Structure-based discovery of the first non-covalent inhibitors of *Leishmania major* tryparedoxin peroxidase by high throughput docking

Margherita Brindisi¹,²*, Simone Brogi¹,²*, Nicola Relitti¹,²,³, Alessandra Vallone¹,², Stefania Butini¹,², Sandra Gemma¹,², Ettore Novellino¹,³, Gianni Colotti⁴, Gabriella Angiulli⁵, Francesco Di Chiaro⁶, Annarita Fiorillo⁵, Andrea Ilari⁴ & Giuseppe Campiani¹,²

¹European Research Centre for Drug Discovery and Development (NatSynDrugs), University of Siena, via Aldo Moro 2, 53100, Siena, ²Dip. di Biotecnologie, Chimica e Farmacia, University of Siena, via Aldo Moro 2, 53100, Siena, Italy, ³Dip. di Farmacia, University of Naples Federico II, Via D. Montesano 49, 80131 Naples, Italy, ⁴Istituto Pasteur Fondazione Cenci-Bolognetti and Istituto di Biologia e Patologia Molecolari IBPM–CNR, c/o Dipartimento di Scienze Biochimiche, Sapienza Università di Roma Piazzale A. Moro 5, 00185 Roma (Italy), ⁵Dipartimento di Scienze Biochimiche, Sapienza Università di Roma, Piazzale A. Moro 5, 00185 Roma (Italy).

*These authors contributed equally to this work.

**Correspondence and requests for materials should be addressed to S.G. (gemma@unisi.it) or A.I. (andrea.ilari@uniroma1.it)**

Leishmaniases is a neglected vector-born disease caused by a protozoan of the genus *Leishmania* and affecting more than 1.300.000 people worldwide. The couple tryparedoxin/tryparedoxin peroxidase is essential for parasite survival in the host since it neutralizes the hydrogen peroxide produced by macrophages during the infection. Herein we report a study aimed at discovering the first class of compounds able to non-covalently inhibit tryparedoxin peroxidase. We have solved the high-resolution structure of Tryparedoxin peroxidase I from *Leishmania major* (LmTXNPx) in the reduced state and in fully folded conformation. A first series of compounds able to inhibit LmTXNPx was identified by means of the high throughput docking technique. The inhibitory activity of these compounds was validated by a Horseradish peroxidase-based enzymatic assay and their affinity for LmTXNPx calculated by surface plasmon resonance experiments. On the basis of these results, the analysis of the enzyme-inhibitor docked models allowed us to rationally design and synthesize a series of N,N-disubstituted 3-aminomethyl quinolones. These compounds showed an inhibitory potency against LmTXNPx in the micromolar range. Among them, compound 12 represents the first non-covalent LmTXNPx inhibitor reported to date and could pave the way to the discovery of a new class of drugs against leishmaniases.

Leishmaniases are a class of infectious diseases caused by protozoan parasites of the genus *Leishmania*, transmitted through the bite of phlebotomine sandflies. According to the World Health Organization, visceral leishmaniases kills more than 20000 people every year and over 310 million people are believed to be at risk of infection¹. Treatment of these diseases is unsatisfactory in terms of safety and efficacy, which sharply contrasts with the therapeutic need in terms of number of people at risk, affected patients, and associated fatalities. This discrepancy is primarily due to the prevalence of these diseases in poor tropical and subtropical countries whose drug development capacity is limited. As a consequence, only a small number of drugs are available, namely antimony-containing compounds (Pentostam) and miltefosine. Both of these drugs present severe side effects and miltefosine is too expensive to be used in underdeveloped countries². Therefore, there is an urgent need to develop new efficacious, less toxic and more affordable drugs.

The trypanothione-dependent hydroperoxide metabolism, characteristic of Trypanosomatids, has been recognized as a promising target for anti-leishmanial drug development since it is absent in the host and most of its components are essential to parasite survival³. Indeed, the defense of these parasites against the reactive oxygen species (ROS) produced by macrophages during the infection depends on the enzymes involved in the unique dithiol trypanothione (N,N8-bis(glutathionyl)spermidine, T(SH)₂) metabolism. T(SH)₂ is synthesized by the Trypanothione Synthetase (TryS) and maintained in the reduced state by Trypanothione Reductase (TR)⁴,⁵. T(SH)₂ participates in crucial thiol-disulfide exchange reactions and serves...
as electron donor in the reduction of hydrogen peroxide to water catalyzed by the tryptaredoxin/tryparedoxin peroxidase couple (TXN/TXNPx). Leishmania TXNPx is a particularly interesting drug target. On the one hand, TXNPx has been proven to be necessary for parasite survival by gene disruption in the Leishmania infantum amastigote; on the other, TXNPx overexpression in Leishmania chagasi (L. infantum) confers H$_2$O$_2$, t-butyl hydroperoxide and peroxyinitrite resistance.

