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Falcipain-2 inhibition by suramin and suramin analogues

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ABSTRACT

Falcipain-2 is a cysteine protease of the malaria parasite *Plasmodium falciparum* that plays a key role in the hydrolysis of hemoglobin, a process that is required by intraerythrocytic parasites to obtain amino acids. In this work we show that the polysulfonated napthylurea suramin is capable of binding to falcipain-2, inhibiting its catalytic activity at nanomolar concentrations against both synthetic substrates and the natural substrate hemoglobin. Kinetic measurements suggest that the inhibition occurs through an noncompetitive allosteric mechanism, eliciting substrate inhibition. Smaller suramin analogues and those with substituted methyl groups also showed inhibition within the nanomolar range. Our results identify the suramin family as a potential starting point for the design of falcipain-2 inhibitor antimalarials that act through a novel inhibition mechanism.

1. Introduction

Malaria, caused by the *Plasmodium* parasite, is one of the most serious public health problems worldwide. Hundreds of millions of cases of malaria occur each year, resulting in about a million deaths, mostly from *Plasmodium falciparum.*[1] Although malaria is a treatable and curable disease, *P. falciparum* is a highly adaptable parasite,[2] resulting in growing resistance to antimalarial medicines. Currently, the most effective treatment for falciparum malaria is artemisinin-based combination therapy (ACT), but this strategy is now threatened by emergence of parasites with decreased sensitivity to artemisinins in Southeast Asia.[3,4] New antimalarial agents, ideally acting against novel targets, are thus greatly needed.[5–10]

During the complex life cycle of the malaria parasite, only the erythrocytic cycle is responsible for human disease symptoms, and thus the design of drugs to treat active infections focuses on erythrocytic parasites. Intraerythrocytic parasites utilize multiple proteases to hydrolyze hemoglobin in an acidic food vacuole to provide amino acids for parasite protein synthesis.[11,12] The key cysteine proteases involved in hemoglobin digestion are falcipain-2 and falcipain-3[13–16] and these enzymes are validated drug targets against which new classes of inhibitors are being pursued.[12,17–19] Treatment with cysteine protease inhibitors blocks hemoglobin hydrolysis, with the accumulation of undigested

Abbreviations: FP-2, falcipain-2; Suramin, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid; AMC, 7-amino-4-methyl coumarin; NF037, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-phenylenecarbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF127, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-ethylphenylene)-carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF151, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-isopropylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF198, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-isopropylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF157, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-fluorophenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF258, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-chlorophenylene)carbonylimino]]bis [naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF260, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-(metoxyphenylene) carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF222, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino-3,1-(4-(metoxymethyl)phenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF023, 8,8′-[carbonylbis[imino-3,1-phenylenecarbonylimino]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; NF058, 8,8′-[carbonylbis[imino-3,1-(4-methylphenylene)carbonylimino]]bis[naphthalene-1,3,5-trisulfonic acid] hexasodium salt; MK-HU1, 4,4′-[carbonylbis[imino-3,1-phenylenecarbonylimino]]bis[benzene-1,3-disulfonic acid] tetrasodium salt.

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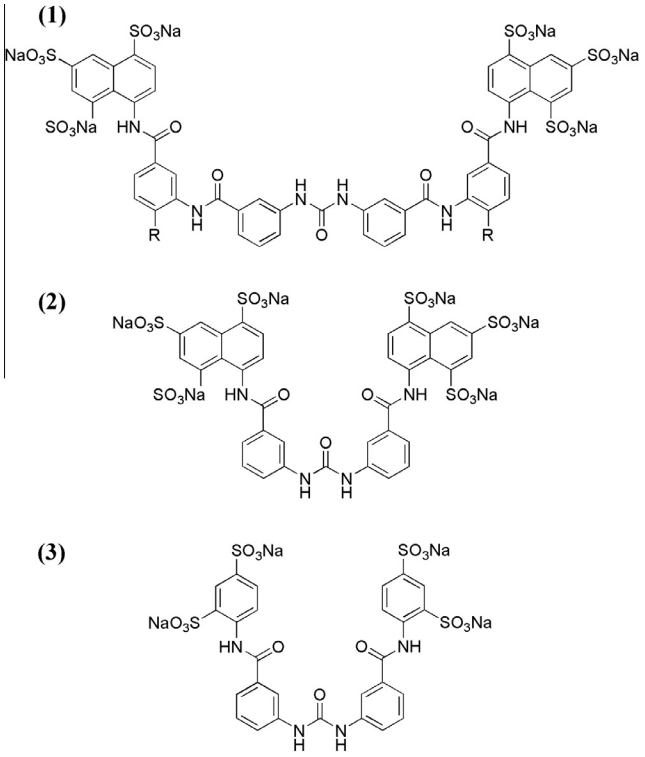

