Development of binding assays to screen ligands for *Plasmodium falciparum* thioredoxin and glutathione reductases by ultrafiltration and liquid chromatography/mass spectrometry

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ABSTRACT

To identify potential lead compounds for malaria drug discovery, ultrafiltration and liquid chromatography and mass spectrometry (UF and LC/MS) based binding assays were developed for the first time for *Plasmodium falciparum* thioredoxin (*Pf*TrxR) and glutathione (*Pf*GR) reductases. In the binding assays, curcuminoids (bis-demethoxycurcumin 1, demethoxycurcumin 2, and curcumin 3) were used to study the binding affinity for *Pf*TrxR and *Pf*GR enzymes. The optimum binding was observed when the curcuminoids mixture (1 μM) was incubated with 1 μM *Pf*TrxR and 0.5 μM *Pf*GR enzymes separately for 60 min at 25 °C. The peak areas of the ligands in the chromatogram corresponding to incubation with active *Pf*TrxR and *Pf*GR enzymes increased by 1.6- and 2.0-fold respectively compared to the chromatogram of test compounds incubated with denatured enzymes. Further, binding assay experiments were carried out for compound 2 under non-competitive and competitive incubation conditions with 1 μM *Pf*TrxR and 0.5 μM *Pf*GR enzymes, separately. The binding affinity of compound 2 was higher for both the enzymes under non-competitive incubation conditions. To validate the binding assay developed, we have tested bis-2,4-dinitrophenyl sulfide (4) which is reported as an inhibitor of *Pf*TrxR and *Pf*GR enzymes. Compound 4 showed greater binding affinity for both enzymes under competitive incubation conditions. The relative peak area of compound 4 increased by 3.2- and 6-fold when incubated with active *Pf*TrxR (1 μM) and *Pf*GR (0.5 μM) enzymes respectively compared to the peak areas of the compound in control experiments. The current method developed has a potential for automated high-throughput screening to rapidly determine the binding affinity of ligands for these enzymes.

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1. Introduction

Malaria is caused by *Plasmodium falciparum* and represents a major worldwide health problem. Due to the high mutational rate of the malaria parasite and the resulting rapid adaptation to environmental changes, geographical distribution of the disease in the world and drug resistance are increasing dramatically [1]. Since the *P. falciparum* during its erythrocytic stages is particularly susceptible to oxidative challenge, the thioredoxin and glutathione redox system represents potential targets for the development of antimalarial drugs [2,3]. Evidence suggests that inhibition of the antioxidant enzymes *Pf*TrxR and *Pf*GR impedes the parasite growth [4,5].

The *Pf*TrxR is structurally and mechanistically closely related to the glutathione reductase system but differs clearly from low molecular weight bacterial TrxRs [2]. The significant structural difference between human TrxR and *Pf*TrxR and their catalytic mechanisms can be exploited for specific drug design [6]. The feature distinguishing *Pf*TrxR from *Pf*GR is an additional C-terminal redox center which is crucial for enzymatic activity [7,8]. In contrast to many high molecular weight TrxRs that have a broad spectrum of substrates, the *Pf*GR specifically reduces glutathione disulfide and has been well characterized [4,9]. The *Pf*GR has sequence similarities with human GR in the core structure, but differs at the ligand binding sites. The knowledge of three-dimensional structures and mechanism of catalysis reported for *Pf*GR and *Pf*TrxR will assist in the discovery of inhibitors that specifically target these enzymes [5,10,11].

Even though a number of *in vitro* spectrophotometric studies to screen thioredoxin reductase and glutathione reductase inhibitors by monitoring the oxidation of NADPH have been reported [12–15], detection of small molecules as ligands with selective binding affinity to a target protein is critical in the drug discovery. With the advent of new ionization techniques, MS-based screening methods have emerged as an important analytical tool in identification and characterization of biologically active compounds for protein

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targets of interest [16–19]. The UF and LC/MS-based approach has been used to screen pure compounds, complex mixtures of compounds and natural product extracts to discover ligands to macromolecular targets such as enzymes [20] or receptors [21]. In the UF and LC/MS-based affinity approach, a huge number of compounds can be screened and compounds that show stronger binding affinities to target molecules will be selected for functional assay studies to assess their inhibitory activity. In the current study, we have developed UF and LC/MS-based binding assays for *P. falciparum* thioredoxin and glutathione reductases for the first time. Pure compounds and compound mixtures of natural products were tested in the present study. To validate the binding assays we have studied the affinity of bis-2,4-dinitrophenyl sulfide (4), reported to be a potential inhibitor for these enzymes [6].

