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Inhibition of Thioredoxin Reductase 1 by Porphyrins and Other Small Molecules Identified By a High Throughput Screening Assay

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Abstract

The selenoprotein thioredoxin reductase 1 (TrxR1) has in recent years been identified as a promising anticancer drug target. A high throughput assay for discovery of novel compounds targeting the enzyme is therefore warranted. Herein, we describe a single-enzyme, dual-purpose assay for simultaneous identification of inhibitors and substrates of TrxR1. Using this assay to screen the LOPAC¹²⁸⁰ compound collection we identified several known inhibitors of TrxR1, thus validating the assay, as well as several compounds hitherto unknown to target the enzyme. These included rottlerin (previously reported as a PKC delta inhibitor and mitochondrial uncoupler) and the heme precursor protoporphyrin IX (PpIX). We found that PpIX was a potent competitive inhibitor of TrxR1 with a $K_i = 2.7 \mu\text{M}$ with regards to Trx1, and in the absence of Trx1 displayed time dependent irreversible inhibition with an apparent second-order rate constant (k_{inact}) of $0.73 \times 10^{-3} \pm 0.07 \times 10^{-3} \mu\text{M}^{-1}\text{min}^{-1}$. Exogenously delivered PpIX was cytotoxic, inhibited A549 cell proliferation and was found to also inhibit cellular TrxR activity. Hemin and the ferrochelatase inhibitor NMPP also inhibited TrxR1 and showed cytotoxicity, but less potently compared to PpIX. We conclude that rottlerin-induced cellular effects may involve targeting of TrxR1. The unexpected finding of PpIX as a TrxR1 inhibitor suggests that such inhibition may contribute to symptoms associated with conditions of abnormally high PpIX levels; such as, reduced ferrochelatase activity seen in erythropoietic protoporphyria. Finally, additional inhibitors of TrxR1 may be discovered and further characterized based upon the new high throughput TrxR1 assay presented here.

Keywords

Screening assay; thioredoxin reductase; inhibitor; substrate; protoporphyrin IX; rottlerin; cancer

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INTRODUCTION

The selenoprotein thioredoxin reductase (TrxR; EC 1.8.1.9) is a FAD containing homodimeric pyridine nucleotide-disulfide oxidoreductase with many cellular roles [1]. Together with NADPH and its prime substrate thioredoxin (Trx) the enzyme forms the core of the Trx system. The mammalian Trx system exerts a wide spectrum of functions including redox regulation, antioxidant defense, regulation of transcription factors as well as support of cell growth and replication [1]. Many of these functions involve the reduction of Trx, which may subsequently reduce a number of different substrates including ribonucleotide reductase [2], peroxiredoxins [3] or NFkB [4,5]. In addition, mammalian TrxR itself has a broad substrate specificity, reducing both protein and non-protein substrates, including low molecular weight compounds such as dehydroascorbate [6], lipoic acid [7], ubiquinone [8] and juglone [9].

In mammals, three separate genes encode TrxR isoforms, namely *TXNRD1* encoding the predominantly cytosolic TrxR1, *TXNRD2* encoding mitochondrial TrxR2 and *TXNRD3* encoding thioredoxin glutathione reductase (TGR), a testis-specific isoform of TrxR with an N-terminal glutaredoxin domain. These genes are intricately regulated, including differential splicing resulting in several different variants, and have been linked to a number of diverse and specific biological functions, as reviewed elsewhere [1]. Several recent studies argue for TrxR1 being an important anticancer drug target. The enzyme is frequently upregulated in a variety of tumors [10–13] presumably leading to an advantage in cell replication and antioxidant defense. The importance of TrxR1 for *in vivo* tumor progression in mice xenograft models was demonstrated by Yoo *et al.*, showing that knockdown of TrxR1 prevented tumor formation [14]. It was also shown that TrxR1 knockdown inhibits self-sufficient growth and DNA replication of cancer cells [15]. It has furthermore been demonstrated that the levels of TrxR1 in tumor cells can influence the outcome of cytotoxic drug treatment in a drug dependent manner; for example, cisplatin shows increased cytotoxicity with high TrxR1 levels compared to lower TrxR1 expression while other redox cycling drugs are conversely less cytotoxic under such conditions [16].

Several drugs in clinical use for anticancer treatment are indeed known to directly target TrxR1. Anticancer agents that inhibit TrxR1 include platinum derivatives (e.g. cisplatin [17,18] and oxaliplatin [19]), arsenic trioxide [20], nitrosoureas [21], nitrogen mustards (chlorambucil and melphalan) [19], and gold compounds (auranofin and aurothioglucose) [22–24]. Most of these drugs contain electrophilic functionalities that easily react with the nucleophilic selenocysteine (Sec) residue of the C-terminal active site of TrxR1 [1]. Thus, TrxR1 targeting may contribute to certain aspects of anticancer drug efficacy in addition to the importance of other drug targets in cancer cells.