Figure 1. Schematic chemical structures of suramin analogues. Substituent (–R) groups are as listed in Table 1. (1) is suramin for R = H, (2) NF023 and (3) is MK-HU1.

hemoglobin in a swollen food vacuole and a block in parasite development.[20–22] The discovery of several specific inhibitors for the falcipain family had been reported in the last years.[19,23] The high resolution crystal structure of falcipain-2 and falcipain-3 has been solved[24,25] and recent effort has gone into the characterization of novel active site inhibitors.[26–30] However, allosteric modulation of the activity of falcipains has not been described.

Upon hemoglobin digestion by falcipains, peptide fragments are released along with heme.[31–34] In solution, heme leads to the generation of toxic oxygen species, which is minimized in the digestive vacuole through detoxification of heme into hemozoin.[33,35,36] Inhibition of both falcipain-2 function and heme crystallization have long been considered as therapeutic approaches for the treatment of malaria.[16,37–42] Indeed, aminoquinolines such as chloroquine act to disrupt the biocrystallization of heme.[43,44]

Suramin is a polysulfonated polyaromatic symmetrical urea (Fig. 1) that is used in the treatment of trypanosomiasis and onchocerciasis.[45] Suramin has also been shown in *P. falciparum* to inhibit invasion of HepG2 cells by sporozoites,[46] merozoite surface protein-1 secondary processing, and erythrocyte invasion by merozoites.[47] In a search for novel ligand prototypes and motifs targeting falcipains, we investigated interactions with suramin, a model polysulfonated compound. Our data show that suramin binds to falcipain-2, modulating its hydrolytic activity through a novel mechanism for this enzyme. Further, we report here an extended evaluation of suramin analogues and discuss implications of allosteric inhibition of falcipains.

2. Results

2.1. Suramin inhibits falcipain-2

To evaluate the effect of suramin on falcipain-2, we first conducted a hemoglobinase assay with the natural falcipain-2 substrate, human hemoglobin. Hemoglobin was hydrolyzed by falcipain-2, as expected, and this hydrolysis was prevented in the presence of 200 nM suramin (Fig. 2A).

We also tested the effect of suramin on the hydrolytic activity of falcipain-2 against the fluorogenic substrate Z-Phe-Arg-AMC. Hydrolysis of the substrate was inhibited, with an IC50 of 95 ± 1.5 nM (Fig. 2B).

2.2. Stoichiometry of binding

In order to characterize the stoichiometry of the suramin/falcipain-2 complex, we performed an isothermal titration of suramin to a fixed amount of falcipain-2 (5 μM), well above the IC_{50} of 95 nM. The tryptophan fluorescence of falcipain-2 was measured following the progressive addition of suramin, revealing a steep decrease in fluorescence due to suppression by suramin (Fig. 3A). Above a molar suramin:falcipain-2 ratio of close to 1:1 we observed loss of dependence of fluorescence suppression on the suramin