2. Experimental

2.1. Chemicals and enzymes

The LC/MS grade solvents were purchased from Fisher Scientific International (Atlanta, GA). A curcumin sample was kindly provided by VWR International (Suwanee, GA). Demethoxycurcumin was purchased from Chromadex (Irvine, CA). Bis-2,4-dinitrophenyl sulfide and other buffer salts were purchased from Sigma–Aldrich (Allentown, PA). Deionized water used in the experiments was freshly generated by a Milli-Q reagent water system (Millipore, MA). All other chemicals were of analytical grade and were purchased from Sigma–Aldrich (Allentown, PA).

*Pf*TrxR (M_r 55 kDa) and *Pf*GR (M_r 114.3 kDa) enzymes were provided as a gift by Prof. Katja Becker, Justus-Liebig University, Giessen, Germany. The recombinant *Pf*TrxR and *Pf*GR enzymes were prepared and purified using silver-stained SDS page according to the procedure published by Kanzok et al. [2] and Farber et al. [22], respectively. The specific activities of *Pf*TrxR (1.9 U/mg) and *Pf*GR enzyme (55 U/mg) were determined by DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] and GR assays developed by Kanzok et al. [2]. Protein concentration of enzymes was determined by the Bradford method [23].

2.2. UF and LC/MS-based P. falciparum thioredoxin reductase (PfTrxR) binding assay

In the binding assay, test compounds (4 μL, 100 μM) were incubated with 3 μL of 1 μM *Pf*TrxR enzyme in 193 μL of assay buffer (PE buffer: 100 mM potassium phosphate and 2 mM EDTA, pH 7.4) at 25 °C for 60 min. The incubation mixture was filtered through a 30 kDa molecular weight cut-off ultrafiltration membrane filter made of regenerated cellulose (Microcon YM-30, Millipore, Billerica, MA) according to the modified method of Liu et al. [21] and then centrifuged at 13,000 × g at 4 °C for 20 min. The enzyme–ligand complex trapped in the membrane was washed with PE buffer (200 μL 3×) and centrifuged at 13,000 × g at 4 °C for 20 min each time. The same ultrafiltration membrane was placed into a new microcentrifuge tube and the ligands were dissociated from *Pf*TrxR enzyme by treatment with 200 μL of methanol for 20 min. The ligand ultrafiltrate was centrifuged at 13,000 × g at 20 °C for 20 min. The ultrafiltrate was then dried under nitrogen using N-VAP 116 Nitrogen Evaporator (Organomation Associates, Inc., Berlin, MA). The ultrafiltrate samples of bis-2,4-dinitrophenyl sulfide (4) and curcuminoids were reconstituted in 100 μL of acetonitrile/0.1% formic acid in water (v/v, 50:50) and methanol/water (v/v, 90:10), respectively. Assays were carried out in duplicate and the control experiments were performed in a similar way with denatured enzyme. The released ligands were then analyzed by LC/MS.

### 2.3. UF and LC/MS-based P. falciparum glutathione reductase (PfGR) binding assay

For this assay, 4 μL of test compound (100 μM) and 185 μL of incubation buffer containing 20.5 mM KH$_2$PO$_4$, 26.5 mM K$_2$HPO$_4$, 200 mM KCl and 1 mM EDTA with pH 6.9, were placed into microcentrifuge. To this, 11 μL of *Pf*GR enzyme (0.5 μM) was added and incubated at 25 °C for 60 min. The incubation mixture was then filtered through a 30 kDa molecular weight cut-off ultrafiltration membrane filter made of regenerated cellulose (Microcon YM-30, Millipore, Billerica, MA) according to the modified method of Liu et al. [21] and then centrifuged at 13,000 × g at 4 °C for 20 min. The enzyme–ligand complex trapped in the filter was washed with assay buffer (200 μL 3×) and centrifuged at 13,000 × g at 4 °C for 20 min each time. The ultrafiltration membrane was placed into a new microcentrifuge tube and the ligands were dissociated from *Pf*GR enzyme by treatment with 200 μL of methanol for 20 min. The ligand ultrafiltrate obtained was centrifuged at 20 °C, 13,000 × g for 20 min. The ultrafiltrate was then dried under nitrogen and the released ligands were reconstituted according to the procedure described in Section 2.2. Assays were carried out in duplicate and the control experiments were performed in a similar way with denatured enzyme.