Leishmania major TXNPx (LmTXNPx) is an obligate homodimer whose active site is formed by the N-proximal peroxidatic cysteine (Cp) from one subunit and a C-proximal resolving cysteine from the two-fold symmetry related subunit (Cr’). According to the generally accepted mechanism, the hydrogen peroxide reduction catalyzed by TXN/TXNPx couple consists of two distinct reactions. In the first reaction, oxidized TXNPx binds TXN, which reduces the inter-subunit disulfide bridge (Cp–Cr’) and, thereafter, is reduced by T(SH)$_2$. In the second reaction, the Cp thiolate of a TXNPx monomer reduces the hydrogen peroxide to water and is oxidized to sulfenic acid (-SOH); the sulfenic Cp then reacts with the Cr’ of the two-fold symmetry related monomer forming a disulfide bridge. The transition of 2-Cys peroxiredoxin from reduced to oxidized state is associated with a conformational change involving the protein regions where Cp and Cr’ are located, namely the Cp loop and the C-terminal arm, respectively. Usually peroxiredoxins in reduced state assume the so-called fully folded (FF) conformation, where: i) Cp is part of an α-helical turn, and is oriented toward the catalytic cavity; and ii) the C-terminal tail is partially folded in an α-helix, and covers the catalytic cavity. Protein oxidation promotes a transition to a locally unfolded (LU) conformation, where: i) the α-helical turn containing Cp unwinds and rotates toward the solvent; and ii) the C-terminal tail unfolds and becomes disordered. The transition from FF to LU conformation is essential for catalysis in most peroxiredoxins, and has been related to changes in protein quaternary structure, from a decameric assembly in FF conformation to a dimeric one in LU conformation. Recently Fiorillo et al. have shown by gel-filtration experiments that LmTXNPx displays a decameric assembly in both the oxidized and the reduced states, and that the LU conformation is the most populated within the low resolution crystal structure of LmTXNPx (PDB ID: 3TUE) obtained under reducing conditions. In this structure, the C-terminal region (residues 169–199) is not visible, even if Cp is reduced. This finding is unusual but not unprecedented since human peroxiredoxin 4 in the reduced state may adopt mixed conformations (PDB ID: 3TKO) and in the structure of Crithidia fasciculata TXNPx in the reduced state (PDB ID: 1E2Y) only three out of the ten monomers forming the decameric assembly assume a correct FF conformation. Conversely, the structure of peroxiredoxin in the oxidized state has been always found to assume the LU conformation. Therefore, structural data suggest that while peroxiredoxins in the oxidized state do assume the LU conformation, peroxiredoxins in reduced state may assume both the FF and LU conformations.

In this paper, we report the X-ray crystal structure of LmTXNPx in FF conformation at high resolution. Structure-based high-throughput docking to the available crystal structures of LmTXNPx identified several compounds as potential enzyme inhibitors. Compounds able to inhibit the activity of TXNPx in an enzymatic assay were characterized in detail by surface plasmon resonance. Based on both the resulting inhibitory activity and examination of the docking models, a family of N,N-disubstituted 3-aminomethyl quinoline derivatives (1–12, Figure 1) was rationally designed, synthesized and experimentally characterized. As a result of this work, we discovered the first non-covalent inhibitors of TXNPx reported to date. These represent suitable leads for the development of novel, effective and safer anti-leishmania drugs.

Results and Discussion

X-ray crystal structure study. We report the high resolution (2.34 Å) three-dimensional (3D) structure of LmTXNPx in the reduced form and FF conformation (LmTXNPx-FF; PDB ID: 4KIF). We previously reported a lower resolution (3.00 Å) structure of the same protein in the reduced form and LU conformation (LmTXNPx-LU; PDB ID: 3TUE). The crystallization conditions used to obtain the two structures were slightly different (see materials and methods), and the unit cell of the LmTXNPx crystal in FF conformation is 6% larger. The significant gain in resolution may be ascribed to the reduced flexibility of the 169–199 region, which is fully visible in the FF conformation structure here presented and absent in the previously reported LU conformation structure.

The overall structure of LmTXNPx-FF is very similar to that of LmTXNPx-LU. Both quaternary structures are pentamers of dimers assembled to form a toroidal ring with an outer diameter of 120 Å and an internal diameter of about 60 Å (Figure 2A, B).

