding exofacial thiols, and suppressing probe reducibility by catalysing dithiolane ring-opening polymerisation. Taken together, we conclude that 1,2-dithiolane is an easily and nonspecifically transthiolated and/or reduced motif, that is not a TrxR-specific substrate.

On the other hand, there may be immediate rewards if 1,2-dithiolane-based reduction probes are properly re-evaluated. This would help maintain a clear literature, avoiding nonspecific electrophilic pan-assay interference compounds (PAINS) being falsely identified as TrxR-substrate hits, and could perhaps allow 1,2-dithiolane probes or produgs instead to find valuable applications as simple, modular systems for thiol-mediated cellular uptake and activation, impacting research in cell penetration and assisted uptake (extended discussion as a Supplementary Note in the Supporting Information).

Diversifying trigger structures to reach redox substrates that are selective for the key oxidoreductases remains a central goal for research in the field. Our modular probe system already ensured zero signal background, excellent hydrolytic robustness, and cellular marking for high-spatial-resolution imaging; these beneficial features might be transitioned to powerful redox probes, if more specific reduction-sensing units can be identified and installed as trigger motifs. In particular, selective substrates for GR, TrxR or Trx would, at last, allow researchers to unveil the dynamics that drive these major dithiol/disulfide-type enzyme systems within cells. By identifying and avoiding problematic and nonselective substrate types, chemical development may instead deliver selective and robust redox chemotypes for bioreductive probe and prodrug research; these will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

Synthesis, analysis, biological evaluations, extended discussion (PDF)

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Cyclic 5-membered disulfides are not selective substrates of thioredoxin reductase, but are opened nonspecifically by thiols

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### Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| AF           | auranofin   |
| Boc          | tert-butoxycarbonyl |
| CA           | cysteamine  |
| COSY         | correlated spectroscopy |
| Cys          | L-cysteine  |
| DAD          | diode array detector |
| DCM          | dichloromethane |
| DDQ          | 2,3-dichloro-5,6-dicyano-1,4-benzoquinone |
| δ            | chemical shift (ppm) |
| DIAD         | diisopropyl azodicarboxylate |
| DIPEA        | diisopropylethylamine |
| DMEM         | Dulbecco's modified eagle medium |
| DMF          | dimethylformamide |
| DMSO         | dimethylsulfoxide |
| dpf          | days post fertilization |
| DTT          | dithiothreitol |
| EC₅₀         | half maximal effective concentration |
| EI           | electron ionisation |
| ESI          | electron spray ionisation |
| ESIPT        | excited-state intramolecular proton transfer |
| ETP          | epidithiodioxopiperazine |
| ex/em        | excitation/emission |
| FACS         | fluorescence activated cell scanning |
| FBS          | fetal bovine serum |
| GFP          | green fluorescent protein |
| GR           | glutathione reductase |
| Grx          | glutaredoxin |
| GSH          | glutathione (reduced form) |
| GSSG         | glutathione disulfide |
| HeLa         | Henrietta Lacks cervical cancer cell line |
| HEPES        | 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid |
| HMBC         | heteronuclear multiple bond correlation |
| HPLC         | high-pressure liquid chromatography |
| hpt          | hours post treatment |
| HRMS         | high-resolution mass spectrometry |
| HSAc         | thioacetic acid |
| HSQC         | heteronuclear single quantum coherence spectroscopy |
| MEDA         | N,N-dimethylcysteamine |
| MEF          | mouse embryonic fibroblasts |
| MsCl         | methanesulfonyl chloride |
| NAC          | N-acetyl L-cysteine |
| NADPH        | β-nicotinamide adenine dinucleotide phosphate |
| NMR          | nuclear magnetic resonance |
| Abbreviation | Full Form |
|--------------|-----------|
| PBS          | phosphate-buffered saline |
| PCR          | polymerase chain reaction |
| PQ-OH        | 6-chloro-2-(5-chloro-2-hydroxyphenyl)quinazolin-4(3H)-one |
| Pr-SH        | a general cellular protein monothiol |
| Rf           | retention factor |
| RPMI         | Roswell park memorial institute medium |
| Ser          | L-serine |
| S-Gem        | disulfide-gemcitabine prodrug |
| SS-50-PQ     | 4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl \(1,2\)-dithiolan-4-yl (methyl)carbamate |
| TCEP         | tris(2-carboxyethyl)phosphine |
| TE           | Tris/EDTA buffer |
| THF          | tetrahydrofuran |
| TRFS         | TrxR fluorescent substrate probe (Fang et al.) |
| TRi          | TrxR inhibitor (Arnér et al.) |
| TRP14        | thioredoxin-related protein of 14 kDa |
| Trx          | thioredoxin |
| TrxR         | thioredoxin reductase |
1. 1,2-dithiolane-based probe strategies

(a) 1,2-dithiolane-mediated uptake probes

- Surface-initiated polymerization
  Matile, Chem. A. Eur. J., 2013

- Cellular uptake of liposomes
  Matile, ANIE, 2017

- Thiol-mediated cellular uptake
  Matile, ANIE, 2015

- Receptor-mediated uptake
  Matile, JACS, 2017

- Enhanced cellular uptake
  Matile, ChemBioChem, 2020

(b) 1,2-dithiolane-based TrxR probes

- Green probe for TrxR
  Fang, JACS, 2014

- Red probe for TrxR
  Fang, ChemComm, 2016

- Non-releasing probe for TrxR
  Fang, Nat. Comm., 2019

- Prodrug for TrxR
  Fang, ANIE, 2018

- 2-photon fluorescent probe for TrxR
  Fang, ChemComm, 2020

**Fig S1** 1,2-dithiolane-based probe strategies: representative recent examples from the literature. (a) 1,2-dithiolanes are used by Matile for strain-promoted thiol-mediated cellular uptake probes based on ring-opening polymerization initiated by exofacial thiols or other nucleophiles. (b) 1,2-dithiolane is employed by Fang as a reduction-sensing motif for cyclisation/release probes and prodrugs for TrxR activity.
2. Evaluation of reduction-mediated release

(a) Calculation of normalized fluorescence increase

(b) Calculation of GSH dose-response

Fig. S2 Evaluation of in vitro performance of SS-50-PQ (10 µM) with the monothiol reductant glutathione (GSH) via reduction-mediated release of solid fluorescent PQ-OH. (a) Calculation of normalized fluorescence increase from raw fluorescence data recorded on a platereader (ex/em 355bp/20/520lp) with respect to a reference experiment with maximum expected signal (TCEP – 100 µM) (b) Calculation of GSH-related dose-response curves of SS-50-PQ upon challenge with increasing GSH concentrations at different timepoints (t = 2 h, 4 h, 6 h).
3. Enzyme specificity/activity study