2.4. LC/MS analysis of curcuminoids (1–3)

The reconstituted ultrafiltrate samples of curcuminoids (5 μL each) were analyzed using an Agilent (Little Falls, DE) 6520 Accurate-Mass Q-TOF mass spectrometer equipped with a 1220 RRLC system. Liquid chromatography analyses of curcuminoids (bis-demethoxycurcumin 1, demethoxycurcumin 2, and curcumin 3) were performed using a ZORBAX Eclipse plus C18 column (2.1 mm × 100 mm, 1.8 μm) with a mobile phase consisting of isocratic solvent system (95% acetonitrile in water/0.1% acetic acid in water–acetonitrile (95:5), 50:50 (v/v)) at a flow rate of 0.2 mL/min. Compounds were detected using negative ion electrospray with a capillary voltage of 3500 V. Nitrogen was supplied as nebulizing and drying gas at flow rates of 25 and 480 L/h, respectively. The drying gas temperature was 350 °C. The fragmentor voltage was optimized to 175 eV. All the samples were analyzed in duplicate. Data were acquired and analyzed using Agilent MassHunter Workstation Qualitative Analysis software, version B.02.00.

2.5. LC/MS analysis of bis-2,4-dinitrophenyl sulfide (4)

The reconstituted ultrafiltrate samples of compound 4 obtained from the binding assay were analyzed using an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer equipped with a 1220 RRLC system. Chromatographic analyses of compound 4 were performed using the ZORBAX Eclipse plus C18 column (2.1 mm × 100 mm, 1.8 μm) with gradient solvent system at 35 °C. The mobile phase consisted of 0.1% formic acid in water v/v (A), and acetonitrile (B). A gradient program was used at a flow rate of 0.2 mL/min. The elution of solvents started from 50% B, linearly increased to 100% B in 5 min and continued same up to 8 min. The mobile phase conditions were returned to 50% B in 10 min and the column was equilibrated further for 3 min. Compound 4 was detected using the negative ion electrospray MS with a capillary voltage of 3200 V. Nitrogen was supplied as nebulizing and drying gas at flow rates of 25 and 600 L/h, respectively. The drying gas temperature was 350 °C. The fragmentor voltage was optimized to 175 eV. Samples were run in duplicate and the data were acquired and analyzed using Agilent MassHunter Workstation Qualitative Analysis software, version B.02.00.
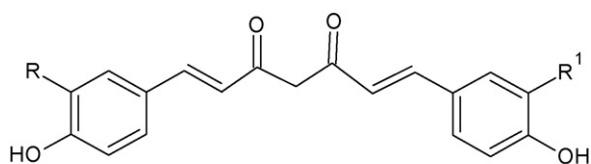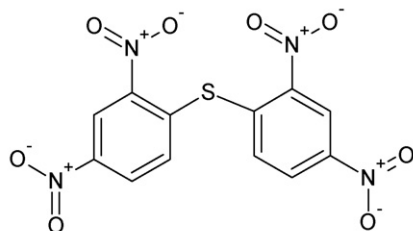
Fig. 1. Structures of curcuminoids (bis-demethoxycurcumin 1, demethoxycurcumin 2, and curcumin 3) and bis-2, 4-dinitrophenyl sulfide (4).

3. Results and discussion

Bio-affinity screening of ligands to the target enzymes is mainly based on the interaction between ligands and the active site of the enzyme. Since the PfTrxR and PfGR enzymes represent important targets for antimalarial therapy, an ultrafiltration mass spectrometry-based binding assay was developed to screen ligands that potentially bind to these enzymes. The curcuminoids (1–3, Fig. 1) were used for the development of the UF and LC/MS-based screening assays. The basic principle involved in the ultrafiltration method has been shown in Fig. 2.