Optimal structure superimposition between LmTXNPx-FF and LmTXNPx-LU highlights that conformational changes occur just in two regions: the 43–53 loop containing Cp (Cys52) and the C-terminal tail (residues 169–199) containing Cr’ (Cys173”). In LmTXNPx-FF Cp is part of the first turn of an α-helix, located in a narrow solvent-accessible pocket that constitutes the active site. Conversely, in LmTXNPx-LU the helix partially unwinds and Cp becomes completely exposed to the solvent and available to be attacked by Cr’ to form a disulfide bridge (Figure 2B, C). As shown in Figure 2 (panels C and D), Cp is hydrogen bonded to the highly conserved Thr49 and Arg128 residues, both of which are essential for catalysis. The C-terminal tail (residues 169–199), which is missing in LmTXNPx-LU, is completely visible in LmTXNPx-FF, thus revealing the complete active site structure of the protein (Figure 2C). Cr’ is part of a loop and its side chain is embedded in a hydrophobic cavity formed by the 170–187 residues. The entrance to this active site cavity is covered by the short C-terminal α-helix (residues 187–195), which protects Cr’ from over-oxidation. Interestingly, different crystallization conditions selected for either LmTXNPx-FF or LmTXNPx-LU, both of which were demonstrated to be present under reducing conditions. Since the oxidized LmTXNPx state is generally associated with the LU conformation, structure comparison between the here reported LmTXNPx-FF and the previously solved LmTXNPx-LU structures provided us with the opportunity to infer the mechanism underlying the transition from FF to LU conformation that occurs upon protein oxidation. Reduced LmTXNPx-FF is ready to exert its catalytic function, i.e., reduction of hydrogen peroxide to water, involving oxidation of Cp to sulphenic acid. Upon Cp oxidation, LmTXNPx undergoes a local conformational change (Figure 2C), consisting in the unwinding of the α-helical turn comprising Cp and disordering of the last 20 residues of the protein comprising Cr’, and resulting in exposure to the solvent of both catalytic cysteines.

Additionally, structural features from both the complete LmTXNPx-FF structure and the LmTXNPx-LU counterpart were
exploited for subsequent molecular dynamics and docking studies, and to design specific molecules able to inhibit the enzyme catalytic activity.

High-throughput docking. In order to select potential inhibitors of LmTXNPx, a high-throughput docking (HTD) procedure was employed. Both LmTXNPx-LU and LmTXNPx-FF crystal structures were exploited by the HTD procedure (GOLD software, Genetic Optimization for Ligand Docking)\textsuperscript{14,15}. First, both structures were analyzed to find suitable binding sites for HTD screening. The SiteMap program (SiteMap, version 2.5, Schrödinger, LLC, New York, NY, 2011) identified a potential large binding site in the LmTXNPx-LU structure (Figure S1). However, access to this binding site might in principle be restricted by the C-terminal protein region, which is not visible in the X-ray structure due to a disordered conformation. In LmTXNPx-FF access to the above-mentioned binding site is hampered by the C-terminal tail, such that a very small volume is solvent accessible (88.39 Å\textsuperscript{3}).

The predicted binding site was used for docking studies (see experimental section for further details). The HTD campaign was performed using two chemical libraries, one of which proprietary (containing around 2,300 small molecules) and the other commercial (Asinex Ltd., 5 Gabricevskogo, St. Building 8, Moscow 125367, Russia) containing around 250,000 small molecules. The scoring function GoldScore was employed to rank the compounds best fitting the predicted binding site. Based on our protocol (see experimental section for further details) twenty potential LmTXNPx ligands were selected and submitted to biochemical evaluation (Table S1). The selection was performed applying a GoldScore cutoff of 65 coupled to cluster analysis and visual inspection.

Evaluation of inhibitory activity by enzymatic assay. To determine the ability of the selected compounds to inhibit the catalytic activity of LmTXNPx, we used the horseradish peroxidase (HRP)-H\textsubscript{2}O\textsubscript{2} competition assay developed by Augusto and co-workers\textsuperscript{16}. HRP is well known to react with H\textsubscript{2}O\textsubscript{2} in a two-electron oxidation process that leads to the formation of HRP compound I (HRP-I), a stable HRP oxidized intermediate that can be followed at 398 nm. Reduced LmTXNPx competes with HRP for the H\textsubscript{2}O\textsubscript{2} substrate, leading to a decrease in HRP-I yield that is proportional to LmTXNPx concentration. The addition of LmTXNPx inhibitors leads to a decrease in free LmTXNPx concentration and consequent increase in the HRP-I yield.

Six out of twenty tested compounds exhibited significant inhibitory activity against LmTXNPx at 100 μM (Table S1).

