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Screening of *Plasmodium falciparum* Iron Superoxide Dismutase Inhibitors and Accuracy of the SOD-Assays

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Abstract—In vitro evaluation of a chemical library of synthetic compounds using two consecutive assays has led to the discovery of fifteen compounds which have the ability to inhibit recombinant *Plasmodium falciparum* iron superoxide dismutase (*Pf*SOD), suggested as a highly selective target for design of antiparasitic drugs. A large number of compounds were in fact excluded, because they were found to significantly interfere with the components of the assays, thus outlining the drawbacks relative to the use of standard SOD-assays for the research of compounds targeting SODs. The best of the selected compounds showed significant antimalarial activities against two strains of *P. falciparum*, including a strain moderately resistant to chloroquine.

Introduction

Superoxide dismutases (SODs) constitute the primary enzymatic defence against the damage caused by the toxic superoxide anion radical $O_2^{-\cdot}$ and its derivatives such as peroxynitrite, by catalyzing its dismutation into hydrogen peroxide and oxygen.[1] Iron-containing superoxide dismutases essentially occur in prokaryotes, algae, higher plants, and in a number of protozoan organisms. They are structurally distinct from the Cu/Zn family of SODs, commonly found in eukaryotes, and, to a lesser extent, from the Mn-SODs family.[2] Many parasitic protozoa are highly susceptible to free oxygen radical-induced oxidative stress, suggesting that Fe-SODs could represent a potential target for chemotherapy.[3] Therefore, specific inhibitors of parasitic Fe-SODs, versus host Cu/Zn- or Mn-dependent enzymes could contribute to the evaluation of Fe-SODs as significant targets of new antiparasitic drugs and to the development of improved chemotherapeutic approaches against malaria. In the present work, we have screened a library of synthetic compounds as potential inhibitors of recombinant Fe-SOD from *P. falciparum* (*Pf*SOD) using successively two different SOD assays. Compounds in the bank were selected owing to their structural diversity and their ability to create stacking (aromatic and akin compounds), polar (nitrogen bearing heterocycles) and hydrophobic (hydrocarbon chains compounds) interactions. The most effective inhibitors were then tested in vitro for their activity against two *P. falciparum* strains expressing different degrees of resistance to the commonly used antimalarial drug chloroquine, the sensitive HB3 and the moderately resistant Dd2.

Results

*Pf*FeSOD was overexpressed in a SODA SODB deficient *Escherichia coli* using pT7-7 vector according to Tabor and Richardson,[4] and purified by ammonium precipitation, gel filtration and ion-exchange chromatography as previously described.[5] The screened library consisted of

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1696 structurally diverse synthetic compounds with molecular weights ranging from 200 to 700 g/mol, which included a large variety of structures including chalcones, piperazines, naphthoquinones, and others.[6] The compounds were tested for their ability to inhibit recombinant *Pf*SOD using an optimized microplate assay based on superoxide-mediated NBT reduction in the presence of an NADH/PMS system.[7] This system offered the advantage that it is independant of an enzymatic superoxide source that might be inhibited by the screened compounds. For this assay, all compounds from the library were tested at a concentration of 50 μM in a phosphate buffer pH 7.0 with 2.5% DMSO as final concentration. As many as 209 compounds, that is 12% of the library, were found to be positive in the enzyme assay. At this concentration, all the compounds inhibited the SOD activity of enzyme by at least 20%. All the positive compounds selected from this first screening were then confirmed for their inhibitory properties using the same NBT-based assay with the spectrophotometric procedure and also another standard SOD assay based on the superoxide-mediated autoxidation of pyrogallol.[8] Fifteen inhibitors (i.e., 0.9% of the library), which did not interfere significantly with components of both SOD-assays, were finally selected (Fig. 1, Table 1). They inhibited partially or totally the SOD activity at 50 μM. The identified inhibitors were mainly chalcones and piperazine derivatives, except for the two compounds **F12** and **SG5281**, which did not show any obvious structural similarity to the other selected compounds. As shown in Table 1, the selected hits inhibited from 25 to 100% the activity of *Pf*SOD at 50 μM, the best results being obtained for **SG5281**, **F12** and **BB29** that inactivated completely the enzyme at this concentration.