The binding assays have been implemented in a total volume of 200 μL of assay buffer to rapidly screen the inhibitors. In the binding assay, the curcuminoids mixture (1 μM) was incubated separately with 0.5, 1 and 5 μM PfTrxR enzyme and 0.25, 0.5 and 1 μM PfGR enzymes, respectively for 60 min. The incubation mixtures were trapped with 30 kDa cut-off ultramembrane filter to dissociate the ligand–enzyme complex. Subsequently, the released ligands were analyzed using negative ion electrospray MS. In the mass spectrum, the curcuminoids mixture displayed ions at m/z 307.0973 $[M-H]^-$, 337.1080 $[M-H]^-$ and 367.1191 $[M-H]^-$ corresponding to the deprotonated molecules of bis-demethoxycurcumin (1), demethoxycurcumin (2) and curcumin (3), respectively.

The relative binding affinities of curcuminoids were lower when incubated with 0.5 and 0.25 μM PfTrxR and PfGR enzymes, respectively as shown in Table 1. The affinity increased in the experiments where curcuminoids were incubated with 1 μM PfTrxR (Fig. 3A) and 0.5 μM PfGR enzymes (Fig. 4A). The peak areas of bis-demethoxycurcumin (1), demethoxycurcumin (2) and curcumin (3) in the curcuminoid mixture incubated with 1 μM active PfTrxR
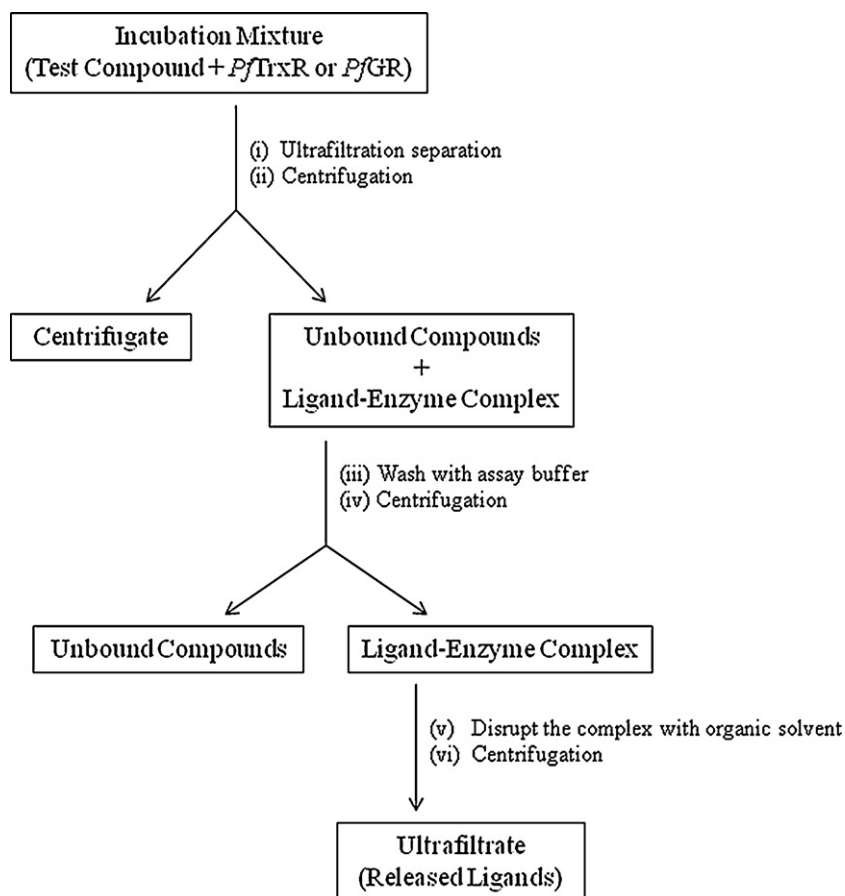
Fig. 2. Schematic representation of ultrafiltration approach.

Table 1
Relative binding affinities of curcuminoids mixture for *Pf*GR and *Pf*TrxR.