In the present study, recombinantly expressed rat TrxR1 was utilized in a new high throughput drug screen to search for both novel substrates and inhibitors in a single assay as a means to discover new drug leads for potential anticancer treatment based upon targeting of TrxR1. A high throughput screen (HTS) of the LOPAC¹²⁸⁰ (Library of Pharmacologically Active Compounds, Sigma) library in quantitative concentration-response mode (qHTS) identified several candidate inhibitors of TrxR1. These included already known inhibitors of the enzyme such as cisplatin, chlorambucil and melphalan, thus validating the accuracy of the assay. Novel inhibitors were also discovered. The effects on the enzyme of two of the most interesting of these new inhibitors, rottlerin (or mallotoxin; 5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromine) and protoporphyrin IX (PpIX; 7,12-diethenyl-3,8,13,17-tetramethyl-21H,23H-porphine-2,18-dipropionic acid) were further characterized.

MATERIALS AND METHODS

Materials

Sec-containing recombinant rat TrxR1 (22.5 U/mg) was produced according to the method of engineering a gene compatible with the bacterial selenoprotein synthesis machinery and using the '2.4/24/24-protocol' [25], here with a BL21(DE3) *gor*⁻ host strain kindly provided by Arne Holmgren, Karolinska Institutet, Stockholm, Sweden. Recombinant human wild-type Trx1 was also generously provided by Arne Holmgren, Karolinska Institutet, Stockholm, Sweden. Production of the Sec-to-Cys mutant of rat TrxR1 was recently described elsewhere [26]. Auranofin was purchased from Alexis Biochemicals, CA, USA. Dimethylsulfoxide (DMSO) Certified ACS grade was from Thermo-Fisher Scientific (Pittsburg, PA). All other reagents or chemicals were obtained from Sigma-Aldrich Chemicals, unless stated otherwise.

Compound Library and Porphyrins

The 1,280-member Library of Pharmacologically Active Compounds (LOPAC¹²⁸⁰, Sigma-Aldrich) was purchased as a set of 10 mM stock solutions in DMSO and formatted as 1,536-well compound plates of 6 concentrations (1:5 dilution) at 5 μ L per well as previously described [27]. For further analyses of porphyrins, PpIX, NMPP and hemin were acquired either from Sigma or from Frontier Scientific, with stock solutions made in either DMSO ($\leq$ 1 mM porphyrin compound) or NaOH (at higher concentrations). With especially hemin easily precipitating, only freshly made solutions were utilized in the follow-up kinetics and cytotoxicity assays.

Dual-purpose TrxR1 assay and LOPAC qHTS

A stepwise description of the qHTS 1,536-well protocol is shown in Table 1. Briefly, 2 μ L of rTrxR1/buffer (90 nM rTrxR1, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.01% Tween-20, final concentrations) was dispensed into Greiner black solid-bottom 1,536-well assay plates using a BioRAPTR (Beckman Coulter, Fullerton, CA, USA) flying reagent dispenser, followed by 1 μ L of NADPH (400 μ M final concentration). After 5 min TrxR1 reduction at room temperature ($\sim$ 22 $^{\circ}$ C), 23 nL of compound solution in DMSO was added via Kalypsys PinTool. The plate was incubated for 15 min at room temperature and then transferred to a ViewLux high throughput CCD imager (PerkinElmer), wherein the substrate capacity of compounds was measured using a kinetic NADPH fluorescence read (λ_{ex} 340 nm/ λ_{em} 450 nm, *see* Read 1 and 2 in Table 1). For inhibitor assessment, a 1 μ L aliquot of sodium selenite (400 μ M final concentration) was dispensed to each well immediately following the substrate kinetic read. Then the plate was directly transferred to the ViewLux where kinetic measurements of NADPH fluorescence were acquired (*see* Read 3 and Read 4 in Table 1). The normalization formula for substrate assessment was as follows: % NADPH consumption = $100 \times [(N-X)/N]$, where X is the fluorescence change in the presence of the test compound and N is the mean of the fluorescence change in no-compound control. The control for the complete, or 100%, consumption of NADPH was based on no-NADPH reaction. The normalization formula for inhibitor assessment was as follows: % inhibition = $100 \times [(N-X)/(N-M)]$, where X is the fluorescence change in the presence of the test compound (change measured after selenite addition), N is the mean of the fluorescence change in no-compound control, and M is the fluorescence change corresponding to the no-enzyme control.

NADPH consumption and DTNB reduction assay

The standard DTNB reduction assay [28] was used to evaluate the hits of interest found in the LOPAC¹²⁸⁰ screen. On a 96-well microtiter plate 15 nM TrxR1 was reduced by 250 μ M

NADPH (AppliChem, Germany) in TE buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA from Merck, Germany) and incubated with the indicated concentrations of the compounds for 30 min at 30°C while following NADPH consumption at 340 nm using a VersaMax microplate reader (Molecular Devices). DTNB was subsequently added to a final concentration of 2.5 mM and the formation of TNB⁻ was followed at 412 nm. For the analysis of PpIX inhibition of DTNB reduction by either wild type TrxR1 or the Sec-to-Cys mutant, note that the latter had to be used at significantly higher concentrations due to its inherently low DTNB reductase activity, as further described in the Results section.