Design and synthesis of optimized compounds. The predicted orientation of representative compounds 1 and NFI442 within the LmTXNPx obtained by the MD and HTD simulations described...
above are reported in Figures 3A in the Main Text and S4 in SI, respectively. Notably, in these models, both compounds were able to interact with residues that have been demonstrated to be crucial for catalysis. In particular, compound 1 (Figure 3A and Table 1) forms H-bonds with Thr49, Thr54 and Val166 and a cation-π interaction with Arg128. For comparison, we docked inhibitor 1 in LmTXNPx-LU binding site (Figure S5 in the SI). In both binding poses the inhibitor occupies the same binding pocket, interacting with the same key residues. However, the p-fluorophenyl ring of 1 in the LmTXNPx-LU structure is solvent exposed (Figure S5), while it interacts with specific residues of the C-terminal tail in the MD frame of the LmTXNPx-FF structure (Figure 3A). SAR studies (see below) are consistent with the proposed binding mode of compound 1. To better investigate the interaction of inhibitor 1 with the highly flexible C-terminal tail, we have submitted the complex 1/LmTXNPx-FF to further 20 ns of MD simulation, starting from the docking pose reported in Figure 3A and following a protocol previously applied for different targets17–19. In the snapshot of the trajectory reported in Figure S6 in the SI, compound 1 directly interacts with Cys173 of the loop at the C-terminal region forming a H-bond interaction from the half to the end of the simulation. Moreover, the compound binds deeper in the binding site, losing the H-bond interactions observed in the previous docking in favor of H-bonding to Gly150 and Arg128. A cation-π stacking with

Figure 3 | Putative binding mode of 1 (A, yellow sticks) and 12 (B, green sticks) obtained by GOLD software (GoldScore values 70.49 and 78.91 for 1 and 12, respectively) into predicted active site of the LmTXNPx-FF enzyme (deep teal cartoon). The key residues of the binding site of the enzyme are represented by sticks. H-bonds are reported by grey dotted lines. The picture was generated by means of PyMOL. Ligand-interaction diagrams are generated by Maestro.
interacting with HRP. The affinity of the compounds for the Lm possible to evaluate the ability of these latter compounds to inhibit ing to Augusto and co-workers16, with the SPR experiments (D 9 inhibition with the exception of 2 compounds showed good to reasonable degrees of enzyme 2 performed and suitable sites for molecular decoration of the hit (compounds 2–12, Table 1) were identified. The synthesis of inhibitors 1–12 is reported in Figure 4. The carbo- oxic acid 13 was activated as the corresponding acyl chloride and then reacted with the suitable benzyamines. Amides 14a,b thus obtained were submitted to a cyclization protocol in the presence of sodium azide and trifluoromethanesulfonic anhydride to afford tetrazoles 15a,b. Hydrazinolysis of the phthalimide moiety afforded primary amines 16a,b that where subsequently submitted to a reductive amination protocol employing the appropriately substi- tuted benzaldehydes in the presence of sodium triacetoxyborohy- dride. Secondary amines 17a–e were then used as substrates of a second reductive amination protocol using quinoline carboxalde- hydes 18a–c (synthesized as described in Scheme S1), affording the final compounds 1, 2 and 7–11. The nitro group of compound 2 was reduced to the corresponding aniline by treatment with tin (II) chloride in ethanol to afford inhibitor 3. The amino group of this latter compound was methylated by using an excess of formaldehyde in the presence of sodium cyanoborohydride as the reducing agent to afford inhibitor 4. Starting from 3, inhibitor 5 was synthesized using the Sandmeyer reaction. Starting from the iodide derivative 5, a Suzuki coupling with phenylboronic acid catalyzed by tetrakis(tri- phenylphosphine)palladium(0) provided the biphenyl derivative 6. Finally, inhibitor 12 was synthesized by reacting intermediate 15a with 1-adamantylcarboxaldehyde to obtain 12, which was then sub- mitted to the second reductive amination reaction as previously described.

Quantitative evaluation of inhibitory activity by surface plasmon resonance and structure-activity relationships. The method of HRP-based competition assay18,20 was used only to establish if the compounds were able to inhibit LmTXNPx. It was not possible to measure directly the Ki with this method since the experiments were not carried out in a steady-state condition and on the other hand the experimental data are difficult to fit due to the presence of different error sources as detailed in the Methods session (see below). To determine the Ki of the enzyme we set up a high-throughput method which combines the HRP–H2O2 competition assay, according to Augusto and co-workers16, with the SPR experiments (KiD values, Table 1 and Figure S7 in the SI). All synthesized compounds showed good to reasonable degrees of enzyme inhibition with the exception of 9 and 10. Indeed, it was not possible to evaluate the ability of these latter compounds to inhibit LmTXNPx since they interfere with the inhibition assay by interacting with HRP. The affinity of the compounds for the hypothesized binding site was improved for all compounds with the exception of 3. The experimental data are consistent with the calculated docking score and free binding energy (∆Gbind) (Table S2, Figure S8 and SAR analysis discussion in the SI). We firstly placed specific substi- tuents at the m-position of the unsubstituted aromatic ring of 1. Accordingly, docking studies predicted that substituents at this position could establish favorable interactions with the protein. However, decoration of the aromatic ring with nitro (2), amino (3), dimethylamino (4), and iodine (5) did not result in a marked increase of binding affinity since the extra-interactions of these substituents slightly shifted the correct orientation of the whole scaffold. This was particularly evident for compound 3 in which the aniline forms a H-bond with the backbone of Glu171 but the key interaction with Thr49 is lost as well as the correct orientation of