Enzyme	Enzyme conc. (μM)	Relative binding affinity of curcuminoids[a]		
		1	2	3
*Pf*GR	0.25	1.4	1.4	1.5
	0.5	3.4	2.7	2.8
	1	2.7	3	3.2
*Pf*TrxR	0.5	1.1	1	1
	1	1.6	1.7	2
	5	2.2	2	2.2
Enzyme	Time (min)	Relative binding affinity of curcuminoids		
		1	2	3
*Pf*GR (0.5 μM)	30	1.1	1.1	1.8
	45	2.9	2.4	2.6
	60	3.3	2.3	2.5
*Pf*TrxR (1 μM)	30	1.1	1.5	1.1
	45	1.9	1.7	2
	60	2.1	2.2	2.4

[a] Relative binding affinity (folds): peak area of compound incubated with active enzyme/peak area of the control experiment in which compound was incubated with denatured enzyme.

enzyme were 1.6-, 1.7- and 2.0-fold, respectively compared to the corresponding control peak areas of the compounds (Fig. 3A and Table 1). Whereas, the peak areas of 1 μM curcuminoids 1, 2 and 3 in the LC/MS chromatogram corresponding to incubation with active 0.5 μM *Pf*GR increased by 3.4-, 2.7- and 2.8-fold respectively

compared to the LC/MS chromatogram of samples incubated with denatured enzyme (Fig. 4A and Table 1).

A slight increase in the relative binding affinities of curcuminoids has been observed in the experiments carried out with 5 μM *Pf*TrxR and 1 μM *Pf*GR enzymes in comparison with incubations
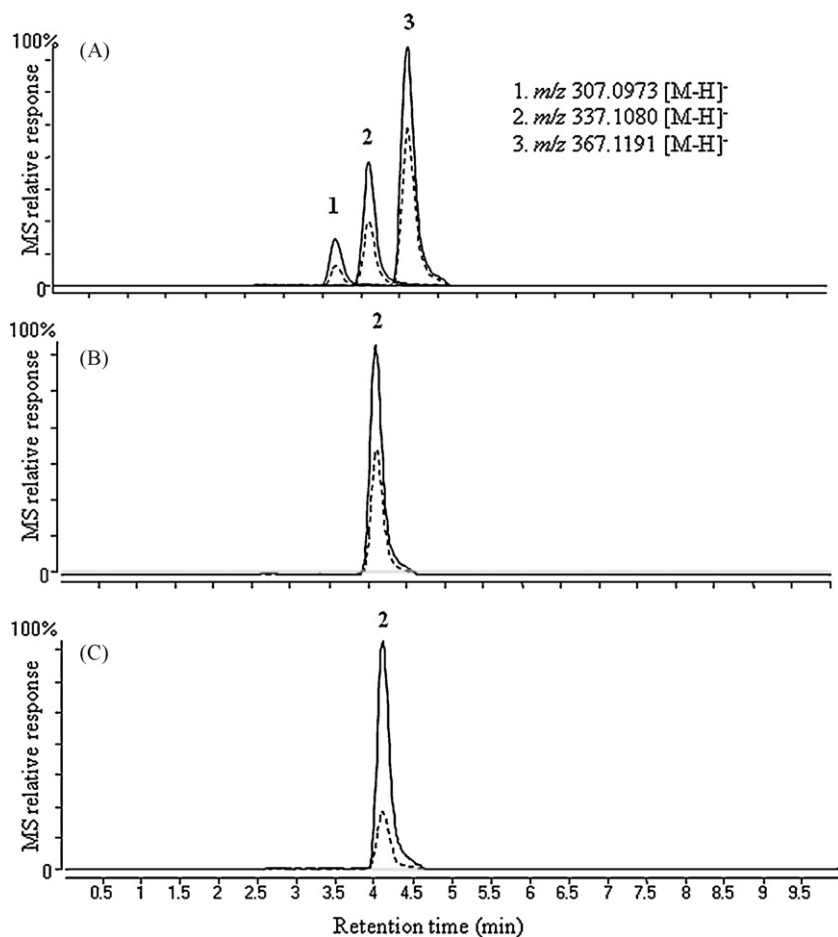
Fig. 3. UF and LC/MS screening of (A) 1 μM curcuminoid mixture (1–3), (B) 1 μM demethoxycurcumin (2), and (C) 0.2 μM demethoxycurcumin (2), incubated with 1 μM *Pf*TrxR enzyme. The solid black line represents the binding experiments using active *Pf*TrxR enzyme, and the dotted black line corresponds to the control incubations using denatured *Pf*TrxR.
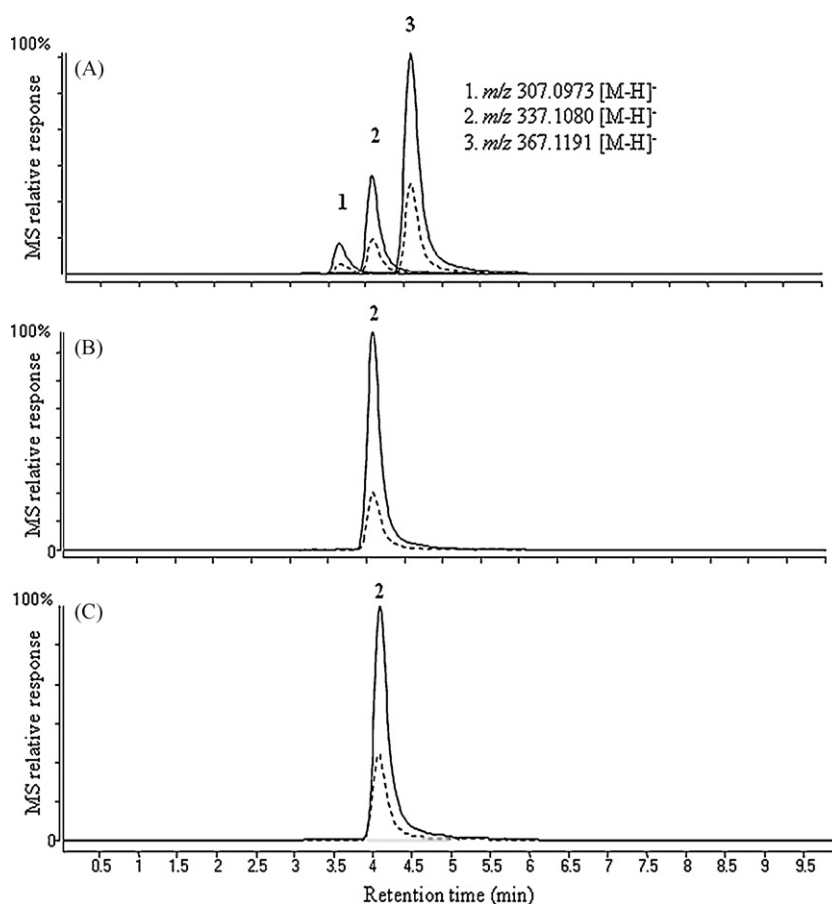
Fig. 4. UF and LC/MS screening of (A) 0.5 μM curcuminoid mixture (1–3), (B) 0.5 μM demethoxycurcumin (2), and (C) 0.1 μM demethoxycurcumin (2), incubated with 0.5 μM *Pf*GR enzymes.