Trx1-coupled insulin assay

For more detailed kinetic analyses, the standard insulin assay [28] was employed in 96-well microtiter plates. Briefly, 0.8 μM TrxR1 was reduced in 200 μM NADPH in TE buffer and incubated at room temperature with increasing concentrations of rottlerin or PpIX for various time points. Duplicate aliquots were removed and added to a master mix containing 20 μM Trx1, 160 μM insulin and 200 μM NADPH in TE buffer. NADPH consumption at 30°C was followed at 340 nm using the VersaMax microplate reader. The natural logarithm of the percentage of inactivation compared to DMSO treated control was plotted against time using GraphPad Prism 5.0a (GraphPad software, Inc.). The resulting slopes, the apparent first-order inactivation rate constants (k_{obs}), were plotted against the inhibitor concentrations resulting in the second-order rate constant k_{inact} .

For analysis of irreversible inhibition, reduced and inhibited TrxR1 (0.8 μM) in the presence of 1 mg/ml BSA was applied to NAP-5 columns (GE Healthcare) and eluted in TE buffer. Enzyme activity was measured before and after desalting using the standard insulin assay as described above.

Glutathione reductase assay

For glutathione reductase activity measurements, a spectrophotometric assay [29] was adopted to microtiter plates as described previously [30]. Briefly, 160 nM yeast GR (Sigma, catalog number G-3664) was reduced by 250 μM NADPH in 0.1 M potassium phosphate buffer, pH 7.0 containing 1 mM EDTA. Rottlerin or PpIX was then added at increasing concentrations and incubated with the enzyme at room temperature. At various time points, aliquots were removed in duplicates and GR activity was measured in the presence of 100 μM NADPH, 1 mM glutathione disulfide (GSSG) and 1 mM EDTA in potassium phosphate buffer at 340 nm using the VersaMax microplate reader.

Cell culture

Human A549 (lung carcinoma), cells were purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, USA), supplemented with 25 nM sodium selenite, 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from PAA Laboratories, Germany). Cells were kept in a logarithmic growth phase at 37°C in humidified air containing 5% CO₂ for all experiments.

Enzyme activity in cell lysates

120 000 A549 cells/well were seeded in 6-well plates the day before treatment to ensure adherence and were subsequently exposed for 24 h to PpIX, NMPP and hemin at concentrations indicated in the text, harvested by trypsinization and washed in cold PBS. The samples were centrifuged at 850 × g for 10 min and the resulting cell pellet was resuspended in lysis buffer (50 mM Tris pH 7.5, 2 mM EDTA pH 8, 0.5 mM PMSF, 1% Igepal CA-630) followed by three rapid freeze/thaw cycles. Centrifugation was performed at

16 000 $\times g$ for 30 min for removal of cell debris whereupon the supernatant was used for enzyme activity measurement, with protein concentrations determined using the Bradford reagent (Bio-Rad Laboratories, Inc., CA, USA). A modification of the standard end-point insulin assay [31] was applied to microtiter plates whereby 3.5 μg of total cellular protein was incubated with 20 μM wild-type Trx1, 275 μM insulin, 1.3 mM NADPH, 80 mM Hepes buffer pH 7.6 and 12.5 mM EDTA for 40 min at 37°C in a volume of 50 μl . As background controls, cell extract without additional Trx1 was incubated in the reaction buffer and lysis buffer without cell extract was incubated with Trx1. The addition of 200 μl 7.2 M guanidine-HCl (Acros Organics, Geel, Belgium) with 1 mM DTNB was used to stop the reaction and detect thiols formed in the sample by measuring absorbance at 412 nm using the VersaMax reader (Molecular devices). The data were plotted as percent of enzyme activity in treated samples compared to controls treated with the same volume of DMSO.

Cytotoxicity assays

Cytotoxicity was assessed by three different methods, based upon detection of either cellular reductive capacity (the MTT assay with formation of formazan salt production), DNA content in cell culture wells (the CyQUANT Cell Proliferation Assay from Invitrogen) or ATP content (the CellTiter-Glo Luminescent Cell Viability Assay from Promega). The CyQUANT and Cell-Titer Glo assays were performed according to the Manufacturer's protocols, with duration of incubation and cell numbers as given in the text. For the MTT assay, typically 8 000 A549 cells/well were seeded onto 96-well microtiter plates the day before treatment. The cells were treated using different concentrations of PpIX, hemin or NMPP as indicated in the text. After treatment, medium was removed carefully, cells were washed in warm PBS and incubated in 150 μl fresh medium with 50 μl sterile-filtered MTT (Thialyl Blue Tetrazolium Bromide, 2 mg/ml in PBS) at 37°C for 4 h. Subsequently, the medium with MTT was carefully aspirated and the formazan crystals were dissolved in DMSO and glycine buffer (pH 10.5) mixed at a ratio of 4:1. Absorbance was then measured at 570 nm using the VersaMax reader (Molecular devices) with background turbidity measured at 650 nm subtracted.