The effect of the selected compounds on the growth of two strains of *P. falciparum* were then studied using the isotopic semi-microdilution method of Desjardins[9] (Table 2). Among the 15 tested products, 14 showed an homogeneous response on the two strains and only one (**BB31**) was moderately active on HB3 strain and inactive on Dd2 strain. Of 12 products exhibiting an inhibitory effect of 79–100% on recombinant *Pf*SOD at 50 μM, 10 are poorly or inactive in vitro on parasites at the same concentration. The two exceptions were products **SP72** and **SP13**. Inversely, the most active compound on parasites (**AB93**) showed only a poor activity on the isolated enzyme. To a lesser extent, **SP59** exhibited also a significant antimalarial activity in vitro and is a modest inhibitor of *Pf*SOD (63% inhibition at 50 μM).

Discussion

For a number of enzymes, the rational design of inhibitors has often led to the development of molecules that structurally mimic the substrate (substrate analogue) or the putative transition state of the reactions they inhibit. In the case of SODs, the substrate is a small negatively charged molecule, and the rational design of competitive inhibitors structurally related to the substrate does not appear to be feasible. Therefore, in the search of new leads with SOD-inhibitory properties, we conducted a random screening of a laboratory chemical library. Most of the compounds selected from the first screening were molecules which interferred significantly with the components of the NBT-based assay, thus revealing either an apparent inhibitory effect or, on the contrary, a 'superoxide dismutase-like' activity. Consequently, a second SOD-assay was necessary to allow a better selection of compounds that inhibited the *Pf*SOD, without significant interference with the components of the two SOD assays. In fact, all known SOD-assays appear limited for the identification of inhibitors because they are based on a continuous generation of very weak quantities of superoxide from an enzymatic (e.g., xanthine oxidase) or a non-enzymatic (e.g., pyrogallol or NBT/PMS system) source. Consequently, all compounds possessing redox-cycling properties, even weak, affect considerablely the redox balance of the assay mixture, and thus the accuracy of the method. The SOD assays using a microplate procedure and set up in this study to search potential SODs inhibitors are not selective or specific enough to be used in high-throughput screenings of chemical libraries.

The mechanism of inhibition of the selected compounds is unknown, but we can hypothesize that they might interact at the entrance or within the channel with residues which attract electrostatically superoxide towards the active site.[10] Some compounds could act also through iron-chelation or by a direct reaction with the metal ion, but in the view of the crystalline structure of Fe-SODs, the narrow channel conducting to the iron would not fulfil the structural requirements to offer the route to bulky molecules. As examples, the powerful iron chelator desferrioxamine does not result in any inactivation of the Fe-SOD from *Crithidia fasciculata*,[3] and bathophenantroline is unable to chelate iron from native *Pf*-FeSOD, but only from the partially denatured enzyme.[5]

In vitro assays on two *P. falciparum* strains showed discrepancies between the inhibitory effect on parasite SOD and the antimalarial properties for a large number of compounds, but many factors have to be taken into account for the interpretation of the results. First, stability of molecules during respective assays has to be considered. The time course of inhibition assays is much shorter (a few min at 25 °C) than in antimalarial tests (48 h at 37 °C). Secondly, the pharmacokinetic profile of the drug appears to be important in intact or parasitized red blood cells, or in culture medium where many components present redox properties and can react with the assayed compound, resulting in its degradation or its inactivation before affecting its potential target. Third, $O_2^{-\cdot}$ is expected to be a major toxic reactive oxygen species and *Pf*SOD is supposed to play an important role for protection against peroxynitrite resulting from reaction of $O_2^{-\cdot}$ and $NO^{\cdot}$ produced during the host reaction. The most expected outcome of a decrease in SOD level in vivo would be a pro-oxidative response due to an elevation of $[\text{peroxynitrite}]_{\text{steady state}}$. However, in in vitro conditions, the main factors encountered in vivo, particularly $O_2^{-\cdot}$ and $NO^{\cdot}$ produced by the host, are lacking. Hence, in the absence of the in vivo physiological conditions, the antimalarial effect of SOD inhibitors observed