(a) Calculation of normalized fluorescence increase

(b) Probe activation by various redox cascades

**Fig. S3** Evaluation of in vitro performance of SS-50-PQ (10 µM) with various members of different redox cascades via reduction-mediated release of solid fluorescent PQ-OH. (a) Exemplary enzyme activity assay: calculation of normalized fluorescence increase from raw fluorescence data recorded on a platereader (ex/em 355bp/20/520lp) corrected by the basal fluorescence caused by autofluorescence of reduced NADPH and normalized to a reference experiment with maximum expected signal (TCEP – 100 µM) (b) Probe activation by various members of the different redox cascades: (1) TrxR1 (20 nM), Trx1 (10 µM), TrxR1+Trx1; (2) TrxR2 (20 nM), Trx2 (10 µM), TrxR2+Trx2; (3) TrxR1 (20 nM), TRP14 (10 µM), TrxR1+TRP14; (4) GR (20 nM), GSH (10 µM), Grx1 (10 µM), GR+GSH, GR+Grx1, GSH+Grx1, GR+GSH+Grx1; (5) GR (20 nM), GSH (10 µM), Grx2 (10 µM), GR+GSH, GR+Grx2, GSH+Grx1, GR+GSH+Grx2.
### 4. Cellular studies

**a**

**inhibitor studies - HeLa cells**

![Graphs showing cellular fluorescence time-courses and dose-response plots for HeLa cells pre-treated with TRI-1, TRI-3, and AF (25 µM SS-50-PQ, 3 h pre-treatment).](image)

**b**

**inhibitor studies - A549 cells**

![Graphs showing cellular fluorescence time-courses and dose-response plots for A549 cells pre-treated with TRI-1, TRI-3, and AF (25 µM SS-50-PQ, 3 h pre-treatment).](image)

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**Fig. S4** Additional evaluation of cellular response of SS-50-PQ influenced by selective TrxR inhibitors TRI-1, TRI-3 and Au(I)-based inhibitor auranofin (AF). (a) Cellular fluorescence time-courses and dose-response plots for HeLa cells pre-treated with TRI-1, TRI-3 and AF (25 µM SS-50-PQ, 3 h pre-treatment). (b) Cellular fluorescence time-courses and dose-response plots for A549 cells pre-treated with TRI-1, TRI-3 and AF (25 µM SS-50-PQ, 3 h pre-treatment).
Fig. S5 Flow cytometry-based workflow for data processing, used to collect single cell statistics of Jurkat T-cells treated with SS-50-PQ. (a) Untreated reference cells: (1) exclusion of debris in FSC-A/SSC-A, (2) selection of single cells in FSC-H/FSC-A. (b) Jurkat T-cells treated only with SS-50-PQ (25 µM) gated according to (1) and (2), followed by (3) live/dead selection applying zombie-staining (ex/em 647 laser / 780bp60), (4) PQ(+) cells out of the zombie(-) cell populations by detecting ELF-97 fluorescence (ex/em: 365 laser / 520lp). (c) Flow cytometry-related total count for an exemplary inhibitor screening experiment: Jurkat T-cells treated with SS-50-PQ (25 µM) optionally after 3 h of pre-incubation with TRI-1, TRI-3 or AF (2 µM). (d) Results from (c) in bar chart representation: comparison live cells (Zombie-negative) as a percentage of all gated single cells; and PQ(+) live cells as a percentage of all live cells.
Fig. S6 (a) Flow cytometry-based single-cell statistics of Jurkat T-cells treated with SS-50-PQ (25 µM) optionally after 3 h of pre-incubation with TRI-1, TRI-3 or AF (1-4 µM) to determine the onset of toxicity with each inhibitor. Comparison of live cells (Zombie-negative) as a percentage of all gated single cells; and PQ(+) live cells as a percentage of all live cells (b) Results with TRI-1, TRI-3 or AF used at nontoxic concentrations (1 µM and 2 µM for all inhibitors) and at moderately toxic concentration (4 µM), with pre-incubations of cells with inhibitor either for 3 h (all concentrations) or 15 h (only 4 µM).
5. *In vivo* animal model: zebrafish study

(a) **zebrafish zygotes (8 hpt)**

![Images of zebrafish zygotes incubated with SS-50-PQ](image)

(b) **zebrafish embryos 3 dpf (2 hpt)**

![Images of zebrafish embryos with SS-50-PQ](image)

**Fig. S7** *in vivo* animal model for reduction-responsive imaging. (a) Epifluorescence stereomicroscopy images of zebrafish zygotes incubated with SS-50-PQ (8 hpt). (b) of zebrafish embryos (3 dpf) with SS-50-PQ (2 hpt).
**Discussion of in vivo imaging in zebrafish**

Activation of fluorescence, as compared to untreated control experiments, was already observed in zebrafish embryos at low SS-50-PQ concentrations (5 µM). Aggregation of solid, fluorescent particles with high brightness inside the living cell was observed. Connected to the release of solid particles, cell death was subsequently observed.

When embryos already at a mobile development stage (3 dpf) were incubated with SS-50-PQ, signal was observed after short treatment time (2 hpt) at very low concentration. A clear response of the animals to the formation of the solid was observed leading to deformation, limitation of mobility and death. At 8 hpt no live animals were observed.

Maximum projection images were taken for the off-to-on signal switch when zebrafish embryos (3 dpf) were incubated with SS-50-PQ (15 µM) and solid particles could be resolved on a single-cell level and localized due to transparency of the organism. Release of solid, fluorescent particles was observed time-dependently after incubation with SS-50-PQ. Presumably, due to aggregation of release solid, the particles itself grow over time. Note that time-resolved confocal microscopy was possible because embryos were embedded in gel (leading to immobilization).
6. Supplementary Note: extended discussion

Specific dithiol/disulfide-exchange reactions are central to biology, and engineered disulfides exploiting these reaction manifolds have applications from chemical biology probes to biophysics and materials chemistry. 1,2-dithiolanes have emerged as substrates of interest in chemical biology, although it has remained contentious whether its reduction is enzyme-selective, or nonspecific. In this paper we answered this question by studying the biochemical and biological performance of the novel 1,2-dithiolane-based redox probe SS-50-PQ. The disulfide was integrated in a stable, modular design that can be adapted to release arbitrary phenols: in this case, a release-activated fluorescent cargo, that we could even use in flow cytometry studies and for cell-resolved imaging in live embryos.