carried out with 1 μM *Pf*TrxR and 0.5 μM *Pf*GR enzymes. A potential reason could be that at high concentration of enzyme, nearly all ligand added is bound to the binding sites of the enzymes. These observations support the enzyme concentrations of 1 μM *Pf*TrxR and 0.5 μM *Pf*GR as required for the optimum binding of curcuminoids to these enzymes.

Based on the above results further incubation experiments were performed using 0.5 μM *Pf*GR and 1 μM *Pf*TrxR enzymes at 30, 45 and 60 min. In all the experiments at the three time intervals, the binding affinities of curcuminoids were increasing in a time-dependent manner until 60 min (Table 1). It was observed that it was necessary to allow sufficient time for ligand–enzyme equilibrium to be reached. Otherwise, the initial complexes might be too weak and misinterpreted as a negative result. As a result a 1-h incubation was used prior to ultrafiltration.

Subsequently, compound 2 (1 μM) was tested against 1 μM *Pf*TrxR enzyme and Fig. 3B shows the similar binding affinity profile to that of 2 observed in a curcuminoids mixture. The peak areas of compound 2 corresponding to incubation with active *Pf*TrxR increased by 1.7-fold compared to the samples incubated with denatured enzyme (Fig. 3B). In order to study the binding affinities under non-competitive conditions, compound 2 was tested further at lower concentration (0.2 μM) with 1 μM *Pf*TrxR enzyme. Interestingly, the relative peak area of compound 2 increased about 4.2-fold compared to the compound peak areas in the control experiments as shown in Fig. 3C.