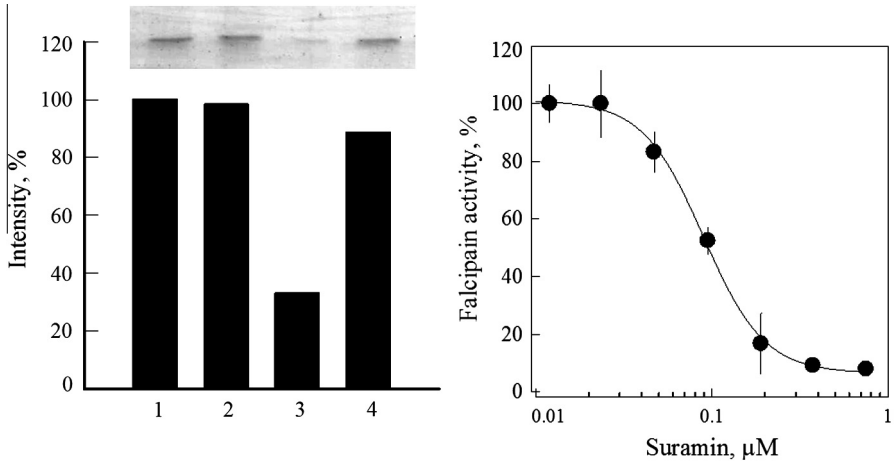

Figure 2. Effect of suramin on falcipain-2 activity. (A) Hemoglobinase activity of falcipain-2. Hemoglobin was incubated with falcipain-2 under the conditions described in Methods, reaction products were resolved by SDS–PAGE, and remaining hemoglobin was quantified by densitometry. (1) human hemoglobin; (2) human hemoglobin + 20 nM falcipain-2 (t = 0); (3) human hemoglobin + 20 nM falcipain-2 (t = 90 min); (4) human hemoglobin + 20 nM falcipain-2 + 200 nM suramin (t = 90 min). (B) Hydrolytic activity of falcipain-2 against the fluorogenic subtrate Z-Phe-Arg-AMC. The peptide was incubated with falcipain-2 under the conditions described in experimental procedure with varying concentration of suramin, and hydrolysis was assessed fluorometrically. Results represent the average of three independent experiments ± SEM and the solid lines represent non-linear regression of data according to Eq. 2.

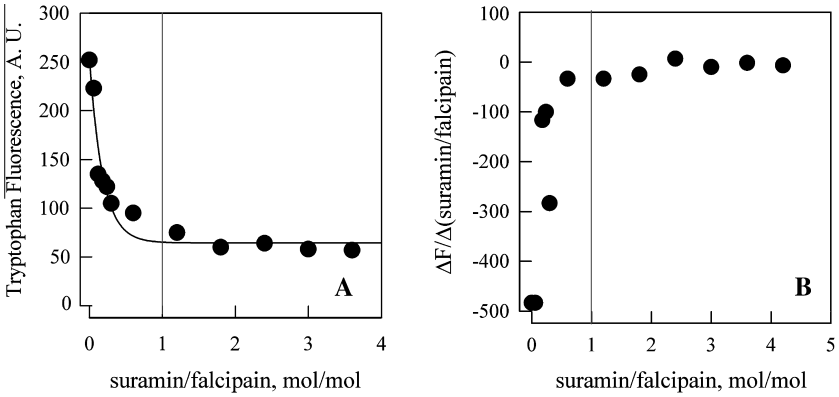

Figure 3. Stoichiometric binding assay for suramin and falcipain-2. The stoichiometry was accessed by monitoring changes in tryptophan fluorescence of falcipain-2 (ex 295 nm, em 340 nm) as a function of increasing suramin concentration. (A) falcipain-2 titration isotherm with suramin monitored by changes in tryptophan fluorescence; (B) first derivative of data in (A), showing the inflection point at a 1:1 molar ratio.

concentration, as more clearly illustrated in the first derivative curve of the titration data (Fig. 3B). These data suggest a binding stoichiometry of one suramin molecule per falcipain-2 molecule.

2.3. Mechanism for suramin inhibition of falcipain-2

In order to shed light on the mechanism of the suramin/falcipain-2 interaction, we evaluated the dependence of Z-Phe-Arg-AMC hydrolysis on falcipain-2 turnover in the presence of varying amounts of suramin. In the absence of suramin, the reaction followed classical Michaelis–Menten kinetics up to 100 μM substrate, with a hyperbolic behavior. In the presence of increasing amounts of suramin the dependence of the hydrolytic activity of falcipain-2 on substrate concentration showed a biphasic behavior, increasing according to the substrate concentration at lower concentrations, followed by a decrease at higher substrate concentrations (Fig. 4A). Lineweaver–Burk plots for substrate hydrolysis in the presence of varying concentration of suramin showed a drift from the linear dependence of 1/V over 1/S when activity was measured in the presence of increasing amounts of suramin (Fig. 4B). The biphasic profile, with a rise in enzyme activity followed by a decrease at higher substrate concentrations is typical for a mechanism involving enzyme inhibition by the substrate.[48–51] Further data analysis allowed the estimation of the suramin IC_{50} for reactions conducted at varying substrate concentration (Fig. 4C) using a linkage plot,[52,53] according to