RESULTS

Design of Dual-Purpose High Throughput TrxR1 Assay

At the onset of this study, we wished to formulate a high throughput, single-enzyme assay system with the dual purpose of simultaneously identifying both substrates and inhibitors of TrxR (Fig 1). For this, we exploited the natural fluorescence of the requisite cofactor NADPH and its consumption upon TrxR1 turnover in the presence of an appropriate electron-acceptor substrate. To determine the capacity of a compound to serve as either a substrate or an inhibitor in the same assay, we coupled a first-step assay of NADPH consumption in the presence of library compound alone, which would reflect substrate reduction, with the TrxR1 inhibition test during the subsequent step of selenite reduction. Thus, when NADPH is added to TrxR1 and a test compound, a decrease in NADPH fluorescence compared to the no-compound background is an indication of a substrate behavior for that particular compound. The incubation of compound with TrxR1 pre-reduced by NADPH will also facilitate identification of inhibitors that lack substrate capacity but may inhibit the enzyme by electrophilic targeting of the Sec residue, e.g. as some prostaglandin derivatives [32]. Following the substrate assessment step, selenite is added to start the main enzymatic reaction (i.e. TrxR1-catalyzed reduction of selenite by NADPH) and the inhibitory activity of the test compound is thereby measured in the same assay, following the change of NADPH fluorescence over time. Unlike the other commonly used TrxR1 substrates DTNB or Trx1, the advantage of using the low molecular weight non-disulfide containing selenite is that, when reduced to selenide by TrxR1, it does not expose

any thiol groups that may interfere with the assay and increase the risk of false positives. However, one disadvantage to fluorescent-based detection methods is the increased opportunity for compound interference effects. To eliminate the identification of such compounds, we utilized a kinetic read format (nine reads every minute) in which the baseline fluorescence for each well is established with the first read. Higher or lower starting fluorescence in comparison to the no-compound control reveals autofluorescent or quenching compounds, respectively. To differentiate quenchers from compounds acting as substrates, changes in fluorescence are measured in the presence of NADPH alone.

The assay was subsequently adjusted to 1,536-well format and 4 μ L reaction volume; robust assay performance was observed (Suppl Fig 1A). Using 90 nM TrxR1 with 400 μ M NADPH and 400 μ M selenite, we found excellent stability of the assay reagents (up to 50 hours), with low well-to-well variability and high Z' -values of ≥ 0.7 (Suppl Fig 1B). Having demonstrated the robustness of the miniaturized protocol, we next sought to validate it with established TrxR1 substrates and inhibitors. The result (Fig 2A) showed that the assay readily detected the substrate behavior of the known TrxR1 substrate juglone in a selenite-free combination of the compound, TrxR1, and NADPH. Further, when the complete enzymatic reaction was assembled upon selenite addition, we were able to determine the apparent IC_{50} for the gold-based TrxR1 inhibitor auranofin in the same assay (representing the second step in the assay). The IC_{50} of inhibition was determined to be approximately 100 nM using 90 nM dimeric TrxR1 (Fig 2B) with approximately one Sec residue per dimer [33]. Since auranofin is believed to stoichiometrically target the Sec-containing active site in each monomer of dimeric TrxR1, these results were in line with the expected readout and validated the overall assay set-up in a 1,536 well format.

Quantitative High Throughput Screening (qHTS) of LOPAC¹²⁸⁰

To assess the utility of this dual-purpose assay, the LOPAC¹²⁸⁰ collection was screened in qHTS mode, with each individual compound being tested at six concentrations in the range of 5 nM to 77 μ M for substrate assessment and 3.7 nM to 57.5 μ M for inhibitor assessment (concentration range shift resulting from the reaction volume increase upon selenite addition). The assay performance remained robust throughout the screen, producing an average Z' -factor of 0.58 (Suppl Fig 2A). The intra-plate auranofin concentration-response curves were also remarkably consistent with an average IC_{50} of 115 ± 0.022 nM (Suppl Fig 2B).

Several previously reported TrxR1 inhibitors, such as the anticancer alkylating agents cisplatin, melphalan, and chlorambucil, as well as the naturally occurring flavonoid myricetin were detected in the screen (Fig 3). Fewer than 10 potential candidate substrates were detected which all displayed NADPH depletion responses close to background; the potential substrate yielding the strongest response was 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) which shares obvious chemical features with known redox cyclers. A weak inhibitor, 7,7-dimethyleicosadienic acid (DEDA), displaying only a partial concentration response but in addition showing a response in the substrate-detection step, was also identified. More interestingly, we detected a number of compounds acting as inhibitors that had not previously been known to target TrxR1, including rottlerin, a claimed PKC- δ inhibitor which also has a large number of additional non-PKC related effects in cells including mitochondrial uncoupling [34]; and protoporphyrin IX, an endogenously synthesized intermediate in heme synthesis. Structures of both new and old TrxR1-targeting compounds are summarized in Fig 3. The complete screening dataset has been deposited in PubChem (PubChem Assay ID for the substrate screen is 488772 while it is 488773 for the inhibitor screen).