A rigorous methodology of reductant titrations, enzyme panel screenings, and inhibitor/knockout cell experiments conclusively demonstrated the nonspecific reduction-based cleavage of the 1,2-dithiolane. While 1,2-dithiolane can indeed be rapidly opened by TrxR (Fig S3), it is also rapidly opened by GSH at sub physiological concentrations (Fig S2). Therefore, in cells, it is highly unlikely to selectively report on TrxR, instead of on more abundantly present reductants. The cellular data show that neither TrxR knockout, nor TrxR inhibition by recently developed Au-free inhibitors (Fig S4-6), greatly modulate the cellular signal obtained from the 1,2-dithiolane probe. We pool literature references with our data to show that inhibition of cellular activation of probes based on 5-membered cyclic disulfides by treatment with auranofin can result from at least two plausible, preceded processes that do not involve TrxR: i.e., delivery inhibition by binding exofacial thiols, and suppressing probe reducibility by catalysing dithiolane ring-opening polymerisation. Taken together, we conclude that 1,2-dithiolane is an easily and nonspecifically transthiolated and/or reduced motif, that is not a TrxR-specific substrate. Beyond the dithiolane-relevant references in the introduction to this paper, we also refer the interested reader to excellent work by Lothrop, Ruggles and Honda12, which shows that even when the unique selenothiol N-terminal reaction centre of TrxR is deleted, 1,2-dithiolane can still be reduced, further weakening the argument that dithiolane has any intrinsic selectivity for TrxR over dithiols.

This research also calls more generally for caution when claiming cellular mechanisms of probe activity based on inhibitor studies with the potential for direct inhibitor-probe interactions, spontaneous probe degradation, and known non-specific probe-activating mechanisms (such as 1,2-dithiolane exofacial thiolyis). In general, most biological literature using auranofin interprets AF assays in light of TrxR inhibition. Here, we have drawn attention to the thiol/disulfide reactivities of AF to caution that it cannot be used for mechanistic evaluation of cellular targets of strained disulfides, whereas recent gold-free TrxR inhibitors13 may be better suited for this purpose. Crucially, with a nuanced view of both AF and of dithiolanes, two promising avenues of research are immediately suggested.

Firstly, if the FDA-approved auranofin shows substantial inhibition of disulfide-mediated uptake not only in monolayer cell culture but also in 3D models and in vivo, this could make it interesting for repurposing, or as a therapeutic lead. The cellular uptake of some viruses and toxins is mediated by thiols, and inhibitors of this process are valuable targets, with the most potent inhibitors reported14 apparently being comparable to what we have observed with auranofin. In an illustrative recent example, AF was reported to slow SARS-COV-2 replication by 95%, although inhibition of thiol-mediated uptake was not considered as a potential basis for this inhibition15.

Secondly, in recent years, a range of 1,2-dithiolane-based probes have been reported as TrxR-specific substrates; they have been used as such for mechanistic validations during screening10 and in disease characterisation.7,16 Most if not all of these reports interpret cellular 1,2-dithiolane probe readouts as monitoring TrxR activity. These reports may benefit from a careful re-evaluation for alternative interpretation and application. Since 1,2-dithiolane probes such as TRFS-green are commercialised, they may continue to be used in assays without appreciation that their cellular readouts might not uniquely monitor TrxR-activity. However, research that assumes they are TrxR-selective, risks false outcomes in the same ways that have been reviewed for pan-assay interference compounds.17 Indeed, the set of 13 compounds that was reported to be validated as TrxR inhibitors on the basis of comparison to a 1,2-dithiolane probe16, consists entirely of hydrophobic PAINS compounds (9 Michael acceptors, 2 naphthoquinones, and 2 polyphenolic flavones) including the archetypical PAINS, curcumin18 and myricetin19, while the 20 new inhibitors also reported included no single compound that is not a typical or known PAINS candidate (ortho- or para-quinones, Michael acceptors, catecholic polyphenols/flavonoids, and the known thiol-reactive PAINS disulfiram20). The cautionary results and stepwise methodology we report can also prevent mistakes in the future. Both will be useful for systematising redox probe development.
7. General methods

7.1 Methods for in vitro evaluation

7.1.1 Cell-free chemoreductant assays

Cell-free in vitro assays were performed to evaluate the activation and release performance for **SS-50-PQ** on reduction-mediated release of the water-insoluble dye **PQ-OH** (precipitation-based induction of ESIPIT-based fluorescence monitored with ex/em 355bp10/520lp). The molecular process was monitored by fluorescence intensity increase based on the off-to-on mechanism. We noted that upon standing **SS-50-PQ** probe stocks had reduced capacity to generate fluorescence in reductant assays. Consequently, their probe stocks were prepared freshly from the solids for all assays.

In a black 96-well plate with black bottom, 80 µL of a diluted solution (12.5 µM in aq. TE, pH 7.4, 1.25% DMSO) of **SS-50-PQ** (final concentration 10 µM) was mixed with a solution of selected chemical reductants (20 µL of a solution (50 µM to 50 mM in aq. TE, pH 7.4). As reductants were used tris(carboxyethyl)phosphine (TCEP) at 100 µM final concentration; dithiothreitol (reduced, DTT), glutathione (reduced, GSH), glutathione (oxidized, GSSG), L-Cysteine (Cys), L-Serine (Ser), mercaptoethyl-dimethylamine (MEDA), cysteamine (CA) and N-acetyl L-Cysteine (NAC) all at 1 mM final concentration. For cell-free inhibition experiments 80 µL of a diluted solution (62.5 µM in aq. TE, pH 7.4, 1.25% DMSO) of **SS-50-PQ** (final concentration 50 µM) was pre-treated with auranofin (from ethanolic stock solutions) with final concentrations 0.5 µM, 1.5 µM and 4.0 µM 15 h before maximum reduction was induced by addition of 20 µL TCEP (at 100 µM final concentration).