![Figure 4 | Synthesis of Inhibitors 1–12.](image-url)
the p-fluorophenyl ring in the hydrophobic cavity. On the other hand, introduction of an aromatic ring at this position led to one of the most potent hit of the series (6, Kₐ = 52 μM). Accordingly, the extra-phenyl ring occupies a hydrophobic sub-pocket and forms a strong interaction with Arg158 by a double π-π stacking. Secondly, we modified the substitution pattern of the quinoline ring by removing the 7-OMe group, which, according to our computational predictions, is not involved in H-bond interactions with the enzyme. Gratifyingly, the resulting 6-OMe derivative 7 showed an improved Kᵩ with respect to our early hit 1 as predicted by the calculated (ΔGbind). The improved activity can be explained by the formation of stronger interactions with Arg128 and Thr54. Notably, removing both OMe groups from the quinoline ring (8) resulted in a decrease in enzyme affinity due to an overall alteration of the binding mode. In a further round of SARs, we assessed the role of the fluorine substituent on the second aromatic ring of 1 by replacing it with H (9), Cl (10) and Br (12); all these modifications resulted in an increase of affinity. From our docking studies, removal of the F substituent allows the formation of a stacking interaction with Phe50. No relevant differences were found with the replacement of F with Cl and Br since both compounds showed a similar binding mode. However, inhibitor 10 forms better interactions with His169 and Thr54 than 11. The best compound of the series was identified when the p-fluorophenyl ring was replaced by a bulky aliphatic adamantyl system (inhibitor 12). The docking output of 12 is reported in Figure 3B. Compound 12 shows a binding mode comparable to that found for the hit compound 1, and it is also able to form a H-bond with the backbone of His169, preserving the other H-bond interactions previously observed in the docking of 1. Replacement of the fluorophenyl ring with an adamantyl moiety resulted in the formation of optimized hydrophobic contacts with Val51, Val128, Ile78 Pro186, Pro188, Pro53 and Phe50. In silico calculation performed by QikProp (Table S3) showed that this class of compounds presents interesting predicted drug-like properties such as clogP, clogS and absorption.

Conclusions

To the best of our knowledge, this paper describes the first non-covalent inhibitors of tryptaxodin peroxidase I of Leishmania parasite. These were developed by a comprehensive approach encompassing X-ray structure determination and a combination of HTD and preliminary hit optimization. Compound 12 was identified as the most effective in both the HRP-H₂O₂ competition assay and SPR experiments. Upon optimization, these non-covalent inhibitors could pave the way to the discovery of new potential drugs against leishmaniasis. Moreover, the combination of the HRP-H₂O₂ assay with SPR measurements appears to be an effective strategy to obtain complementary qualitative information about the compound ability to inhibit enzyme activity and quantitative thermodynamic parameters describing enzyme binding. Studies aimed at obtaining more potent inhibitors that might be tested against leishmania in vitro are currently ongoing.

Methods

Chemistry. General methods. All starting chemicals and solvents were purchased from commercial vendors and used without additional purification. Silica gel 60 F254 (0.040–0.063 mm) with UV detection was used for checking the reaction progress. Column chromatography was performed on silica gel (0.063 mm). 1H NMR and 13C NMR spectra were recorded on a Varian 300 MHz or Bruker 400 MHz spectrometer. The residual signal of the deuterated solvent was used as internal standard. Splitting patterns are expressed as singlet (s), doublet (d), triplet (t), quartet (q), and broad (br); the value of chemical shifts (δ) are given in ppm and coupling constants (J) in hertz (Hz). Mass spectra were recorded utilizing electron spray ionization (ESI). Yields refer to purified products and are not optimized. All reactions were run in an inert atmosphere using oven-dried glassware and anhydrous solvents. Combustion analysis (CHN) was used to confirm the purity (>95%).

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3-(((1-Benzyl-1H-tetrazol-5-yl)-methyl)(4-fluorobenzyl)amino)methyl)-6,7-dimethoxyquinolin-2(1H)-one (1). Compound 17a (50.0 mg, 0.1 mmol) was suspended in a mixture of MeOH/AcOH 1% (5 mL). After 3 days, the mixture was filtered and concentrated in vacuo. The crude material was purified by flash chromatography on silica gel (33% DCM in CH2Cl2) to give 17b (23 mg, 95%) as a yellow oil. 1H NMR (300 MHz, CDCl3) δ 7.75–7.77 (m, 2H), 7.47–7.51 (m, 2H), 7.27–7.30 (m, 2H), 7.13–7.15 (m, 2H), 7.09–7.11 (m, 2H), 7.02–7.04 (m, 1H), 3.90 (s, 2H), 3.69 (s, 2H), 1.90 (br s, 1H). MS (ESI) m/z 358 [M + Na]+; analysis (calcld., found for C22H19FN3O3: C (72.86, 72.92), H (5.09, 5.16), N (16.77, 16.83).