Follow-up studies

Of the hits found in the LOPAC¹²⁸⁰ screen, nine compounds, comprising known inhibitors (myricetin, melphalan, chlorambucil and cisplatin), novel candidate inhibitors displaying complete as well as incomplete dose responses (wortmannin, PpIX and rottlerin), and candidate substrates (DMNQ and DEDA), were acquired as dry powders and retested using the NADPH-consumption assay to assess their potential substrate properties, as well as the standard DTNB reduction assay to detect inhibitory activities. As suggested from the screen data, none of the inhibitory compounds promoted NADPH consumption in this follow-up test (Fig 4A). Of the two compounds selected as positives during the substrate-detection portion of the screen, only 2,3-dimethoxy-1,4-naphthoquinone yielded a weak response above the background. Within the same assay set-up, juglone showed a strong activity consistent with its recognized efficient substrate nature with TrxR1 [9]. While no additional or novel readily-processed substrates were identified from this screen, the detection and subsequent confirmation of the weak naphthoquinone molecule nonetheless indicates that the assay possesses adequate sensitivity for future searches of TrxR1 substrates within larger compound libraries.

When further tested in the standard DTNB reduction assay, wortmannin, 7,7-dimethyleicosadienic acid and 2,3-dimethoxy-1,4-naphthoquinone did not display any significant inhibition of TrxR at 100 μ M after 30 min preincubation with the enzyme at room temperature, consistent with their very weak inhibitory response measured during the screen (Fig 4B). All other tested compounds showed clear inhibition of TrxR1. Of those, myricetin, melphalan, chlorambucil and cisplatin have previously been identified as good inhibitors of the enzyme [19,35], thus validating our assay methodology. PpIX and rottlerin were here also confirmed as potent inhibitors of TrxR1 at 10 or 20 μ M, leading to an inhibition of 93.4 % or 86.4 % of enzyme activity, respectively. As these compounds were novel inhibitors, we proceeded with a more detailed analysis of the inhibition using Trx1 as the substrate of TrxR1 in the Trx1-coupled insulin reduction assay [31].

We found that PpIX acted as a competitive inhibitor towards Trx1 with a K_i -value of 2.7 μ M, but it was not towards NADPH under the conditions used here (Fig 5). However, the effect of rottlerin on TrxR1 activity in this assay was only very slight and could not be validated (data not shown). In the following experiment, we verified a time dependent inactivation of TrxR1 by either rottlerin or PpIX (Fig 6). In this assay, TrxR1 was reduced with NADPH and preincubated with the compounds (400 μ M) in the presence of 1 mg/ml BSA for up to 120 min. Unbound inhibitor was then removed by applying the samples to a NAP-5 desalting column. Assaying the TrxR1 activity in the protein fraction after desalting, we found that rottlerin and PpIX inactivated TrxR1 in a time-dependent manner, likely through irreversible binding to the reduced enzyme.

In order to quantify the potency of TrxR1 inhibition by rottlerin and PpIX, the second-order rate constants (k_{inact}) were determined by measuring the percentage of TrxR1 inhibition at increasing concentrations of rottlerin or PpIX over time and plotting the natural logarithm of these data (Fig 7). Thus, first-order inactivation rate constants (k_{obs}) were determined and were plotted against inhibitor concentrations for determination of apparent second-order rate constants k_{inact} (Fig 7). This showed that both rottlerin and PpIX were potent inhibitors of TrxR1. PpIX, however, displays stronger inhibition than rottlerin with a k_{inact} of $0.73 \times 10^{-3} \pm 0.07 \times 10^{-3} \mu\text{M}^{-1}\text{min}^{-1}$ compared to a k_{inact} of $0.30 \times 10^{-3} \pm 0.04 \times 10^{-3} \mu\text{M}^{-1}\text{min}^{-1}$ for rottlerin. Identical analyses were performed to investigate possible inhibition of the structurally closely related non-selenoprotein enzyme glutathione reductase by either rottlerin or PpIX. At concentrations of 40 μ M for each compound and a preincubation time in the presence of NADPH for up to two hours, no inhibition of GR was found (data not shown).

PpIX is the natural precursor of heme, lacking an iron atom. We therefore also tested hemin (oxidized form of iron-containing PpIX) in the TrxR1 activity assay, as well as the ferrochelatase inhibitor NMPP that is a PpIX derivative with a methyl group in the center of the porphyrin ring. These porphyrins also inhibited TrxR1, but NMPP with a k_{inact} value approx. 30% compared to that for PpIX. Hemin showed an inhibition at a potency between that of PpIX and NMPP, but was difficult to determine with certainty due to precipitation at higher concentrations and complex kinetics (Fig 7).

With the low or absent inhibition of GR by PpIX but potent inhibition of TrxR1 by PpIX and its competitive inhibition pattern with regards to Trx, this suggested to us that PpIX targeted the C-terminal Trx-reducing and Sec-containing active site of TrxR1. To probe this possibility, we analyzed the PpIX inhibition of a Sec-to-Cys mutant of TrxR1. The Sec-to-Cys mutant shows about 10% the activity of the wild type enzyme using DTNB as a substrate, through a N-terminal dithiol/disulfide motif in the enzyme [26,36]. This analysis revealed that the Sec-to-Cys mutant of TrxR1 was virtually resistant to inhibition by PpIX in its DTNB reduction (Fig 8), indeed further suggesting that the Sec-containing Trx reducing active site of TrxR1 is the site of PpIX inhibition.