The reaction mixtures were incubated at 37 °C and 100% humidity for 6 h. Time-course measurements were conducted to determine kinetics of the reduction-mediated release. Data was interpreted by calculating the absolute, time-dependent fluorescence intensity \( F(t) \) normalised to the plateau signal observed in a reference maximum-fluorescence experiment \( F_{\text{max}}(t) \) that used incubation with 100 µM of TCEP. For monothiol-reductant concentration-dependent **SS-50-PQ** was challenged with increased concentrations of glutathione (reduced, GSH) at 10 µM, 30 µM, 100 µM, 300 µM, 1 mM, 3 mM and 10 mM and the dose-response relation was later calculated from data representation on a \( \log_{10} [\text{GSH/probe}] \) vs. \( F/F_{\text{max}}(t = 6h) \) graph.

7.1.2 Cell-free bioreductant assays

Cell-free in vitro enzyme specificity assays (bioreductants) were performed to evaluate the specificity and reaction kinetics of **SS-50-PQ** to enzymatic reduction based on the reduction-mediated release of **PQ-OH** after incubation with bioreductant cascade model systems. The molecular process was equally monitored by fluorescence intensity increase (ex/em 355bp10/520lp).

In a black 96-Well Plate with black bottom, 70 µL of a diluted solution (14.3 µM in aq. TE, pH 7.4, 1.43% DMSO) of **SS-50-PQ** (final concentration 10 µM) was mixed with 10 µL of a solution (0.2 µM in aq. TE, pH 7.4) of a of the selected oxidoreductase enzyme (TrxR1, TrxR2, GR to reach 20 nM final concentration) and/or 10 µL of a solution (100 µM in aq. TE, pH 7.4) of its respective native substrate (Trx1, Trx2, TRP14, Grx1, Grx2 to reach 10 µM final concentration). The biochemical reaction was started by addition of 10 µL of a solution of NADPH (1 mM in aq. TE, pH 7.4 to reach 100 µM final concentration).

Human recombinant thioredoxin (Trx1 and Trx2) (lyophilized) human recombinant glutaredoxin (Grx1 and Grx2) (lyophilized from 10 µL TE-buffer, pH 7.5), human recombinant thioredoxin-related protein of 14 kDa (TRP14), human thioredoxin reductase (TrxR1 and TrxR2) (1.5 mM/L in 50% glycerol/TE-buffer, pH 7.5) and human recombinant glutathione reductase (GR) (100 µM in 50% glycerol/TE-buffer, pH 7.5) were obtained from IMCO Corp., Stockholm (Sweden) or produced and purified as previously described.\(^{21-24}\)

The reaction mixtures were incubated at 37 °C and 100% humidity for 6 h. Time-course measurements were conducted to determine kinetics of the enzyme-mediated release. Data was interpreted by calculating the absolute, time-dependent fluorescence intensity \( F(t) \) corrected by the absolute, time-dependent background fluorescence \( F_{\text{NADPH}}(t) \) caused by autofluorescence of reduced NADPH normalised to the plateau signal observed in a reference maximum-fluorescence experiment \( F_{\text{max}}(t) \) with 100 µM of TCEP.

S13
7.2 Cell culture methods

7.2.1 General cell cultivation methods

HeLa/A549 Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM: L-glucose (4.5 g/L), L-glutamine, L-pyruvate, phenol-red, NaHCO₃ (2.7 g/L)) purchased from PAN Biotech, Aidenbach (Germany). Jurkat cells were grown in RPMI-1640 medium (L-glutamine, sodium bicarbonate) purchased from Sigma-Aldrich Life Science by Merck KGaA, Darmstadt, Germany. Media were supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL) and NaSeO₃ (100 nM). For washing and resuspending steps PBS Dulbecco buffer from Merck GmbH, Darmstadt (Germany) was used and for trypsination, TrypLE™ Express from gibco Life Technologies Inc., Massachusetts (USA) was used. Centrifugation steps were conducted using a centrifuge 5810R from Eppendorf GmbH, Hamburg (Germany) was used. Cells were grown at 37 °C under 5% CO₂ atmosphere and cell growth was confirmed using a Nikon Eclipse Ti microscope from Nikon Corp., Minato (Japan).

7.2.2 Cell lines

HeLa (DSMZ; ACC 57), A549 (DSMZ; ACC 107), Jurkat (DSMZ; ACC 282) cell lines were purchased from the German Collection of Microorganisms and Cell Cultures. TrxR knockout and reference mouse embryonic fibroblasts (MEF) are a kind gift from Marcus Conrad. Briefly, MEFs isolated from conditional TrxR1 knockout mouse embryos, were immortalised by lentiviral transduction. In vitro deletion of TrxR1 was achieved by Tat-Cre induced recombination and verified by PCR and Immunoblotting for TrxR1. All cell lines are tested regularly for mycoplasma contamination and only mycoplasma negative cells are used in assays.

7.2.3 Cellular activation and inhibition assays

For evaluation of cellular processing of SS-50-PQ cells were seeded in 96-well plates (Microplates, 96-Well, F-Bottom, black, FluoTrack, high binding) from Greiner bio-one GmbH, Kremsmünster (Austria) at a medium level of 100 µL. Cell medium was treated with SS-50-PQ (from stock solution in 100% DMSO) to reach 10 µM, 25 µM 50 µM or 100 µM final concentrations at maximum final levels of 1% DMSO. For inhibition experiments cells were pre-treated with the selected TrxR inhibitors TRi-1 and TRi-3 (from DMSO stock solutions) and auranofin (from ethanolic stock solution), 3h or 15 h before further treatment with SS-50-PQ.

Treated cell plates were grown at 37 °C under 5% CO₂ atmosphere and time-course fluorescence measurements were conducted to determine kinetics of cellular processing. Fluorescence readout of cell-free activity and/or cell assays was performed either using a Fluostar Omega plate reader from BMG Labtech, Ortenburg (Germany) (ex/em 355bp20/520lp) or a Tecan Infinite M200 plate reader from Tecan, Maennedorf (Switzerland) (ex/em 355bp10/520lp recording fluorescence intensity. Data was interpreted by representing the absolute, time-dependent fluorescence intensity F(t).

7.2.4 Fluorescence microscopy

Intracellular formation of solid green-fluorescent particles corresponding to a time-dependent increase F(t) was confirmed using a Nikon Eclipse Ti2 upright microscope from Nikon Instruments Europe BV, Amsterdam (Netherlands) (ex/em 355bp50/410lp; or transmitted light, as appropriate). Images were processed using FIJI (ImageJ)²⁷ open-source image processing software.

