A cell-based high-throughput screen validates the plasmodial surface anion channel as an antimalarial target[§]

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Abbreviations:

PSAC, plasmodial surface anion channel; PhTMA$^+$, phenytrimethylammonium cation.

Abstract

The plasmodial surface anion channel (PSAC) is an unusual small conductance ion channel induced on erythrocytes infected with plasmodia, including parasites responsible for human malaria. Although broadly available inhibitors produce microscopic clearance of parasite cultures at high concentrations and suggest PSAC is an antimalarial target, they have low affinity for the channel and may interfere with other parasite activities.   To address these concerns, we developed a miniaturized assay for PSAC activity and carried out a high-throughput inhibitor screen. Approximately 70,000 compounds from synthetic and natural product libraries were screened, revealing inhibitors from multiple structural classes including two novel and potent heterocyclic scaffolds.   Single channel patch-clamp studies indicated that these compounds act directly on PSAC, further implicating a proposed role in transport of diverse solutes. A statistically significant correlation between channel inhibition and *in vitro* parasite killing by a family of compounds provided chemical validation of PSAC as a drug target. These new inhibitors should be important research tools and may be starting points for much-needed antimalarial drugs.

Introduction

P. falciparum malaria is a leading infectious burden on global health, with over 500 million people infected and more than 1 million deaths each year. Estimates of the economic burden on developing countries are also astounding. Despite ongoing trials, a globally useful vaccine is still years away. Aside from preventative measures such as insecticide-treated bednets (Maxwell et al., 2002), treatment of malaria cases with drugs targeting one or more parasite activities is the only meaningful option at present. Evolving resistance to nearly all available antimalarials renders this option imperfect and has motivated workers to identify new parasite targets and small molecule inhibitors. Despite this drive, less than 1% of the ~5600 proteins encoded by the parasite have been explored as therapeutic targets.

Most of the clinical sequelae of malaria result from blood stage parasites that invade, grow, and replicate asexually in erythrocytes. For this reason, identification and characterization of novel blood stage parasite activities represents an important approach to antimalarial drug discovery and development. The plasmodial surface anion channel (PSAC), an unusual ion channel on the infected erythrocyte membrane, is an important new target. Although the genetic basis of this channel activity remains unknown, studies from several groups suggest that it may play an essential role in intracellular parasite survival. Consistent with this hypothesis, transport studies including patch-clamp reveal strict conservation in all plasmodia (Lisk and Desai, 2005). One possibility is that PSAC functions in uptake of nutrient solutes from serum and may also help to discharge parasite metabolic waste products (Desai et al., 2000). An important example of a nutrient requiring uptake is isoleucine, the only amino acid that cannot be obtained through hemoglobin degradation (Liu et al., 2006; Martin and Kirk, 2007). The vitamin pantothenic acid is also essential for parasite growth and requires uptake (Saliba et al., 1998).

4

Biochemical studies suggest this channel has a number of unusual functional properties not present in known mammalian channels. One example is that PSAC efficiently excludes the small Na^+ ion even though it mediates transport of bulky solutes of various sizes and charge (Cohn et al., 2003). Another is that patch-clamp studies reveal a surprisingly small single channel conductance for a channel permeant to large organic ions. Other examples include atypical fast-flickering ion channel gating (Desai et al., 2000) and an unexpected interaction between permeating solutes and known inhibitors (Lisk et al., 2007; Bokhari et al., 2008). It should therefore be possible to identify specific inhibitors suitable for clinical use.

A conceptual advantage of PSAC over intraerythrocytic parasite targets is its surface location on infected erythrocytes. Because inhibitors do not need to cross multiple lipid membranes to reach this target, this location relaxes drug design constraints on inhibitor membrane permeability. The location also largely excludes parasite resistance resulting from energy-dependent extrusion of unmodified drugs, which has been implicated in treatment failures with chloroquine (Fidock et al., 2000) and possibly other antimalarials (Sanchez et al., 2008).

Although these advantages are now well recognized, development of antimalarials targeting parasite-induced transport has not been pursued because it is not known whether the channel serves an essential function for the parasite. Moreover, existing inhibitors have low specificity and affinity, making the drug discovery path unclear. Finally, some electrophysiological surveys have detected multiple distinct ion channels on infected erythrocytes (Staines et al., 2007), raising questions about the relative contributions of PSAC and other routes. To address these and other concerns, we have now developed a miniaturized assay for organic solute uptake by infected cells and performed a high-throughput screen for small molecule inhibitors. Our studies identified novel compounds with improved affinities for transport inhibition, confirmed

the central role of PSAC in the parasite-induced uptake of solutes, and validated this ion channel

as an antimalarial drug target.