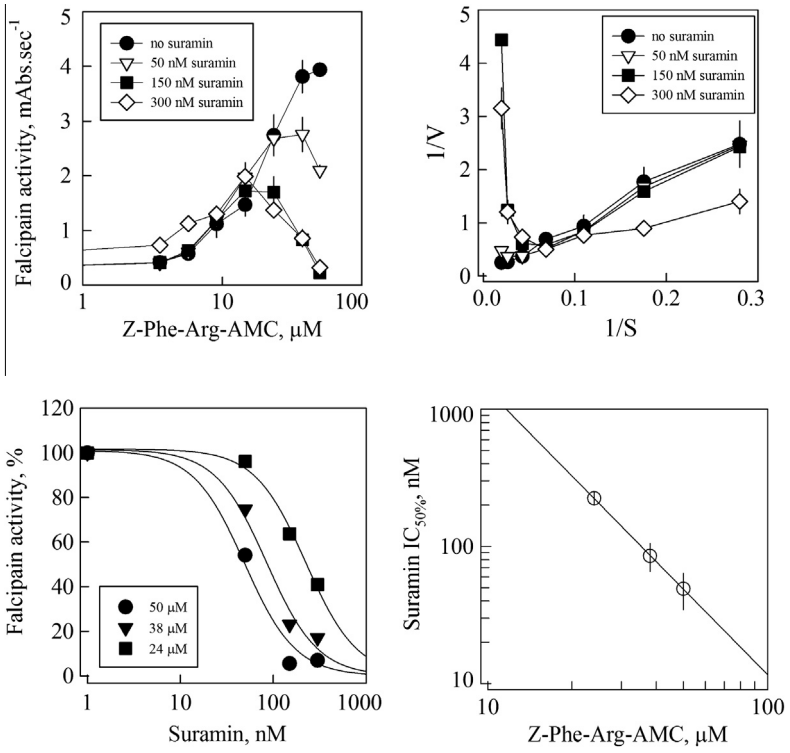

Figure 4. Effect of suramin on falcipain-2 catalytic activity. (A) Z-Phe-Arg-AMC hydrolysis by falcipain-2 (20 nM) in the presence varying concentration of suramin. (B) Lineweaver–Burk plots of data from (A). (C) Dependence of falcipain-2 inhibition by suramin on substrate concentration (data from (A). Lines correspond to the non-linear regression with Eq. 2. (D) Wyman's linkage plot of the logarithm of the suramin IC_{50} over falcipain-2 activity as a function of the logarithm of the substrate concentration. The IC_{50} corresponds to values calculated as depicted in (C). The solid line corresponds to the best fitting of Eq. 1 to the data. Symbols represent the average of three independent experiments ± SD.

$$\delta \log IC_{50} / \delta \log \text{substrate} = \Delta N_{\text{substrate}} \quad (1)$$

The slope of log IC_{50} versus log [substrate], the log of substrate concentration, gives $\Delta N_{substrate}$, the number of substrate molecules associated with falcipain-2 upon treatment with suramin. Figure 4D shows the decrease in IC_{50} with increasing substrate concentration, suggesting different substrate association states for each falcipain-2/suramin association condition. By adjusting Eq. 1 to the experimental data in Figure 4D we calculated the change in substrate association state $\Delta N_{substrate}$ that follows falcipain-2 inhibition by suramin at 2 units. Thus, in the range of suramin and substrate concentrations evaluated (where substrate inhibition occurs), the cooperativity of suramin and substrate binding appears to be linked, meaning that a conformational equilibrium holds in falcipain-2 upon suramin binding, eliciting a novel regulatory substrate binding site in the enzyme. These data appear to unveil a novel inhibition mechanism for falcipain-2, through the binding and stabilization of the enzyme/substrate/inhibitor complex, a characteristic feature of uncompetitive inhibition, but which allows the subsequent allosteric binding of a second substrate molecule to falcipain-2, resulting in its inhibition.