Confocal time lapse microscopy was performed on live HeLa cells seeded in 8-well ibiTreat µ ibidi slides from ibidi GmbH, Martinsried (Germany). Dishes were placed on the motorized stage of a Leica SP8 laser-scanning confocal microscope (Wetzlar, Germany), treated with SS-50-PQ at indicated concentrations and immediately imaged for one hour at ex/em: 405/530bp20 comparing the fluorescence and the brightfield image. Acquired images were processed using FIJI (ImageJ)²⁷ open-source software.
7.2.5 Flow cytometry-based single-cell statistics

For single-cell resolved statistical analysis of intracellular solid green-fluorescent particles resulted from reduction-mediated activation/release of SS-50-PQ, a flow cytometry-based method was developed and implemented.

After respective treatment with TrxR inhibitors and/or SS-50-PQ, cells were harvested and stained with a fixable viability dye according to manufacturer’s recommendations (zombie NIR™ Fixable Viability Kit, BioLegend). Cells were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min and either stored in PBS or immediately resuspended in a wash buffer containing PBS with 1% bovine serum albumin (BSA) and 1 mM EDTA Disodium salt (0.1 mol/L or 0.2 N).

Flow cytometry was conducted at the BioMedical Centre Core Facility of the LMU Munich on a BD LSRFortessa instrument (BD Bioscience) using the BD FACSDiva Software. The following excitation/detection settings were used: zombie ex/em 647/780bp60; PQ fluorescence (ex/em: 355/530bp30). Data was processed using FlowJo v.10.7.1(BD).

An unstained sample was used to exclude cell debris and doublets. Zombie dye was used to exclude dead cells. PQ-positive gate was set, so that 0% of cells were PQ positive in an unstained sample. Cell debris, singlets, Zombie and PQ gates were set on an appropriate sample in each experiment and applied to all samples. General gating strategy is shown Fig S6.

7.3 Methods for in vivo animal model

7.3.1 Animal model

Adult zebrafish were housed in groups of 20-40 individuals in a fish facility (Aquaneering) maintaining approx. 700 mS, pH 6.9 – 7.1 and 28°C with a 14/10 h light/dark cycle as outlined by common zebrafish handling guidelines. All experiments used fertilized eggs from ab wild-type parents, grown in 30% Danieau medium (0.12 mM MgSO₄, 0.21 mM KCl, 0.18 mM Ca(NO₃)₂, 17.4 mM NaCl, 1.5 mM HEPES, pH 7.2) at 28°C, again with a 14/10 h light/dark cycle. To suppress melanin pigmentation, 150 µM phenylthiourea (PTU) was added to the 30% Danieau medium at 8-10 hpf when needed.

All procedures involving animals were carried out according to EU guidelines and German legislation (EU Directive 2010_63, licence number AZ 325.1.53/56.1-TU-BS).

7.3.2 In vivo activation experiments

SS-50-PQ was prepared as stock solutions (10 mM in DMSO) and stored at 4°C and briefly warmed to 37°C before use. 5 µM, 15 µM and 45 µM dilutions were freshly prepared in 30% Danieau supplemented with DMSO to 1% (final concentration). At 3 dpf, embryos were transferred to each well of a 6-well plate, and the 30% Danieau medium exchanged for the test dilutions (5 ml per well). Embryos were incubated at 28°C until the formation of solid green-fluorescent particles was observed.

7.3.3 Imaging

Successful signal development was confirmed on a stereofluorescence microscope (Leica M205FA or MDG41, from Leica Microsystems, Wetzlar, Germany) equipped with a UV filter (ex/em: 360bp40/420lp). Next, the embryos were embedded in lateral position in 1.2% ultra-low gelling agarose (type IX-A), overlaid with the test solution. The embryos were transferred to a confocal microscope (Zeiss Airyscan from Carl Zeiss microscopy, Jena, Germany) equipped with a 405 nm diode and several laser lines, with a Life Imaging Services heating chamber set to 28°C. Whole embryos were recorded using a 10× objective and the 405 nm diode for excitation, with subsequent tile/grid stitching in FIJI/ImageJ. Image acquisition was controlled by the Zen Black software from Carl Zeiss microscopy, Jena, Germany.
7.4 Laboratory techniques

All solvents, reagents and building blocks were purchased from standard commercial sources. Anhydrous solvents obtained in septum-capped bottles and analytical grade or higher quality solvents were used without purification. Industrial grade solvents were distilled prior to use. Unless otherwise stated, reactions were performed at room temperature without precautions regarding air or moisture and were stirred using a magnetic Teflon®-coated stir bars. Air or moisture sensitive reactions were conducted in dry Schlenk glassware.

Flash column chromatography was performed on Geduran® Si 60 silica gel from Merck GmbH, Darmstadt (Germany) an optionally conducted using a Biotage® Select automated column chromatography system from Biotage GmbH, Uppsala (Sweden). Unless stated otherwise, and thin layer chromatography to monitor reactions and determine Rf values was performed on silica coated aluminium sheets with fluorescent indicator (TLC Silica gel 60 F254 from Merck GmbH, Darmstadt, Germany) with visualisation by UV irradiation (254 nm/360 nm) or staining with KMnO4 solution (3.0 g KMnO4, 20 g K2CO3, 0.30 g KOH, 0.30 L H2O).

7.4.1 Mass spectrometry

High resolution mass spectrometry (HRMS) was conducted either using a Thermo Finnigan LTQ FT Ultra Fourier Transform ion cyclotron resonance spectrometer from ThermoFisher Scientific GmbH, Dreieich (Germany) applying electron spray ionisation (ESI) with a spray capillary voltage of 4 kV at temperature 250 °C with a method dependent range from 50 to 2000 u or a Finnigan MAT 95 from Thermo Fisher Scientific, Dreieich (Germany) applying electron ionisation (EI) at a source temperature of 250 °C and an electron energy of 70 eV with a method dependent range from 40 to 1040 u. All reported m/z values refer to positive ionization mode, unless stated otherwise.