Assessing cytotoxicity of exogenously added PpIX, NMPP or hemin to A549 cells, we found a rather complex pattern of response. Only PpIX and not NMPP or hemin gave a strong decrease of absorbance in an MTT assay performed after 24 hours or 48 hours of incubation, with even a tendency to increase in absorbance over control in the 24 hour cultures treated with NMPP or hemin (Fig 9A). When cytotoxicity was assessed using a CyQUANT assay, based upon fluorometric quantitation of DNA content, PpIX again gave clearly a more cytotoxic response than NMPP or hemin; however, all three porphyrins showed increased cytotoxicity with increasing concentrations (Fig 9B). Finally, when assessing cytotoxicity using a CellTiter-Glo assay, based upon detection of cellular ATP levels, PpIX, NMPP and hemin gave similar increases in toxicity that as correlated to either concentration or time (Fig 9C). Measuring the total TrxR activity in cell extracts from A549 cells treated under these conditions for 24 hours, we found that PpIX was a potent inhibitor of cellular TrxR activity, reaching almost 90% inhibition at 10 μM PpIX (Fig 10). Also NMPP and hemin gave inhibition of TrxR activity, but at higher concentrations than PpIX (Fig 10).

DISCUSSION

A TrxR1 dual-purpose assay for the discovery of novel substrates and inhibitors of the enzyme was here successfully developed, optimized and validated in a 1,536-well format. The initial concentration-response screen of the LOPAC¹²⁸⁰ collection yielded both previously reported and unreported TrxR1-targeting molecules, among which rottlerin and PpIX were found as two TrxR1 inhibitors that were selected for further characterization. The results of this study should thus have importance both for future development of high capacity drug screens as well as for the facilitated understanding of effects that may involve TrxR targeting among selected compounds of the LOPAC¹²⁸⁰ library, particularly rottlerin and PpIX.

The obvious advantage of the assay presented here is the capacity to concomitantly determine if a compound is either a substrate or an inhibitor of TrxR1, thus obviating the need for downstream deconvolution experiments. This was achieved by the simple implementation of dual measurements of NADPH consumption via fluorescence detection, first in the presence of compound alone (i.e. substrate assessment) and then during selenite reduction (i.e. inhibitor assessment). We judge that the use of selenite as a substrate should have many advantages over DTNB or Trx-coupled assays for TrxR activity in this particular

screening assay, mainly due to the non-disulfide nature of selenite, which aids in overcoming compound interference and false positives. In addition, no target deconvolution assays are necessary because TrxR1 is the only enzyme present in the samples.

Rottlerin and PpIX were here found to be two irreversible inhibitors of TrxR1 but not inhibitors of the structurally related non-selenoprotein glutathione reductase. As we also found that PpIX was a competitive inhibitor of TrxR1 with regards to Trx1 and that the Sec-to-Cys mutant of TrxR1 was resistant to PpIX inhibition. This reflects a pronounced specificity in TrxR1 targeting of PpIX and its cellular effects may clearly in part be due to its effects on TrxR. Rottlerin, which has been widely used in research, initially as a selective protein kinase C δ (PKC δ) inhibitor, may clearly also exert some of its cellular effects through targeting of TrxR. Recently, an increasing number of studies have suggested that rottlerin may not necessarily inhibit PKC δ directly but rather induce cellular changes resembling those caused by an indirect inhibition in addition to several other effects. The compound was also reported to be a colloidal aggregator, inhibiting enzymes such as AmpC beta-lactamase in the absence of detergent [37]. Cellular actions of rottlerin that are independent of direct inhibition of PKC δ activity were summarized by Soltoff [34] and include the uncoupling of mitochondria as well as effects on phosphorylation and thus activity of multiple other proteins. The inhibition of TrxR1 by rottlerin has to our knowledge been unknown so far and may thus have additional implications on the use of this compound in research and for the interpretations of prior results based on rottlerin usage.

PpIX is formed in the heme biosynthetic pathway as the direct precursor of heme (iron-PpIX) in a reaction catalyzed by ferrochelatase (EC 4.99.1.1/FECH). As a mitochondrial metabolite, it would not be envisioned to be able to target cytosolic TrxR1 under normal conditions. However, accumulation of PpIX in blood, erythrocytes and tissues occurs in patients suffering from erythropoietic protoporphyria (EPP), a genetic disorder due to a partial deficiency of FECH with a prevalence of 1:75,000 to 1:200,000 worldwide [38]. Symptoms of EPP manifests mainly in the skin and liver. Red blood cells loaded with PpIX pass through dermal capillaries where they are exposed to short wavelength light and PpIX is released into the vessels, excited to a triplet state and forms free radicals damaging the surrounding tissues [39]. The resulting inflammation causes severe erythema and oedema, stinging and burning sensations with the severity of these symptoms depending on the intensity of the sunlight and exposure time. Here we found that in addition to forming free radicals, PpIX also directly inhibits TrxR1, known as an important antioxidant enzyme. This targeting may thus contribute to the gravity of the symptoms of EPP, diminishing the removal of reactive oxygen species or damaged biomolecules. As PpIX is excreted from the liver, high levels of PpIX may also cause chronic liver disease. This hepatic toxicity is independent of the photosensitized radical formation; however, the underlying mechanisms are still unknown [38]. Thus, inhibition of liver TrxR1 might also play a role in the hepatic toxicity of PpIX. Similarly, with hemin evidently inhibiting TrxR1 in both the pure enzyme and cellular context, this suggests that targeting of TrxR1 should also be considered as a potential mechanism mediating some of the consequences of increased cellular hemin concentrations.