Table 1
Inhibitory activity of suramin analogues. IC_{50} ± standard error were calculated by adjusting Eq. 2 to the data in Figure 4

Compound	–R	IC_{50} (nM)
1a (NF037)	–H	36 ± 7
1b (Suramin)	$-CH_3$	95 ± 1.5
1c (NF127)	$-CH_2CH_3$	80 ± 21
1d (NF151)	$-CH(CH_3)_2$	111 ± 5
1e (NF198)	$-C_6H_5$	62 ± 11
1f (NF260)	$-OCH_3$	67 ± 5
1g (NF222)	$-CH_2OCH_3$	60 ± 11
1h (NF258)	–Cl	78 ± 11
1i (NF157)	–F	42 ± 7
2 (NF023)	–H	167 ± 5
3 (MK-HU1)	–H	98 ± 32

2.4. Characterization of the activity of suramin analogues against falcipain-2

To further characterize the molecular mechanism of falcipain-2 inhibition by suramin, we evaluated compounds with the replacement of the methyl groups of suramin by a series of non-polar (e.g., alkyl, phenyl, halogen and methoxy substituents) or polar (with halogen heteroatoms) substituents (Table 1, Fig. 5).

Regardless of the nature of the modification, all compounds tested showed similar inhibition potency to that of suramin, all with IC_{50}s in the nanomolar range (Fig. 5, Table 1). The most effective inhibitor was **1a**, which bears a hydrogen substituent instead of the methyl group of suramin. These data suggest that the presence of substituents with larger volume at this position might exert a negative influence on the inhibition profile.

We also examined the effect of decreasing the polysulfonated character of the polyaromatic symmetrical urea of suramin, as well as reducing its size. Compounds **2** and **3** displayed somewhat higher IC_{50} compared to its analogue **1a**. These data indicate that the oversulfonated character of suramin (**1b**) and some analogues is not a determining factor for falcipain-2 binding and inhibition. Therefore, a minimal chemical entity comprising the sulfonated phenyl-amide might be sufficient for the inhibitory activity of suramin analogues.