7.4.1 NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker Avance (600/150 MHz, with TCI cryoprobe) or a Bruker Avance III HD Biospin (400/100 MHz, with BBFO cryoprobeTM) from Bruker Corp., Billerica (USA) either at 400 MHz or 500 MHz. 18F-NMR spectra were recorded on a Bruker Avance III spectrometer (400 MHz for 1H; 377 MHz for 18F). NMR-spectra were measured at 298 K, unless stated otherwise, and were analysed with the program MestreNova 12 developed by MestreLab Ltd., Santiago de Compostela (Spain). 1H-NMR spectra chemical shifts (δ) in parts per million (ppm) relative to tetramethylsilane (δ = 0 ppm) are reported using the residual protic solvent (CHCl3 in CDCl3: δ = 7.26 ppm, DMSO-d6 in DMSO-d6: δ = 2.50 ppm, CHD2OD in CD3OD: δ = 3.31 ppm) as an internal reference. For 13C-NMR spectra, chemical shifts in ppm relative to tetramethylsilane (δ = 0 ppm) are reported using the central resonance of the solvent signal (CDCl3: δ = 77.16 ppm, DMSO-d6: δ = 39.52 ppm, CD3OD: δ = 49.90 ppm) as an internal reference. For 1H-NMR spectra in addition to the chemical shift the following data is reported in parenthesis: multiplicity, coupling constant(s), number of hydrogen atoms and, if available, assignment. The abbreviations for multiplicities and related descriptors are s = singlet, d = doublet, t = triplet, q = quartet, p = pentuplet or combinations thereof, m = multiplet and br = broad. The numbering scheme used for the assignments is specified in each case in a figure depicting the respective molecular structure and does not follow any convention. The reported assignments are supported by 2D-NMR experiments (HMBC, HSQC, COSY). Where known products matched literature analysis data, only selected data acquired are reported.

7.4.1 HPLC(−MS) analysis

Analytical high pressure liquid chromatography (HPLC) analysis was conducted either using an Agilent 1100 system from Agilent Technologies Corp., Santa Clara (USA) equipped with a DAD detector and a Hypersil Gold HPLC column from ThermoFisher Scientific GmbH, Dreieich (Germany) or a Agilent 1200 SL system Agilent Technologies Corp., Santa Clara (USA) equipped with a DAD detector, a Hypersil Gold HPLC column from ThermoFisher Scientific GmbH, Dreieich (Germany) and consecutive low-resolution mass detection using a HCT ultra PTM discovery system applying ESI from Bruker Corp., Billerica (USA). For both systems mixtures of water (analytical grade, 0.1 % formic acid) and MeCN (analytical grade, 0.1 % formic acid) were used as eluent systems.
8. Synthetic Procedures

6-chloro-2-(5-chloro-2-hydroxyphenyl)quinazolin-4(3H)-one (PQ-OH, 2)\(^{26}\)

![Chemical structure](image)

A mixture of 5-chloro-salicylaldehyde 7 (1.92 g, 12.3 mmol), 2-amino-5-chlorobenzamide 8 (2.15 g, 12.6 mmol), TsOH (0.23 g, 1.2 mmol) and anhydrous EtOH (250 mL) was heated to reflux for 1 h. Then, the mixture was cooled to 0 °C. DDQ (2.72 g, 12.0 mmol) was added portionwise and stirring was continued at 0 °C for 1 h. The resulting precipitate was collected, washed with cold, anhydrous EtOH and dried under reduced pressure to afford PQ-OH (3.32 g, 10.5 mmol, 88%) as a faint yellow solid.\(^{26}\)

Bright green fluorescence was observed, when the solid PQ-OH (2) was exposed to UV light.

**HRMS (EI):** C\(_{14}\)H\(_{14}\)Cl\(_2\)N\(_2\)O\(_2\). [M-e] calc. 305.9958, found 305.9955. \(^1\)H-NMR (400 MHz, DMSO-d\(_6\)): \(\delta\) (ppm) = 13.36 (s, 1H), 12.62 (s, 1H), 8.29 (d, \(J = 2.6\) Hz, 1H), 8.10 (d, \(J = 2.3\) Hz, 1H), 7.90 (dd, \(J = 8.7, 2.4\) Hz, 1H), 7.85 (d, \(J = 8.8\) Hz, 1H), 7.50 (dd, \(J = 8.9, 2.6\) Hz, 1H), 7.05 (d, \(J = 8.9\) Hz, 1H).

**SS-50 (HCl) precursor synthesis**

![Chemical structure](image)

**N-Boc serinol (9)**\(^{10}\)

2-amino-1,3-diol 3 (2.50 g, 27.4 mmol) was dissolved in a 1:1 mixture of dioxane:H\(_2\)O (200 mL, 0.13 M) and NEt\(_3\) (4.85 mL, 35 mmol) was added. The mixture was cooled to 0 °C and a solution of Boc\(_2\)O (7.04 g, 32.2 mmol) dissolved in dioxane (35 mL) was added, the reaction mixture was allowed to warm to r.t. and was then further stirred for 15 h. The solution was acidified to pH 4 using 2 M aq. HCl, then saturated with potassium sodium tartrate and extracted with EtOAc (3×200 mL). The combined organic layers were dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure to give 9 (5.03 g, 26.3 mmol, 96%) as a colourless solid and used without further purification.\(^{10}\)

**TLC R\(_f\) = 0.50 (EtOAc).** The product matched literature analysis data.\(^{10}\) Selected data acquired: **HRMS (EI):** C\(_{16}\)H\(_{17}\)NO\(_3\)Cl\(_2\).[M-e] calc. 317.1046, found 317.1046. \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \(\delta\) (ppm) = 5.29 (d, \(J = 6.7\) Hz, 1H), 3.81 (dd, \(J = 11.1, 4.4\) Hz, 2H), 3.74 (dd, \(J = 11.1, 4.5\) Hz, 2H), 3.67 (s, 1H, NH), 2.89 (s, 2H, OH), 1.44 (s, 9H).

**N-Boc 2-amino-1,3-bis(acetyllthio)propane (4)**\(^{31}\)

**Protocol A:**

To a solution of PPh\(_3\) (13.50 g, 51.5 mmol) in anhydrous THF (300 mL) under N\(_2\)-atmosphere at 0 °C was dropwise added DIAD (10.0 mL, 50.8 mmol). The mixture was stirred for 30 min and precipitation of a colourless crystalline solid was observed. Then, a solution of N-Boc serinol 9 (4.70 g, 24.3 mmol) in anhydrous THF (25 mL) and HSAc (3.8 mL, 53.5 mmol) were added under vigorous stirring. The reaction mixture was stirred at 0 °C for 1 h, was then allowed to warm to r.t. and further stirred for 16 h, before being concentrated under reduced pressure. The residue was dry-loaded on activation silica gel (Ceduran®) and purified by flash column chromatography (isohecan:EtOAc, 90:10 to 0:100) to give and compound 4 (6.63 g, 21.6 mmol, 88%) as a colourless solid.\(^{31}\)

**Protocol B:**

**Step 1:** To a solution of N-Boc serinol (77 mg, 0.40 mmol), and pyridine (157 \(\mu\)L, 1.95 mmol) in anhydrous DCM (10 mL) under N\(_2\)-atmosphere at 0 °C was added MsCl (31 \(\mu\)L, 0.40 mmol). The
mixture was stirred for 30 min, then warmed to r.t. and stirred further for 1.5 h. After diluting with DCM (20 mL), the mixture was washed with sat. aq. NaCl (2×20 mL) and the aq. washings were extracted with DCM (2×10 mL). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated under reduced pressure to $N$-Boc 2-aminopropane-1,3-diyl dimethanesulfonate as a colourless oil, which was used without further purification.