3-(((1-Benzyl-1H-tetrazol-5-yl)-methyl)(4-fluorobenzyl)amino)methyl)-6,7-dimethoxyquinolin-2(1H)-one (1). Reaction was stirred for 8 h, then the mixture was cooled to 0 °C and concentrated under reduced pressure. The crude product was purified by flash chromatography on silica gel (3% MeOH in DCM) to give 7a (14 mg, 35%) as a white solid. 1H NMR (300 MHz, CDCl3) δ 7.72–7.73 (m, 2H), 7.32–7.33 (m, 2H), 7.07–7.10 (m, 2H), 7.03–7.05 (m, 2H), 6.97 (t, J = 7.3 Hz, 2H), 6.89–6.90 (m, 2H), 6.74 (s, 1H), 5.35 (s, 2H), 3.96 (s, 2H), 3.72 (s, 2H), 3.71 (s, 2H). MS (ESI) m/z 613 [M + Na]+; analysis (calcld., found for C30H24N3O3: C (78.46, 78.52), H (5.69, 5.75), N (14.23, 14.29).
purified by flash chromatography on silica gel (2% MeOH in CHCl3) to give 19 as a colorless oil (50 mg, 70%). 1H NMR (CDCl3): δ 6.75-7.22 (m, 3H), 3.72-3.74 (m, 2H), 5.70 (s, 2H), 3.90 (s, 2H), 2.18 (s, 2H), 1.94-1.89 (m, 3H), 1.72-1.58 (m, 7H), 1.47-1.44 (m, 5H).

3-(((Benzyl-H-1-tetrazol-5-yl)methyl)(1-adamantyl methyl)amino)(methyl)-6,7-dimethoxyquinolin-2(1H)-one (12). Starting from 19 (50 mg, 0.2 mmol) the title compound was prepared following the same procedure of 10. The crude material was purified by flash chromatography on silica gel (33% DCM in MeOH:CHCl3) to give 12 as a yellow oil, (20 mg, 25%). 1H NMR (CDCl3): δ 12.06 (br s, 1H), 7.89 (s, 1H), 7.10-7.08 (d, J = 7.0 Hz, 3H), 6.94-6.90 (d, J = / 12.4 Hz, 3H), 6.79 (s, 1H), 5.62 (s, 2H), 3.99 (s, 2H), 3.78 (s, 2H), 2.39 (s, 2H), 1.94-1.89 (m, 3H), 1.72-1.58 (m, 7H), 1.47-1.44 (m, 5H; analysis (calcld., found for C36H39NO7) C (69.29, 68.93), H (6.91, 6.64), N (15.15, 14.90).

Crystallization, data collection and processing. Crystals of LmTXNPx-F/F were grown by hanging drop vapor diffusion method at 293 K. LmTXNPx sample was concentrated to about 12 mg/mL in 20 mM Tris-HCl buffer (pH 7.5) in presence of 50 mM DTT. Aliquots (0.1 µL) of protein solution with 50 mM DTT were mixed with an equal volume of the reservoir solution composed of 20-22% (w/v) PEG3350, 100 mM Tris-HCl buffer (pH 7.0), 0.2 M NaClO4. Crystals of reduced LmTXNPx grew in few days at 293 K and belong to the C2221 space group with the following cell dimensions: a = 111.8 Å, b = 226.2 Å, c = 91.7 Å.

The crystals were cryo-protected by adding 20% glycerol (v/v) to the mother liquor, mounted in nylon loops and flash-frozen by quick submersion into liquid N2 for transport to the synchrotron-radiation source. All X-ray diffraction data were collected at 100 K on the beam line XRD1 at ELETTRA transport to the synchrotron-radiation source. All X-ray diffraction data were collected at 100 K on the beam line XRD1 at ELETTRA transport to the synchrotron-radiation source.

Table 2 | Crystal parameters, data collection statistics and refinement statistics of LmTXNPx

| Space group | C2221 |
|----------------|----------------|
| Cell dimensions | [a, b, c] |
| a (Å) | 111.81 |
| b (Å) | 226.203 |
| c (Å) | 91.719 |
| Data reduction | [Unique reflections, Resolution shells (Å), Completeness, Rmerge, I/σ(I), Redundancy, CC(1/2)] |
| Unique reflections | 49113(5000) |
| Resolution shells (Å) | 2.34-50 [2.34-2.48] |
| Completeness | 98.9% [94.7%] |
| Rmerge | 0.1621 [1.07] |
| I/σ(I) | 10.261 [6.15] |
| Redundancy | 5.48 [5.48] |
| CC(1/2) | 99.37 [71.2] |
| Refinement | [Rvalue, Rfree, Rms bond lengths (Å), Rms bond angle (°), Ramachandran Plot analysis, Residues in the most favored region (%), Residues in allowed region (%)] |
| Rvalue (%) | 19.6 [22.10] |
| Rfree (%) | 24.7 [27.6] |
| Rms bond lengths (Å) | 0.015 |
| Rms bond angle (°) | 1.53 |
| Ramachandran Plot analysis | 97 |
| Residues in allowed region (%) | 3 |