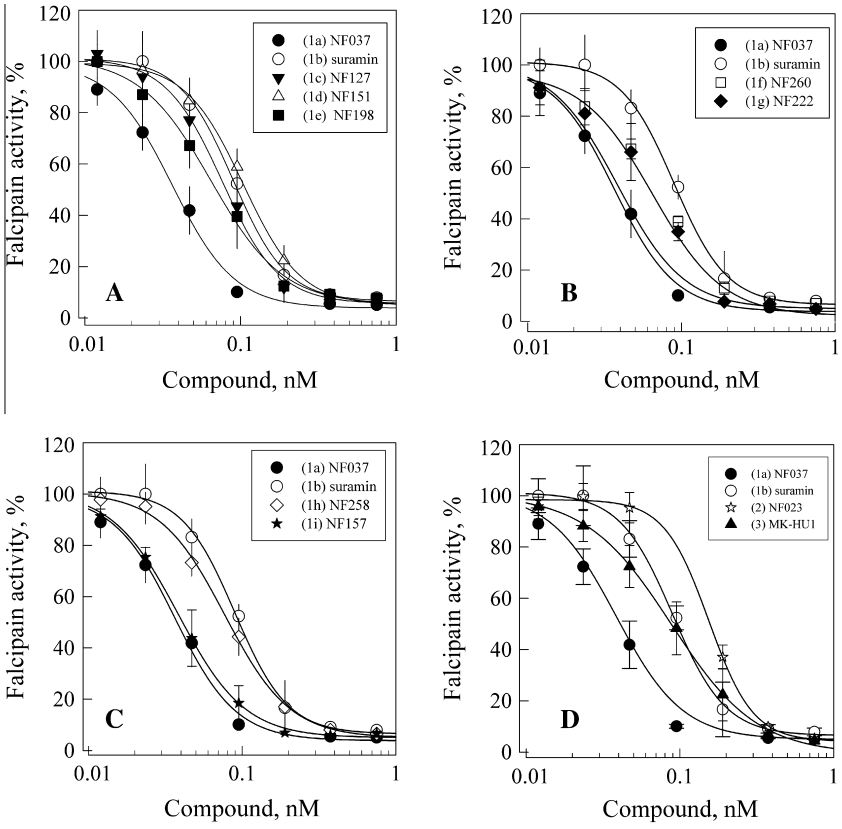

Figure 5. Inhibitory activity of suramin analogues against falcipain-2. Assays were performed in the presence of 50 μM Z-Phe-Arg-AMC and 20 nM falcipain-2, in the presence of varying concentration of suramin and suramin analogues. (A) Analogues with apolar substituents; (B) analogues with polar substituents; (C) analogues with a halogen substituent; (D) short urea analogues. Symbols represent the average of three independent experiments ± SD and the solid line represents the best non-linear curve fit with Eq. 2.

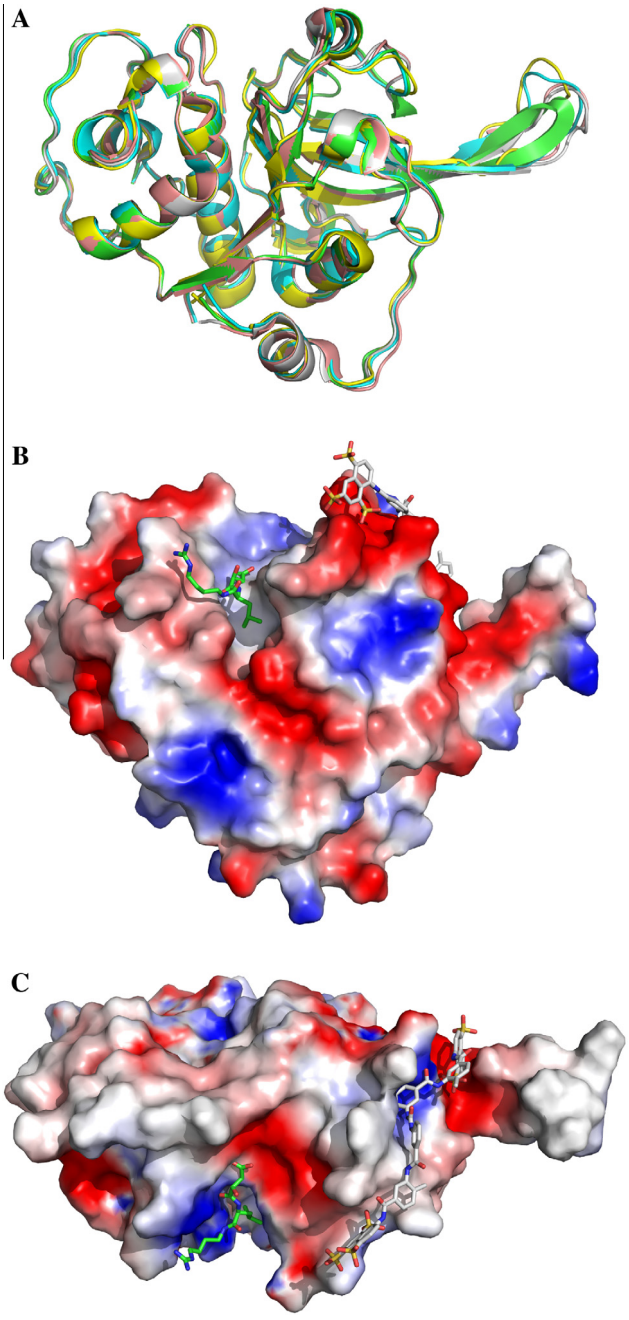

Figure 6. Structural representation of falcipain-2-suramin interaction. (A) Structural alignment of the five available falcipain-2 crystal structures. Structural models were superposed with PyMOL[68] and are displayed in ribbons, showing the similarity between the structures despite the dissimilar ligands used in crystallization, as follows: E64 (PDB entry 3BPF), iodoacetamide (PDB entry 2GHU), chagasin (PDB entry 2OUL), PbiCP (PDB entry 3PNR) and cystatin (PDB entry 1YVB). (B) falcipain-2 in complex with E64 (carbons in green) and docked with suramin (from PDB entry 2H9T, carbons in grey) using HEX.[67] (C) the structure shown in (B), rotated 90° in the *x*-axis.