On the other hand, the results of binding assay of compound 2 incubated with 0.5 μM *Pf*GR enzyme under competitive and non-competitive incubation conditions has been shown in Fig. 4B and C. The peak areas of compound 2 in the LC/MS chromatogram cor-

responding to incubation with active *Pf*GR under competitive and non-competitive conditions increased by 3.7- and 3.1-fold (Fig. 4B and C), respectively in comparison with the LC/MS chromatogram of samples incubated with denatured enzyme. This evidence indicated that compound 2 binds specifically to the *Pf*GR enzyme.

From ancient times curcuminoids have been widely used in Ayurvedic medicine and are known for a wide spectrum of biological activities. Studies have demonstrated their antiprotozoal [24,25] and antimalarial properties [26]. The inhibitory activity of curcumin and their analogues for mammalian thioredoxin reductase by DTNB-based spectrophotometric functional assay have been reported [27–29]. In contrast, the main focus of our study was to screen pure compounds and complex mixtures to identify the binding affinities for these enzymes prior to submission to the functional assays. Based on the binding affinity ranking, the compounds will be pursued further for testing in functional assays to determine their inhibitory activities. The relative binding affinities of curcuminoids for *P. falciparum* thioredoxin and glutathione reductases have been described for the first time in this report. In view of the abundance, non-toxic nature and demonstrated therapeutic effects in several human diseases, curcuminoids appear to be promising natural products to study further for their potential as low-cost botanical dietary supplement.

To validate the UF and LC/MS-based binding assay experiments developed in the current study, we have examined the binding affinity of a synthetic compound bis-2,4-dinitrophenyl sulfide (4) which is reported as an inhibitor of *Pf*TrxR and *Pf*GR enzymes [4]. Compound 4 (1 μM) was incubated with 1 μM *Pf*TrxR and 0.5 μM *Pf*GR enzyme and the trapped ligand was released and analyzed by negative ion electrospray MS. The study of nitro aryl derivatives
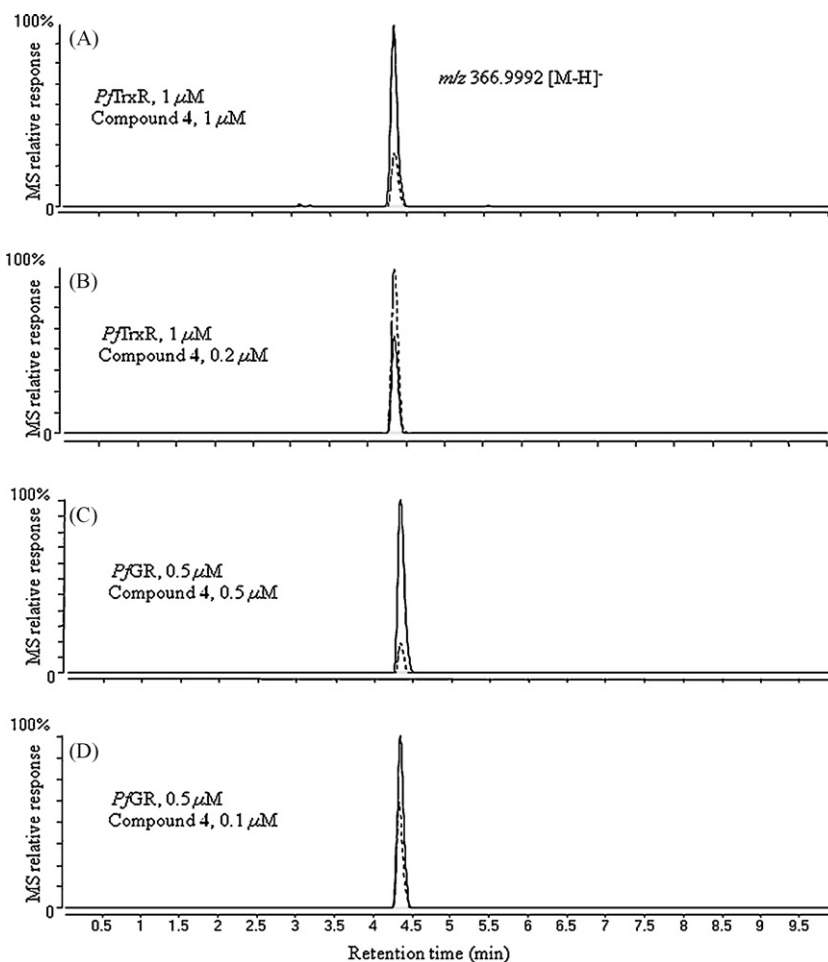
Fig. 5. UF and LC/MS screening of (A) 1 μM bis-2,4-dinitrophenyl sulfide (4), (B) 0.2 μM bis-2,4-dinitrophenyl sulfide (4) incubated with 1 μM *Pf*TrxR enzyme, (C) 0.5 μM bis-2,4-dinitrophenyl sulfide, and (D) 0.1 μM bis-2,4-dinitrophenyl sulfide (4) incubated with 0.5 μM *Pf*GR enzyme.

