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Targeting *Plasmodium falciparum* protein kinases with adenosine analogue–oligoarginine conjugates

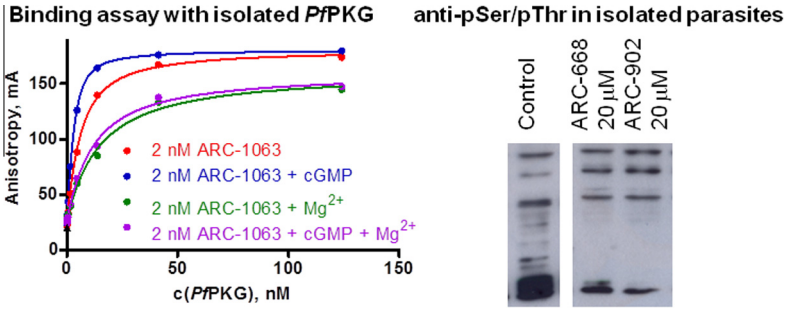
Darja Lavogina [a], Alexandre Budu [b], Erki Enkvist [a], Christine S. Hopp [c], David A. Baker [c], Gordon Langsley [d], Celia R.S. Garcia [b,*], Asko Uri [a,*]

[a] Institute of Chemistry, University of Tartu, Estonia
[b] Department of Physiology, Institute of Biosciences, University of Sao Paulo, Brazil
[c] Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom
[d] Institut Cochin, INSERM U1016, CNRS UMR 8104, Université Paris Descartes Cité Sorbonne, Paris, France

HIGHLIGHTS

- Adenosine–oligoarginine conjugates (ARCs) were targeted to kinome of *P. falciparum*.
- ARCs demonstrated high affinity towards *P. falciparum* cGMP-dependent protein kinase.
- ARCs decreased remarkably the general phosphorylation levels in isolated parasites.
- ARCs represent novel bisubstrate scaffolds for biochemical studies in *P. falciparum*.

GRAPHICAL ABSTRACT



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ABSTRACT

During the last decade, a vast number of inhibitors, ligands and fluorescent probes have evolved for mammalian protein kinases; however, the suitability of these compounds for studies of evolutionarily divergent eukaryotes has mostly been left beyond the scope of research. Here, we examined whether adenosine analogue–oligoarginine conjugates that had been extensively characterized as efficient inhibitors of the human protein kinases are applicable for targeting *Plasmodium* protein kinases. We demonstrated that ARCs were not only able to bind to and inhibit a representative member of *Plasmodium falciparum* kinome (cGMP-dependent protein kinase) in biochemical assay, but also affected the general phosphorylation levels in parasites released from the infected red blood cells upon saponin treatment. These findings urge advantaging of already existing biochemical tools, whose initially generic, but intrinsically "tunable" selectivity profiles could be used for dissection of signaling pathways outside the initially defined group of biological targets.

1. Introduction

Malaria belongs to the group of major human parasitic diseases. The parasitic protozoan *Plasmodium falciparum* is responsible for most of the lethal cases of malaria, causing annually over halfmillion deaths (World Health Organization, 2012). In recent years, several studies have revealed the utmost importance of phosphorylation balance for the progression of the malaria parasite life cycle (Koyama et al., 2009; Solyakov et al., 2011; Talevich et al., 2012),

Abbreviations: ARC, adenosine analogue–oligoarginine conjugate; *Pf*PK, *Plasmodium falciparum* protein kinase; *Pf*PKAc, catalytic subunit of *Plasmodium falciparum* cAMP-dependent protein kinase; *Pf*PKG, *Plasmodium falciparum* cGMP-dependent protein kinase; PK, protein kinase; PKI(14–22)-Myr, myristoylated truncated version of thermostable protein kinase inhibitor (amino acid residues 14–22); RBC, red blood cell.

* Corresponding authors. Address: Department of Physiology, Institute of Biosciences, University of Sao Paulo, Rua do Matao, Travessa 14, No. 321, 05508-900 Sao Paulo, SP, Brazil. Fax: +55 11 30918095 (C.R.S. Garcia). Address: Institute of Chemistry, University of Tartu, Ravila 14a, 50411 Tartu, Estonia. Fax: +372 737 5275 (A. Uri).
E-mail addresses: cgarcia@usp.br (C.R.S. Garcia), asko.uri@ut.ee (A. Uri).

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and thus enhanced the importance of protein kinases of *P. falciparum* (*Pf*PKs) as druggable targets.

According to the generally accepted concept, the selectivity of a potential drug candidate is of utmost importance to minimize possible off-target effects. The divergence of *Pf*PKs from those of mammals thus unequivocally increases their attractiveness as potential drug targets (Ward et al., 2004). On the other hand, as several *Pf*PKs have homologues in mammalian organisms (Budu and Garcia, 2012; Dorin et al., 2005; Haste et al., 2012; Hopp et al., 2012; Vaid and Sharma, 2006), the inhibitors developed against mammalian PKs might serve as initial "templates" for the design of *Pf*PK-selective compounds. Additionally, there is growing evidence that host PKs might also contribute to parasite survival (Doerig, 2004; Doerig et al., 2010; Taoufiq et al., 2008). Apart from the potential therapeutic outcome, the development of inhibitors targeting *Pf*PKs might lead to generation of probes for investigation of *Pf*PK pathways and pinpoint the signaling "nodes" crucial for parasite survival. Overall, taking into consideration that only a relatively limited number of disclosed compounds have been shown to possess high affinity and inhibition potency towards *Pf*PKs (Zhang et al., 2012), the application of PK inhibitors for malaria treatment is still largely unexplored.