2.5. Molecular docking between suramin and falcipain-2

The structures of falcipain-2 solved so far are similar, with superposition Cα RMSD of less than 1 Å. Comparative analysis does not allow the identification of large differences between them (Fig. 6A), and no detectable novel binding motif belongs to any particular structure. Macromolecular docking of suramin (PDB entry 2H9T) to falcipain-2 (PDB entry 3BPF, for a falcipain-2-E64 complex) resulted in a best score with the suramin molecule docked in the vicinity of the falcipain-2 active site (Fig. 6B and C), in a cleft located between the active site and the falcipain-2 'arm'. E64 is an inhibitor of cysteine proteases, binding irreversibly to the active site. Suramin interaction with falcipain-2 in the docked complex spans from His197 and Lys184 to Asn173 and Gln171, and these two amino acids are located adjacent to the guanidine moiety of the E64 ligand in the falcipain-2 crystal structure used in this docking. These data allow us to obtain an overall estimation of the extent of surface interaction between falcipain-2 and suramin, which is also indicative of the existence of a region in the falcipain-2 structure for interaction with synthetic substrates, hemoglobin or hemoglobin fragments.

3. Discussion

We have found that suramin binds to and inhibits falcipain-2 at nanomolar concentrations. It has been shown that suramin might exert control over several biological functions, as it is a first-line drug to treat African trypanosomiasis[54] and it acts on a variety of processes in cancer, hemostasis, among others.[47,55–61] Despite having some good characteristics for pharmaceutical use, such as metabolic stability and long half life in plasma, suramin also interacts nonspecifically with several targets, leading to significant toxicity. Therefore suramin, and likely suramin analogues are not appropriate for treatment of malaria. However, suramin proved useful for the identification of novel molecular features in falcipain-2.

This report describes the ability of suramin and some of its analogues to interact with recombinant falcipain-2. We have shown that suramin inhibits activity of falcipain-2 against both a peptide substrate and its natural substrate, hemoglobin. Suramin analogues with modulation of the main substituent at the methyl group of suramin and with varying degrees of sulfonation and length had activities similar to that of suramin. Kinetic measurements revealed that falcipain-2 is subject to substrate inhibition once an allosteric effector—in this case suramin—binds to the enzyme. Suramin forms a uni-molecular complex with falcipain-2, which is inhibited at high concentration of substrate. The substrate inhibition effect has not been previously reported for falcipain-2, and it was only observed here when the activity was conducted in the presence of the non-competitive inhibitor suramin. It is likely that the binding of suramin to falcipain-2 unveils a second substrate binding site, resulting in the allosteric inhibition of falcipain-2. We do not exclude that this phenomenon may occurs in the absence of suramin or any other heterotropic ligand, although suramin assists in unveiling this effect.

The hydrolysis of hemoglobin and hemoglobin fragments by falcipain-2 occurs at the active site. Substrate inhibition precludes the existence of a second substrate binding site, distinct from the active site. The falcipain-2 'arm', which is located well apart from the active site, has been characterized as a hemoglobin binding exosite.[25,62] In light of our present results, we suggest that the falcipain-2 arm may function as the negative heterotropic effector over falcipain-2 catalytic activity.