Inhibition assay. Competition kinetics have been performed for determining the activity of the compounds selected by HTD. The ability of the compounds to inhibit the H2O2 reduction by LmTXNPx has been determined by competition approach utilizing the well-known reactivity of H2O2, with horseradish peroxidase (HRP) and ferricyanide (Fe(CN)63−/4−). H2O2 reacts with HRP in a pH 7.4 buffer containing 10 mM HEPES, pH 7.4; 150 mM NaCl, 0.005% surfactant P20.

HRP has a heme-containing peroxidase, which uses hydrogen peroxide (by reducing it to water) to oxidize different organic substrates to free radicals. The reaction takes place in three steps:

1) PFe(III) + H2O2 → P*Fe(IV) + O2 + H2O
2) P*Fe(IV) → O2 + X → X + PFe(III)
3) PFe(IV) = O + X + 2H2 → PFe(III) + X + H2O

Where PFe(III) is HRP (the native protein), P*Fe(IV) = O is the HRP compound I (HRP-I), PFe(III) = O is the HRP compound II (HRP-II) and X is a generic substrate. The formation of HRP-I results in a change in light absorption in the visible range (λmax = 403 nm instead of 403 nm (wavelength corresponding to the maximum of HRP absorbance) in order to avoid interferences arising from HRP-I reduction to HRP-II. This method is usually used to determine the second order reaction constant of 2Cys-Peroxiredoxin reaction with hydrogen peroxide and peroxynitrite. Thus, H2O2-mediated HRP oxidation is a two-electron oxidation process, leading to the formation of HRP-I, followed at 398 nm. The addition of increasing concentrations of pre-reduced LmTXNPx led to a lower yield in HRP-I since two reactions take place in solution:

HRP + H2O2 → HRP-I + H2O

As described by Fiorillo et al.24 the αA can be written as a function of the HRP and LmTXNPx concentrations:

ΔαA = \frac{k_{HRP}[HRP]+k_{LmTXNPx}[LmTXNPx]}{k_{HRP}[HRP]+k_{LmTXNPx}[LmTXNPx]} (equation 1)

where kHRP and kLmTXNPx are the second-order rate constants for the combination of HRP and LmTXNPx with hydrogen peroxide and display the following values: kHRP = 2.0 × 107 M−1 s−1 and kLmTXNPx = 3.4 × 107 M−1 s−1 and ΔαA is the change in absorbance due to the HRP-I formation in the absence of LmTXNPx.

Thus, the inhibitor in addition to the reaction 1 and 2 the following equilibrium exists in solution:

LmTXNPx + HRP-I → LmTXNPx + HRP (reaction 3)

Where I indicates the inhibitor and LmTXNPx-I indicates the complex between LmTXNPx and the inhibitor. The addition of active LmTXNPx inhibitors led to a decrease in the free LmTXNPx and the consequent recover in the HRP-I yield and therefore to a partial or total recovering of the ΔαA. Thus, the reaction 1 can still be used but [LmTXNPx] should be expressed as a function of inhibitor concentration and the dissociation constant of the LmTXNPx-I complex (which is equal to the inhibition constant):
Where \( T \) is the LmTXNPx concentration in the absence of the inhibitor, \([I]\) is the concentration of the inhibitor in the free form, and \(K_p\) is the dissociation/interaction constant.

The HRP-based indirect kinetic assays to test the inhibitors was performed in the following way: HRP 2 \( \mu \)M was exposed to H2O2 0.25 \( \mu \)M in sodium phosphate buffer pH 7.4 and 25 °C, either in the absence or in the presence of the indicated concentrations of reduced LmTXNPx and in the absence or in the presence of inhibitors. The relative changes in absorbance at 398 nm (\( \Delta A/\Delta t \)), reflecting HRP-I formation, were determined.

The equation 2 was arranged as follow to tentatively fit the data:

\[
y = \frac{(x + a)}{(x + b)} = \frac{(a \cdot x + b)}{(a \cdot x + b)}
\]

where \( y = \Delta A/\Delta t, x = [I] \), a = Kd and b = (1 + [HRP]/T_HRP). Many attempts to fit the experimental data have been done but the reasons are reported below:

1) High inhibitor concentrations (>10 \( \mu \)M). When the concentration of the inhibitors is high, non-specific interaction with both HRP-I and HRP-II may take place thereby affecting the spectroscopic signal.