**Step 2:** The material obtained in step 1 was dissolved in acetone (5 mL), potassium thioacetate (recrystallized from EtOH, 137 mg, 1.20 mmol) was added and the mixture was heated to reflux for 2 h. Then the volatiles were removed under reduced pressure and DCM (10 mL) and H$_2$O (10 mL) were added. The layers were separated and the aq. layer was extracted with DCM (2×10 mL). The combined organic layers were dried over Na$_2$SO$_4$, filtered over a short layer of silica gel and concentrated under reduced pressure to give compound 4 (110 mg, 0.36 mmol, 89%) as a colourless solid.

**TLC** $R_f = 0.36$ (n-pentane:Et$_2$O, 3:1). **HRMS (ESI$^+$):** C$_{12}$H$_{11}$NNaO$_2$S$_2$ $[M+Na]^+ $ calc. 330.08042, found 330.80393. **$^1$H-NMR** (400 MHz, CDCl$_3$): $\delta$ (ppm) = 4.88 – 4.62 (m, 1H), 3.99 – 3.80 (m, 1H), 3.07 (d, $J = 7.0$ Hz, 4H), 2.35 (s, 6H), 1.42 (s, 9H).$^{10}$

**N-Boc 1,2-dithiolan-4-amine (5)$^{10}$**

Bis-thioacetate 4 (4.59 g, 14.9 mmol) was dissolved in a 0.17 M solution of KOH in methanol (500 mL). The mixture was stirred at r.t. open to air for 15 h, concentrated under reduced pressure (at <40 °C) and the remaining yellow oil was dissolved in EtOAc (200 mL). The solution was washed with aq. NaCl (2×50 mL) and H$_2$O (100 mL), dried over Na$_2$SO$_4$, filtered and concentrated under reduced pressure (at T < 40 °C) to afford compound 5 (3.22 g, 14.6 mmol, 98%) as a yellow solid.$^{10}$

**TLC** $R_f = 0.49$ (isohexane:EtOAc, 10:1). **HRMS (ESI$^+$):** C$_{7}$H$_{13}$NNaO$_2$S$_2$ $[M-H]^− $ calc. 220.04714, found 220.04718. **$^1$H-NMR** (400 MHz, CDCl$_3$): $\delta$ (ppm) = 5.06 (d, $J = 3.9$ Hz, 1H, NH$_3$), 4.95 (s, 1H, CH$_3$), 3.21 (dd, $J = 11.6, 4.8$ Hz, 2H), 3.09 (dd, $J = 11.6, 2.0$ Hz, 2H), 1.44 (s, 9H).

**N-Boc N-methyl-1,2-dithiolan-4-amine (10)**

To a mixture of 60% NaH suspension in mineral oil (434 mg, 10.8 mmol), anhydrous DMF (180 mL) and iodomethane (731 µL, 11.7 mmol) under N$_2$-atmosphere at 0 °C was added dithiolane 5 (2.00 g, 9.04 mmol) in one portion. After stirring at 0 °C for 40 min, H$_2$O (500 mL) was added and the mixture was extracted with EtOAc (3×250 mL). The extracts were washed with H$_2$O (200 mL) and aq. NaCl (2×200 mL), dried over Na$_2$SO$_4$ and concentrated under reduced pressure.

The remaining yellow oil (3.41 g) was dissolved in methanol (250 mL) and KOH (2.10 g, 37.5 mmol) was added. This “depolymerization mixture” was stirred open to air for 6 days and was extracted four times (after 1 day, 2 days, 4 days and 6 days) to obtain compound 10 (507 + 587 + 307 + 90 mg = 1.49 g, 6.33 mmol, 70%) according to the following procedure:

The depolymerization mixture was extracted with hexanes (2×350 mL). The extracts were washed with half-saturated aq. NaCl (300 mL), dried over Na$_2$SO$_4$ and filtered over a plug of silica gel (32 g). The filtrate was discarded and compound 10 was eluted with a 1:8-mixture of EtOAc:hexanes (360 mL). After evaporating the eluent, compound 10 was obtained as a yellow oil. Additionally, the silica was washed with ethyl acetate (200 mL), the washings were evaporated, the remainder was dissolved in small amount of methanol and added back into the depolymerization mixture.

**TLC** $R_f = 0.49$ (isohexane:EtOAc 10:1). **HRMS (EI):** C$_{6}$H$_{13}$NO$_2$S$_2$ $[M-e]$ calc. 235.0696, found 235.0677. **$^1$H-NMR** (400 MHz, CDCl$_3$): $\delta$ (ppm) = 5.49 – 4.73 (m, 1H, 2-H), 3.30 (dd, $J = 12.0, 7.8$ Hz, 2H, 1-H), 3.03 (dd, $J = 12.0, 5.9$ Hz, 2H, 1-H$'$), 2.82 (s, 3H, 3-H), 1.47 (s, 9H, 6-H). **$^{13}$C-NMR** (101 MHz, CDCl$_3$): $\delta$ (ppm) = 155.3 (C=O, C4), 80.5 (O(CH$_3$)$_2$, C5), 61.2 (CH, C2), 40.5 (CH$_2$, C1), 29.5 (CH$_3$, C3), 28.5 (CH$_3$, C6).
**N-methyl-1,2-dithiolan-4-amine hydrochloride (6)**

![](image)

Dithiolane 10 (118 mg, 0.501 mmol) was dissolved in anhydrous MeOH (0.033 M, 15 mL) and stirred open to air for 1 h before a 1.25 M solution of HCl in methanol (5 mL, 6.25 mmol) was added. The resulting mixture was heated to reflux for 6 h and then evaporated under reduced pressure to yield hydrochloride 6 (85 mg, 0.50 mmol, quant.) as a colourless solid.