N-Methylprotoporphyrin IX (NMPP), a ferrochelatase inhibitor, is a toxic by-product that may be formed during normal metabolism of some drugs [40]. Here we found that this compound can also inhibit TrxR1, which may thus be implicated in the toxic side effects of such drugs. In addition, precursors of PpIX are administered to patients prior to photodynamic therapy. These precursors are metabolized into PpIX in the body [41]. When the area of treatment is excited by light the induction of radical formation by PpIX is here used to e.g. kill tumor cells, which preferentially accumulate PpIX upon administration of

the precursor 5-aminolevulinic acid [42,43]. The impact of potential inhibition of TrxR1 during photodynamic therapy is still unknown and should be studied.

Motexafin gadolinium (MGd, Xcytrin®) is a synthetic metal-coordinating expanded porphyrin, known as a texaphyrin shown to have antitumor activity. It causes oxidative stress due to the oxidation of intracellular reducing equivalents such as NADPH, GSH and ascorbate [44]. Recently, Hashemy *et al.* found that MGd was both a substrate and inhibitor of TrxR1 [45]. This porphyrin containing molecule somewhat resembles the effects of PpIX on TrxR1 found here, but in contrast to MGd, we found that PpIX clearly inhibited TrxR1 without also being a direct substrate for the enzyme (Fig 4).

With PpIX, NMPP, and hemin here found to inhibit TrxR1, this inhibition must hence be considered in relation to porphyrin metabolism and toxicity. An imbalance in PpIX and hemin or heme concentrations, perhaps most so with high PpIX due to a deficiency in FECH, could well have an impact on cellular TrxR1 activity with subsequent consequences for cellular redox signaling, antioxidant defense and growth. This should be of additional interest considering earlier findings suggesting direct functional links between heme oxygenase 1 (HO-1) activity and TrxR1 [46]. It is in this context also interesting to note that hemin acts a well known oxidant *in vivo*, mediated through formation of oxidized LDL [47] that has also been linked to TrxR1 expression [48], and the fact that hemin is a potent inducer of Trx1 expression [49]. Thus, collectively, these findings indicate that heme and porphyrin metabolism seems to be intricately linked with the activities of TrxR1 and the complete thioredoxin system. It should be noted that as we herein measured cytotoxicity by different methods, the extent of porphyrin effects varied between PpIX, NMPP and hemin, but overall, PpIX was the most toxic of the three porphyrins and was also the compound that yielded the strongest TrxR inhibition.

We conclude that the newly developed NADPH-fluorescence based selenite reduction assay for measuring TrxR activity is a method well-suited for high throughput drug screening of large compound libraries. Its simple principle based on the direct reduction of selenite not involving other enzymes such as Trx makes follow-up deconvolution assays unnecessary. Using this method, we here identified both known and previously unknown compounds as TrxR inhibitors. Further use of the dual-purpose assay described herein in qHTS of larger compound libraries including analogues of certain compounds enabling SAR (structure-activity relationship) studies could result in the discovery of new drug leads as substrates or inhibitors of TrxR. Identification of such small molecules may lead to the development of new and potent anticancer agents targeting TrxR1. Previously used compounds with biological effects that have not been known as TrxR1 inhibitors need to be reconsidered in light of this capacity, as found here with rottlerin. In addition, natural compounds may be identified as substrates or inhibitors of TrxR1 with a potential role in physiology or pathology, as here shown for PpIX, NMPP and hemin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

TrxR	thioredoxin reductase
PpIX	protoporphyrin IX
NMPP	N-methylprotoporphyrin
HTS	high throughput screening
DMSO	dimethyl sulfoxide
RFU	relative fluorescence units

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Figure 1. Schematic representation of the TrxR1 dual-purpose assay

TrxR1 catalyzes the transfer of reducing equivalents from NADPH to test compounds acting as substrates or selenite (SeO₃²⁻). The consumption of fluorescent NADPH is monitored at an excitation wavelength of 340 nm and an emission of 450 nm.

Figure 2. Assessment of juglone as a substrate and auranofin as an inhibitor of TrxR1 using the dual-purpose assay in a 1,536-well format

A) Concentration-dependent curve of TrxR1-dependent NADPH consumption in the presence of juglone. B) Inhibition of TrxR1 reduction of selenite in the presence of increasing concentrations of auranofin. Each point represents the mean $\pm$ standard deviation of an independent experiment conducted in duplicates (white and black circles).

Figure 3. Chemical structures and IC_{50} values of selected TrxR1-targeting compounds identified from the qHTS of the LOPAC¹²⁸⁰ collection.