2) Low inhibitor concentrations (<10 \( \mu \)M). The equations 2 and 3 are effective only when the \([I]\) concentration of inhibitor in the free form \( \ll \) [HRP], i.e. when \([I] \ll [HRP] + [TXNPx]. Thus, at a concentration <10 \( \mu \)M which is \( \ll \) [HRP] + [TXNPx], the use of equations 2 and 3 yields big errors.

The compounds ability to inhibit TXNPx has been evaluated by simply measuring the following ratio: \( \Delta A/\Delta t \) where \( \Delta A \) is the difference in absorbance between HRP and compound (\( \Delta A = 0.04 \)) and \( \Delta t \) is the difference in absorbance between HRP and compound I in the presence of LmTXNPx and 100 \( \mu \)M inhibitors. All the compounds have been previously tested for their ability to inhibit HRP at a concentration of 100 \( \mu \)M.

**Computational Details.**

a) **Preparation and binding sites analysis.** The three-dimensional structures of 4K1F and 3TUE were used as the template for docking studies. The structures were then submitted to LigPrep program (version 2.5, Schrodinger, LLC, New York, NY, 2011) to remove chemically impossible moieties and tautomers at cellular pH value (7.0 ± 0.5). The resulting output was saved as sdf file. Asinex 3D-chemical database was downloaded by zincsite (https://zinc.docking.org/) as 25 separate .sdf files.

b) **3D-chemical databases preparation.** Our proprietary database was built in Maestro suite. The structures were minimized by means of MacroModel using the OPLS-AA 2005 as force field. GB/SA model was used in order to simulate the solvent effect applying “no cutoff” for non-bonded interactions. PR CG method (1000 maximum iterations and 0.001 gradient convergence threshold) was employed. Compounds were then submitted to LigPrep program (version 2.5, Schrodinger, LLC, New York, NY, 2011), producing a possible ionization state taking also into account all enantiomers and tautomers at cellular pH value (7.0 ± 0.5). The resulting output was saved as .sdf file. Asinex 3D-chemical database was downloaded by zincsite (https://zinc.docking.org/) as 25 separate .sdf files.

c) **HTD protocol.** High-Throughput Docking was performed by means of GOLD 5.0.2 program from CCDC, UK using the Genetic algorithm (GA). This technique permits a partial flexibility of receptor and full flexibility of compounds. 125000 GA operations were executed for each of the 50 independent GA runs. A search efficiency of 100% was employed. For docking calculation, the active site were defined through XYZ coordinates by adopting a radius of 8 \( \AA \). The maps were saved as .ccp4 files and visualized in PyMOL.

d) **Molecular Dynamics simulation.** According to Desmond guidelines, after the above-mentioned preparation, the LmTXNPx and the 1-LmTXNPx complex were subjected to MD simulation. These calculations were performed by means of Desmond 3.0 package via maestro GUI. We have used an orthorhombic box full of water molecules represented by TIP3P model. OPLS_2005 force field "27" was adopted for the calculations. Ions (Na+ and Cl-) were implemented in the box in order to obtain a resulting salt concentration of 0.15 M that represents the biological concentration of monovalent ions. A constant temperature of 300 K and a pressure of 1.01325 bar were used, employing NPT as ensemble class. For integrating the equations of motion, RESPA24 integrator was applied with 2 fs as inner time step. Constant temperature and pressure were maintained during the simulations employing Nose-Hoover thermostats and the Martyna–Tobias–Klein method22. PME technique22 was used to calculate long range electrostatic contacts. 9 \( \AA \) was used as cut-off for van der Waals and short-range electrostatic contacts. Systems were equilibrated by default parameters with subsequent controlled minimizations and MD simulations applied to slowly relax the selected systems. Consequently, a single trajectory for LmTXNPx of 20 ns was calculated. Concerning 1-LmTXNPx complex the MD simulation (20 ns) was performed in duplicate in order to improve the reliability of the presented model of interaction. The obtained trajectory files were examined by using both tools (Quality Analysis and Simulation Event Analysis Simulation) provided in Desmond. The above-mentioned tools were employed to produce all plots regarding MD simulations reported in this paper.

e) **Estimated free-binding energies.** Prime/MM-GBSA technique provided in Prime software22 consists in computing the variation between the free and the complex state of both the ligand and the protein after energy minimization. The method was applied on the docking complexes of the ligands developed in the present work. The program was used to assess the free-binding energy (\( \Delta G_{\text{bound}} \)) as formerly described by uwG24,25,26.
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Author contributions

M.B., S.G., N.R., A.V. and S.B. designed the synthetic strategy, synthesized and analyzed the compounds. Si.Br. and E.N. performed molecular modelling studies. G.C., G.A. and F.D. purified the protein and performed the SPR experiments and enzyme assays. A.I., A.F. and F.D. solved the X-ray structure and performed structural analysis. S.G., G.C. and A.I. wrote the manuscript. All authors reviewed the manuscript.

Additional information

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