Adenosine analogue–oligoarginine conjugates (ARCs) have been developed as bisubstrate inhibitors and fluorescent probes of mammalian PKs; the most efficient representatives of ARCs possess picomolar affinity and inhibition potency towards several basophilic PKs (Lavogina et al., 2012; Uri et al., 2010). All ARCs consist of a nucleoside-mimicking fragment targeted to the ATP-site of a PK, and a peptidic fragment targeted to the protein substrate site of a PK (Lavogina et al., 2010a). The latter moiety incorporates multiple arginine residues and endows ARCs with cell plasma membrane-penetrative properties (Vaasa et al., 2010). The structural diversity and the high affinity of ARCs has enabled the application of ARC-based fluorescent probes in a variety of *in vitro* assays both in the biochemical format as well as in living cells and tissues (Enkvist et al., 2011; Vaasa et al., 2009, 2010, 2012).

In the present work, we set out to investigate: (A) whether ARCs are able to target *Pf*PKs *per se*, and (B) whether ARCs are applicable as research tools for studies of blood stage *P. falciparum*. For that, we performed our initial experiments in biochemical assay with a purified *Pf*PK (represented by cGMP-dependent protein kinase), and then proceeded to tests with the proteins expressed by the parasites in red blood cell culture and parasites isolated from red blood cells upon saponin treatment.

2. Materials and methods

2.1. Cell culture

P. falciparum-infected red blood cells (RBCs) were cultured in flasks with RPMI 1640 medium according to the protocol described in literature (Trager and Jensen, 1976). RBCs were obtained from the healthy volunteers according to the procedures approved by the São Paulo University Ethics Committee.

2.2. Synthesis of ARCs

Synthesis of ARC-type inhibitors and chemical labeling of ARCs with fluorescent dyes (5-FITC, Bodipy FL-SE, 5-TAMRA-SE, Alexa-647-SE) were performed according to the previously described procedures (Enkvist et al., 2006, 2009, 2011; Lavogina et al., 2009, 2012; Vaasa et al., 2010). All compounds except **ARC-660**, **ARC-684** and **ARC-3004** have been previously described; the synthesis of novel ARCs is presented in the Supplementary methods. All compounds used in biological tests were >95% pure by HPLC and possessed predicted m/z values according to the mass-spectrometric measurements.

2.3. Binding/displacement assay with PfPKG

The full length *P. falciparum* cGMP-dependent protein kinase (*Pf*PKG) and the N-terminally truncated version of *Pf*PKG (*Pf*PKG(286-853)) were produced according to the previously described protocol (Deng et al., 2003). The detailed description of cloning, expression and purification of constructs is presented under Supplementary methods.

The binding/displacement assay based on detection of fluorescence polarization/anisotropy was performed as previously published (Vaasa et al., 2009). Briefly, in binding format, fluorescent probe **ARC-1063** (total concentration of 0.5 1, 2 or 5 nM) was added to the dilution series of the full-length recombinant *Pf*PKG and the fluorescence anisotropy was measured after 10 min incubation at 30 °C. Subsequently cGMP, and/or magnesium acetate (total concentrations of 15 μM and 20 mM, respectively) were added to the samples and the measurement was repeated. The data was analyzed with GraphPad Prism 5 software using the previously defined equation (Vaasa et al., 2009).

In displacement format, dilution series of inhibitors were prepared and the complex consisting of *Pf*PKG and **ARC-1063** (total concentrations of 2.4 and 2 nM, respectively) was added to each sample. The anisotropy was measured after 10 min incubation at 30 °C. Subsequently, cGMP (total concentration of 15 μM) was added to the samples and the measurement was repeated. The data was analyzed with GraphPad Prism 5 software using sigmoidal dose–response equation with variable slope. The displacement constant K_d values were calculated with the aid of online K_i calculator (Nikolovska-Coleska et al., 2004; The K_i calculator for fluorescence-based competitive binding assays, 2004).

2.4. Determination of parasitemia

The incubation of *P. falciparum*-infected RBCs (*ca* 5% parasitemia, *ca* 2.5% hematocrit) with inhibitors was performed for 24 h at 37 °C in 96-well plates in a closed chamber with the controlled atmosphere (5% CO_2, 5% O_2 and 90% N_2) in the stationary conditions. The untreated incubations contained the PBS buffer used for the dilution of inhibitors. Subsequently, the cells were washed with PBS, fixed with 2% formaldehyde solution in PBS overnight at room temperature, washed again with PBS and incubated for 30 min at 37 °C with solution containing 0.1% Triton X-100 and 0.5 mg/mL RNase A in PBS. Finally, YOYO-1 solution was added to the samples (final concentration of 10 nM), and the RBCs were passed immediately into the cytometer (BD FACSCalibur). The data was analyzed with GraphPad Prism 5 software (unpaired t test). Results represent data from a duplicate or a triplicate experiment.

2.5. Confocal microscopy

The data acquisition was performed with a Zeiss confocal microscope (LSM 510; MicroImaging, Inc.) with 488 nm excitation (Ar laser) for Bodipy FL- or 5-FITC-labeled compounds, 544 nm excitation for 5-TAMRA-labeled compounds, 633 nm excitation for Alexa-647-labeled compounds, and excitation at 351 and 364 nm (UV laser) for the nuclear stain DAPI. The image analysis was performed with LSM 510 software, version 2.5 (Carl Zeiss MicroImaging, Inc). For sample preparation, see Supplementary material.