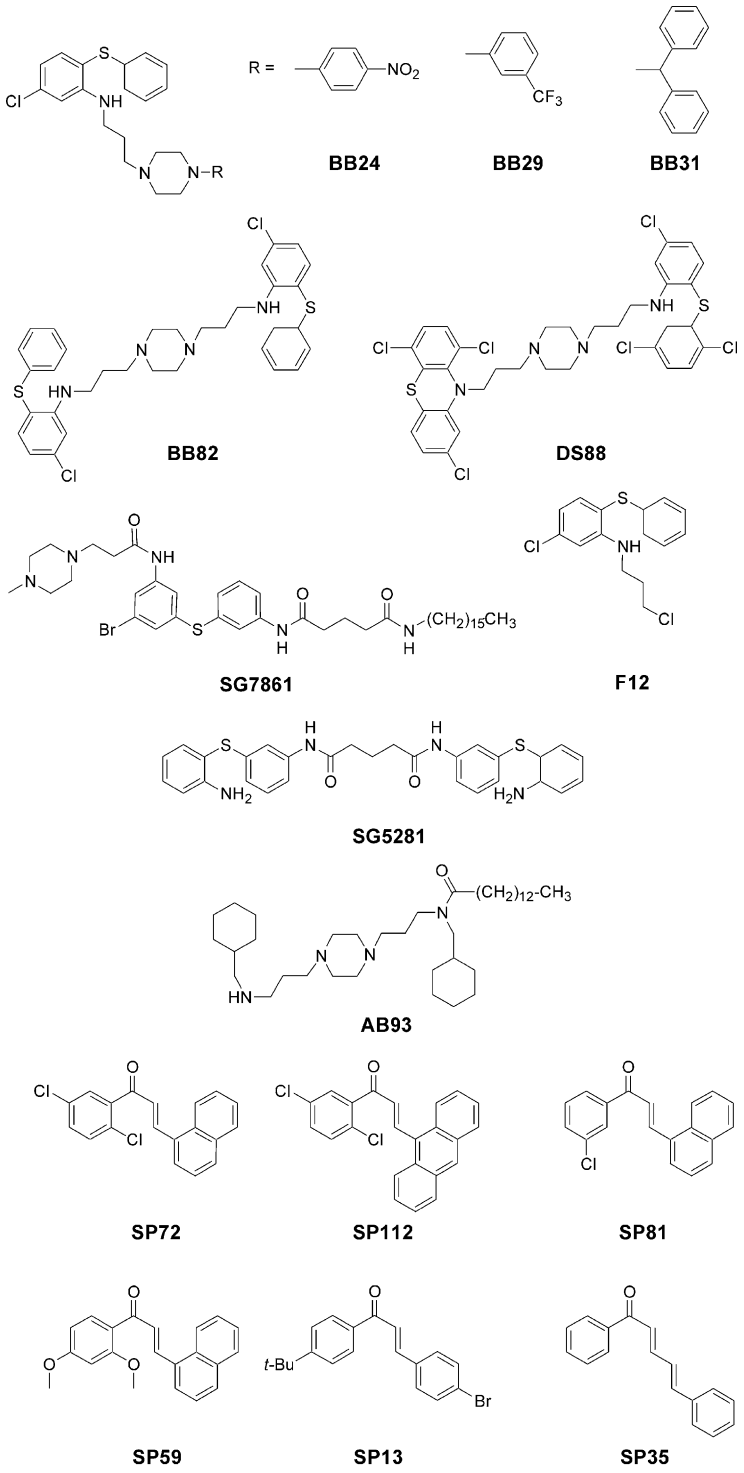

Figure 1. Chemical structures of the selected Fe-SOD inhibitors.

in vitro would be likely more uniformly distributed with regard to response.