Falcipains, along with other proteases in the *P. falciparum* food vacuole, are responsible for the digestion of hemoglobin, and inhibition of falcipain-2 and related cysteine proteases blocks parasite development.[12] Cleavage of falcipain's natural substrate, hemoglobin, generates free heme, which is known to be toxic and a cause of oxidative stress. A negative-feedback pathway, in which the binding of a second hemoglobin molecule or fragments to the enzyme could reduce falcipain turnover, may facilitate the conversion of heme into hemozoin. We envision that inside the food vacuole falcipain-2 may not be fully active. Instead, falcipain-2 may be partially inhibited by hemoglobin and its peptide fragments and by non-crystallized heme. We speculate that free heme moieties might behave as allosteric effectors, inhibiting of falcipains, and resulting in a mechanism for protection from toxic species of heme

in solution. Upon conversion of heme into hemozoin, inhibition would cease, and falcipain-2 would reach higher activity levels, releasing peptides and additional heme, in a tightly balanced mechanism, potentially minimizing the risks for oxidative damage that would result in death of the parasite. However, this hypothesis remains to be further investigated. The design of novel allosteric, non-competitive inhibitors targeting falcipain-2 at the suramin binding site will be helpful for further characterization of this regulatory site and potentially to facilitate the discovery of novel antimalarial therapeutics.

4. Experimental procedures

4.1. Material

Suramin was from Sigma Chemicals (St. Louis, MO). Suramin analogues were synthesized as described elsewhere.[57] All other reagents were of analytical grade. All buffers and solutions were prepared just prior to use.

4.2. Construction, expression and purification of recombinant falcipain-2

The 241-amino-acid mature domain of falcipain-2 was expressed in *Escherichia coli*, purified by high-affinity Ni column chromatography, and refolded to its active form as described elsewhere.[13,63]

4.3. Assays of falcipain-2 hemoglobinase activity

Assays of hemoglobinase activity were carried out in the presence of 0.1 mg/ml of human hemoglobin (Sigma–Aldrich, Brazil) in 100 mM sodium acetate buffer, 10 mM DTT, 10% glycerol, pH 5.5, 20 nM falcipain-2, in the presence or absence of suramin (200 nM) at 37 °C for 90 min. The reactions were quenched by addition of denaturing SDS sample buffer and products were resolved by 15% SDS–PAGE and stained with Coomassie Blue R-250, followed by densitometry with ImageJ.[64]

4.4. Assays of falcipain-2 peptidolytic activity

Assays of falcipain-2 (20 nM) activity were carried out as previously described[65] in 100 mM sodium acetate buffer, 10 mM DTT, 10% glycerol, pH 5.5,[66] in a final volume of 200 μl containing the fluorogenic substrate Z-Phe-Arg-AMC (50 μM). The hydrolysis was continuously monitored as the fluorescence of released AMC (excitation 355 nm, emission 460 nm) over 30 min at room temperature (23 ± 2 °C) with a SpectraMax M5 (Molecular Devices®) spectrofluorimeter. At least three independent experiments were used for each curve.

4.5. Measurement of the half maximal inhibitory concentration (IC_{50}) for suramin and suramin analogues

The activities were measured in 96-well microtitre opaque plates at room temperature, as described above, in the presence of varying concentrations of suramin analogues. The IC_{50} values were calculated from the percent enzyme activity as a function of inhibitor concentration, and data points were fitted to a logistic four-parameter curve with Sigma Plot software (Jandel Scientific) according to the equation:

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{IC_{50}}\right)^{-\text{Hillslope}}} \quad (2)$$

where y is the enzyme normalized activity, min is the minimum activity value and max is the maximum activity value observed.

At least three independent experiments were used to determine the half maximal inhibitory concentration (IC_{50}) values for each compound.

4.6. Tryptophan fluorescence measurements of falcipain-2

Suramin binding to falcipain-2 was assessed by measurement of the fluorescence of falcipain-2 tryptophan residues in a Jasco FP-6300 spectrofluorimeter (Jasco Inc, USA). Assays included 5 μM falcipain-2 in 100 mM sodium acetate buffer pH 5.5%, 5% glycerol, 10 mM DTT, and varying concentrations of suramin from a stock solution in the same buffer, with excitation at 295 nm and emission at 340 nm. The maximum dilution was 10%, and fluorescence and protein concentrations were corrected accordingly.

4.7. Molecular docking between suramin and falcipain-2

Molecular docking was performed using the program Hex[67] through its webserver (http://hexserver.loria.fr/) and structures for falcipain-2 (PDB entry 3BPF, for falcipain-2/E64 complex)[25] and suramin (PDB entry 2H9T),[59] with 360° rotational dock in 7.5° steps, using either surface or surface + electrostatic complementarities functions.

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