**1H-NMR (400 MHz, DMSO-d6):** δ (ppm) = 8.49 (s, 2H, 3-H), 3.35 (p, J = 5.1 Hz, 1H, 2-H), 2.58 (dd, J = 5.2, 1.7 Hz, 4H, 1-H), 1.74 (s, 3H, 4-H). **13C NMR (101 MHz, DMSO-d6):** δ (ppm) = 63.3 (CH, C2), 40.4 (CH2, C1), 31.1 (CH3, C4).

**4-chloro-2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)phenyl (1,2-dithiolan-4-yl) (methyl)carbamate (SS-50-PQ, 1)**

**Step 1:** To a solution of PQ-OH (57.2 mg, 0.187 mmol) in anhydrous DCM (5 mL) under nitrogen atmosphere at 0 °C was added a solution of triphosgene (60.5 mg, 0.204 mmol) in anhydrous DCM (1 mL), followed by a solution of DIPEA (38 µL, 0.221 mmol) in anhydrous DCM (2 mL). The resulting mixture was stirred at 0 °C for 30 min, then warmed to r.t. and stirred further for 30 min, before being evaporated under high vacuum (the volatiles were condensed in a liquid nitrogen trap and treated with 2M aq. NaOH as well as piperidine to discharge residual phosgene). The remainder was dissolved in anhydrous DCM (12.5 mmol) and this solution of PQ-OH-chloroformate was directly used in the next reaction.

**Step 2:** To a suspension of compound 6 (29.2 mg, 0.170 mmol) in anhydrous DCM (2 mL) was added DIPEA (34 µL, 0.20 mmol), the resulting solution was added dropwise at 0 °C to the solution of PQ-OH chloroformate obtained in step 1 and the mixture was stirred at 0°C for 15 min. Then, additional DIPEA (29 µL, 0.17 mmol) was added, before the mixture was warmed to ambient temperature. After stirring for 30 min, the mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (isohexane:EtOAc, 90:10 to 0:100) to give compound 1 (39.0 mg, 0.083 mmol, 49%) as a colourless solid.

Only weak, blue residual fluorescence was observed upon irradiation with UV-light, confirming that the obtained material was not contaminated with PQ-OH.

**TLC Rf = 0.26 (isohexane:EtOAc, 3:1). HRMS (ESI+):** C19H16Cl2N2O2S2+ [M+H]+: calc. 468.00046, found 468.00049.

Individual rotamers were observed by NMR spectroscopy at 298 K (ratio = 2/1) and time-averaged spectra were obtained at 373 K.

**1H-NMR (400 MHz, tetrachlorethan-d2, 373 K):** δ (ppm) = 8.24 – 8.22 (m, 1H, 15-H), 7.95 (d, J = 2.6 Hz, 1H, 9-H), 7.71 (d, J = 1.9 Hz, 2H, 17-H; 18-H), 7.50 (dd, J = 8.7, 2.7 Hz, 1H, 7-H), 7.20 (d, J = 8.7 Hz, 1H, 6-H), 5.09 (p, J = 6.7 Hz, 1H, 1-Hax), 3.32 (dd, 2J2-2 = 12.1 Hz, 3J2-1 = 7.6 Hz, 2H, 2-Hax), 3.10 (dd, 2J2-2 = 12.1 Hz, 3J2-1 = 5.6 Hz, 2H, 2'-Hax), 3.04 (s, 3H, 3-H). **13C-NMR (101 MHz, tetrachlorethan-d2, 373 K):** δ (ppm) = 160.1 (C=O, C13), 153.7 (C=O, C4), 149.0 (C=N, C11), 147.6 (C=Ar, C5), 147.4 (C=Ar, C14), 135.3 (C=Ar, C18), 133.6 (C=Ar, C19), 132.3 (C=Ar, C7), 132.1 (C=Ar, C8), 130.4 (C=Ar, C9), 129.7 (C=Ar, C17), 128.0 (C=Ar, C10), 126.1 (C=Ar, C15), 125.2 (C=Ar, C6), 122.4 (C=Ar, C16), 62.9 (CH, C1), 40.7 (CH2, C2), 30.6 (CH3, C3).

S19
9. NMR Spectra

*N-Boc 1,2-dithiolan-4-amine (5)*

**$^1H$-NMR**

![NMR Spectrum 1](image)

δ (ppm)

**$^{13}C$-NMR**

![NMR Spectrum 2](image)

δ (ppm)

S20
$N$-Boc $N$-methyl-1,2-dithiolan-4-amine (10)

$^1$H-NMR

$\begin{array}{c}
\text{SS} \text{N} \text{O} \\
\text{10}
\end{array}$

$\begin{array}{c}
\text{m} \quad 5.13 \\
\text{dd} \quad 3.03
\end{array}$

$\begin{array}{c}
\text{dd} \quad 3.30 \\
\text{s} \quad 2.82 \\
\text{s} \quad 1.47
\end{array}$

$^1$C-NMR

$\begin{array}{c}
\text{SS} \text{N} \text{O} \\
\text{10}
\end{array}$

$\begin{array}{c}
\text{-}155.4 \\
\text{-}80.5 \\
\text{-}61.3 \\
\text{-}40.5 \\
\text{<}29.5 \\
\text{<}28.6
\end{array}$
$N$-methyl-1,2-dithiolan-4-amine hydrochloride (6)

**^1H-NMR**

![^1H-NMR spectrum](image)

**^13C-NMR**

![^13C-NMR spectrum](image)
SS-50-PQ (1)

$^1$H-NMR (373 K)

\[
egin{array}{ccc}
\text{(m)} & \text{(d)} & \text{(d)} \\
8.23 & 7.71 & 7.20 \\
\text{(d)} & \text{(dd)} & \\
7.95 & 7.50 & \\
\end{array}
\]

\[
\begin{array}{cccc}
1 & 2 & 3 & 4 \\
5 & 6 & 7 & 8 \\
9 & 10 & 11 & 12 \\
13 & 14 & 15 & 16 \\
17 & 18 & \\
\end{array}
\]

$^{13}$C-NMR (373 K)

\[
\begin{array}{cccc}
160.1 & 153.0 & 147.6 & 135.3 \\
132.3 & 129.4 & 128.7 & 128.0 \\
126.1 & 125.2 & 122.4 & 62.9 \\
40.7 & 30.6 & \\
\end{array}
\]

\[
\begin{array}{cccc}
1 & 2 & 3 \\
4 & 5 & 6 \\
7 & 8 & 9 \\
10 & 11 & 12 \\
13 & 14 & 15 \\
16 & 17 & 18 \\
\end{array}
\]
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