by different mass spectrometric techniques has attracted great attention and interest in recent years because of their fragmentation process involving skeletal rearrangements [30]. A number of detection methods for nitro aryl and diaryl sulfides using positive and negative ion electrospray mass spectrometry experiments have been reported in the literature [31–33]. In this study, we report a new LC/MS method to detect compound 4 using negative electrospray MS. Initially, direct infusion experiments to the MS detector were performed to study ionization of compound 4. To facilitate the ionization, the samples were prepared in a mixture of acetonitrile and 1% formic acid in water (50:50) and infused to the MS. Subsequently, separation conditions for compound 4 were optimized as described in Section 2.5 to obtain a better LC/MS chromatogram. The deprotonated molecule of compound 4 was observed at m/z 366.9992 $[M-H]^-$ corresponding to the molecular formula $C_{12}H_7N_4O_8S$ (DBE: 11) in its negative electrospray MS. This ion formation may be possible due to the stabilization of nitro groups in the aryl moiety [34]. The main fragment observed at m/z 198.9814 $[M-H]^-$ in the MS spectrum with molecular formula $C_6H_3N_2O_4S$ (DBE: 6) is proposed due to the loss of the dinitrophenyl unit ($C_6H_3N_2O_4$) from the molecule. The electron withdrawing substituents such as nitro groups in the aryl unit may facilitate the S-aryl bond fission, and this phenomenon in some of the nitropyridylaryl nitro sulfides has been reported [30].

The relative peak area of compound 4 increased by 3.2-fold when incubated with active *Pf*TrxR with respect to the compound peak area in the control experiments (Fig. 5A). In the *Pf*GR binding assay, the peak area of 4 increased about 6-fold when incubated at

a concentration of 0.5 μM with 0.5 μM enzyme as shown in Fig. 5C. However, the peak areas of compound 4 in control experiments increased when it was incubated under non-competitive conditions with *Pf*TrxR and *Pf*GR enzymes as shown in Fig. 5B and D. These results clearly demonstrate that compound 4 showed specific binding affinity but the affinity decreases as the concentration of compound decreases.

4. Conclusions

P. falciparum TrxR and GR reductases represent validated and highly interesting drug targets to develop new antimalarial drugs [4,35,36]. High-throughput screening against target enzymes has now become one of the primary methods for the discovery of naturally occurring small molecules with therapeutic potential [37,38]. Drug discovery programs generally rely on large libraries of plant extracts that are tested in an increasing variety of biological assays used to identify active extracts. But the complexity of typical natural product extracts results in the difficult and time consuming task of identifying and isolating the compounds responsible for the activity using conventional purification methods. The UF and LC/MS approach can be applied to screen pure compounds and natural products extracts. In these methods the detection of ligands using mass spectrometry methods takes place as soon as the ligand–enzyme complex is trapped into ultramembrane filter followed by dissociation. In our study, the relative binding affinity for demethoxycurcumin (2) did not vary much when tested as a pure compound alone or as a component of the curcumin mixture.

Based on this we would like to emphasize that this approach may be useful to screen complex mixtures for potential ligands prior to the conventional isolation and purification procedures. Also, the UF and LC/MS-based bioassays integrate the binding affinity selection by ultrafiltration, analysis and identification by LC/MS in the entire screening process. The methods are specific and fast and need very low amounts of test compounds due to the low detection limits of the LC/MS. In conclusion, the current methods developed have a potential for automated high-throughput screening to discover potential ligands for *Pf*TrxR and *Pf*GR enzymes. Further functional assay experiments are necessary to understand the inhibitory activity of these compounds for *Pf*TrxR and *Pf*GR reductases.

Acknowledgements

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