For product **AB93**, which is the most powerful antimalarial among all tested, the target may not be *Pf*SOD itself, but other components in the parasite cell in accordance with antimalarial effects previously reported for different members of this series.[11] We cannot exclude also a concentration process in the digestive vacuole at low pH values of the parasitized red blood cell which results in a very high drug concentration and a consecutive significant SOD inhibition.

Among the products presently tested, **SP72**, **SP13** and **SP59** have a significant antimalarial action, which might be related to their inhibition properties on parasite SOD, but such a proposal has to be deeply documented. Many chalcones have already been described for their high antiparasitic—antimalarial and antitrypanosomal—activities, likely through a Michael

Table 1. *Plasmodium falciparum* SOD-inhibition percentages of selected compounds at 50 μM using the assay based on pyrogallol autoxidation

Cpd	*Pf*-SOD[a] (%)	Cpd	*Pf*-SOD[a] (%)
BB24	85	**AB93**	25
BB29	100	**SP72**	97
BB31	82	**SP112**	89
BB82	93	**SP81**	79
DS88	96	**SP59**	63
F12	100	**SP13**	83
SG7861	80	**SP35**	43
SG5281	100		

[a]Conditions: 50 mM Tris buffer pH 8.2 containing 1% DMSO, EDTA (1 mM), pyrogallol (0.24 mM), and enzyme (approx. 43 nM).

Table 2. In vitro antimalarial activity of selected compounds on the chloroquine sensitive *Plasmodium falciparum* strain HB3 and the moderately chloroquine resistant strain Dd2[a]

Compd	IC_{50} *P. f.* HB3	IC_{50} *P. f.* Dd2
Chloroquine	21.2 nM	54.9 nM
BB24	30% inhib at 50μM	Inactive at 50 μM
BB29	Inactive at 50 μM	Inactive at 50 μM
BB31	30±3.5 μM	45% inhib. at 50 μM
BB82	45±5	20% inhib. at 50 μM
DS88	Inactive at 50 μM	Inactive at 50 μM
F12	Inactive at 50 μM	Inactive at 50 μM
SG7861	Inactive at 50 μM	37% inhib. at 50 μM
SG5281	50±20	38% inhib. at 50 μM
AB93	3±0.7 μM	2.5±0.9
SP72	18±4 μM	22±2.5
SP112	50±3 μM	N.D.
SP81	37% inhib. at 50μM	47% inhib. at 50 μM
SP59	27±6 μM	18±6 μM
SP13	37±4 μM	27±10 μM
SP35	20% inhib. at 50 μM	50±12 μM

[a]Results are given as mean of three experiments±standard deviation.

addition of nucleophilic species to the double bond of the enone.[12–14] Here, the chalcones-induced decrease of SOD activity could be attributable to covalent modification occurring at positively charged residues responsible for the electrostatic control of substrate diffusion, located at the entrance of the channel conducting towards the active metal ion (i.e., lysine 29 or arginine 170).

In conclusion, during our search for specific *P. falciparum* FeSOD inhibitors, it appeared that current methods available to measure SOD activity are not adequate for use in high-throughput screenings of chemical libraries because many interferences can occur. Direct methods for measuring SOD activities such as stopped-flow and pulse radiolysis, allowing the direct visualization of the superoxide anion decay, might be more adapted for the discovery of inhibitors, but would not be applicable for a screening purpose. Among the 15 molecules selected from the library screening, some of them (**SP72**, **SP13** and **SP59**) could be considered both as significant SOD inhibitors and to have an antimalarial activity and one, **AB93**, likely has an additional target. The absence of activity of many molecules may be related to problems of chemical stability or of bioavailability and not to the absence of relevance between their intrinsic value as SOD inhibitors and their antimalarial properties. As IC_{50} value determined from in vitro parasite cultures are in the micromolar range, rather far from values currently admitted for lead molecules (10–100 nM range), this work represents a first step of research of drugs targeting *P. falciparum* FeSOD. However it allowed selecting several leads, which can now be optimized, based on a more rational approach from the X-ray structure of *Pf*SOD that has been recently solved.[15]

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