Figure 4. NADPH consumption and subsequent DTNB reduction by TrxR1 in the presence of hits identified in the qHTS

A) TrxR1 (15 nM) was reduced by NADPH and incubated with the different compounds or DMSO over 30 min while following NADPH consumption at 340 nm. B) Subsequently, DTNB was added and the reduction to TNB⁻ was followed spectrophotometrically at 412 nm. Each bar represents the mean value and standard deviation of duplicates.

Figure 5. Evaluation of competition between NADPH and PpIX (A) or Trx1 and PpIX (B–D)
 (A) No competition between NADPH and PpIX was observed at concentrations of up to 10 μM PpIX using the Trx-coupled insulin assay with varying NADPH concentrations. Simultaneous incubation of TrxR1 with Trx1 and increasing concentrations of PpIX however displayed competitive inhibition as shown by Michaelis-Menten (B) and Lineweaver-Burk (C) plots. The inhibition constant K_i of 2.7 μM was determined in (D). All data points represent the mean value and standard deviation of duplicates. Concentrations of PpIX were as follows: 0 μM ($\circ$, solid line); 1.25 μM ($\blacksquare$, dashed line); 2.5 μM ($\blacktriangle$, dotted line); 5 μM ($\blacktriangledown$, solid line); 7.5 μM ($\blacklozenge$, dashed line) and 10 μM ($\bullet$, dotted line).

A) Rottlerin **B) PpIX** **Figure 6. Inhibition of TrxR1 is irreversible**

NADPH-reduced TrxR1 (0.8 μ M) was incubated with 400 μ M rottlerin (A) or PpIX (B), respectively, in the presence of 1 mg/ml BSA. TrxR activity was monitored using the standard insulin assay as described in 'Materials and Methods'. After clear inhibition of the enzyme was achieved, unbound rottlerin or PpIX was removed using a NAP-5 desalting column. The activity of eluted TrxR1 was measured according to the same procedure as above.

Figure 7. Inactivation kinetics and rate constants for rottlerin, PpIX, NMPP and hemin towards TrxR1

0.3 μM TrxR1 was reduced by 250 μM NADPH and incubated with various concentrations of the selected inhibitory compounds, as indicated in the figure. At different time points small aliquots were removed and assayed for TrxR1 activity at 30°C using the standard insulin assay as described in ‘Materials and Methods’. The percentage of residual enzyme activity was plotted as its natural logarithm (upper graphs). The apparent first-order inactivation constants (k_{obs}) were determined using linear regression for these data points. A theoretical second-order rate inactivation constant k_{inact} was then determined by plotting the k_{obs} against inhibitor concentration (lower graph, k_{inact} given below the plots). The k_{inact} for hemin was not determined due to its complex kinetics, clearly preventing linear regression analyses in the plots for higher concentrations (dotted lines), which at least in part was due to precipitation of the compound.

Figure 8. PpIX inhibits TrxR1 but not its Sec-to-Cys mutant

TrxR1 (20 nM) or its Sec-to-Cys mutant (U498C, increasing concentrations) were incubated with 250 μ M NADPH with or without addition of PpIX (10 μ M) for 30 min at 25°C, whereupon reduction of DTNB was measured as increase in absorbance at 412 nm. In contrast to TrxR1, no sign of inhibition could be detected with the U498C mutant. The plot shows the mean $\pm$ standard deviation of three separate experiments.

Figure 9. Cytotoxicity and inhibition of cell proliferation by PpIX, NMPP or hemin in A549 cell cultures

Cells were plated in 96-well plates at either 8,000 or 4,000 cells per well for 24 hours, or 48/72 hour treatment time, respectively. Cells were treated in triplicate with the compounds at indicated concentrations. A) MTT assay for assessment of cellular reductive capacity; B) CyQUANT assay for assessment of cell proliferation by measurement of DNA content, and C) CellTiter-Glo Assay for quantification of total ATP content. Graphs show mean $\pm$ standard deviation of an independent experiment conducted in triplicates.

Figure 10. Total cellular TrxR activity upon treatment of A549 cells with PpIX, NMPP or hemin Cells were cultured in 6-well plates for 24 hours, whereupon TrxR activity was measured in cell extracts using a Trx-linked insulin reduction assay, as described in the text. Values were normalized to non-treated controls.

Table 1

TrxR1 assay protocol

Sequence	Parameter	Value	Description
1a	Reagent	2 μ L	rTrxR1 (90 nM final concentration) or buffer for background control
1b	Reagent	1 μ L	NADPH (400 mM final concentration)
2	Compound	23 nL	Test Compound
3	Time	15 min	RT incubation
4	Detector	λ_{ex} 340 nm/ λ_{em} 450 nm fluorescence	Substrate assessment read: kinetic mode $\Delta 8$ min: Read 1 and Read 2
5	Reagent	1 μ L	Selenite (400 mM final concentration)
6	Detector	λ_{ex} 340 nm/ λ_{em} 450 nm fluorescence	Inhibitor assessment read: kinetic mode $\Delta 8$ min: Read 3 and Read 4