Materials and Methods

Reagents. Compounds **1** through **9** were obtained from ChemDiv (San Diego, CA). All other

reagents were obtained from Sigma Aldrich (St. Louis, MO) or Thermo Fisher Scientific

(Waltham, MA).

Osmotic lysis. Osmotic lysis experiments were performed as previously described (Wagner et

al., 2003). Trophozoite-infected RBCs were enriched by percoll/sorbitol separation to > 95%

parasitemia. The cells were then washed in PBS (150 mM NaCl, 20 mM Na_2HPO_4, pH 7.5), and

resuspended at 0.05-0.1% hematocrit in lysis solution (280 mM sorbitol, 20 mM Na-HEPES, and

0.1 mg/mL BSA, pH 7.4) supplemented with PSAC inhibitors as indicated. Experiments

examining uptake of other organic solutes used the same protocol except for iso-osmotic

replacement of sorbitol by the solute or Cl^- salt. Osmotic swelling and lysis was then

continuously followed by recording transmittance of 700 nm light through the cell suspension

(DU640 spectrophotometer with Peltier temperature control, Beckman Coulter, Fullerton, CA).

Each suspension was manually resuspended at 7 min intervals to minimize effects of cell

settling. Control experiments indicated that uninfected erythrocytes do not undergo lysis in PBS

or sorbitol with or without PSAC inhibitors.

High-throughput screen. The above osmotic lysis assay was used for high-throughput

screening after miniaturization to a 384-well format with the following modifications. Infected

6

erythrocytes were enriched, washed, and suspended in PBS at 1% hematocrit before dispensing

20 µL/well into 384-well plates with a microplate dispenser (Matrix Wellmate, Thermo

Scientific), yielding 2 x 10^6 trophozoite-infected cells/well. Compounds from the available

screening collections were then dispensed by a 100 nL pin transfer of a 10 mM DMSO stock into

separate wells and incubated with the cells for a minimum of 20 min. Sorbitol transport and

osmotic lysis were then initiated by addition of 80 µL of the lysis solution. The final compound

screening concentration was therefore 10 µM. Plates were immediately vortexed and then

incubated at room temperature before reading absorbance without resuspension of cells at 30

min, 2 h, and 18 h (700 nm light, Synergy HT, BioTek). Although parasite viability is quickly

lost in saline incubations at room temperature, our previous studies have shown that PSAC-

mediated osmotic fragility of infected cells does not require live parasites (Alkhalil et al., 2004).

This observation is consistent with the unchanged single channel properties for many ion

channels upon excision of cell-attached patches or on reconstitution in artificial bilayers. We

also found that readings after the 18 h timepoint were additionally useful for distinguishing

amongst the most potent hits.

Plates run in duplicate revealed highly reproducible results for all compounds. In plate

negative control wells received DMSO by pin transfer. With each experiment, separate positive

control plates were identically run except that the lysis solution was replaced by PBS

supplemented with 2 mM furosemide. The activity of each screening compound was calculated

at each reading timepoint according to:

$$\% \ \text{block}(t) = 100 * (A_{cpd} - \overline{A}_{neg}) / (\overline{A}_{pos} - \overline{A}_{neg}) \qquad \qquad \text{Eq. 1}$$

where A_{cpd} represents the absorbance from the compound well and $\overline{A}_{neg}$ and $\overline{A}_{pos}$ represent the

mean absorbances of negative and positive control wells run in parallel. At each timepoint, this

parameter represents a quantitative measure of activity against PSAC-mediated transport.

As described previously (Zhang et al., 1999), the Z' statistic was used to evaluate assay

signal-to-noise and was calculated according to:

$$Z' = 1 - 3 * (\sigma_{neg} + \sigma_{pos}) / (\overline{A}_{pos} - \overline{A}_{neg}) \qquad \text{Eq. 2}$$

where σ_{neg} and σ_{pos} represent the standard deviations of control absorbance measurements.

According to this equation, a perfect noise-free assay yields a *Z'* of 1.0; assays having values >

0.5 are generally considered robust and suitable for single replicate high-throughput screening.

Electrophysiology. Cell-attached patch-clamp recordings on trophozoite-stage infected RBCs

were obtained in symmetric bath and pipette solutions of (in mM): 1000 choline-Cl, 115 NaCl,

10 $MgCl_2$, 5 $CaCl_2$, 20 Na-HEPES, pH 7.4. This hypertonic recording solution improves the

signal-to-noise ratio for single PSAC detection; chloride concentration dependences of single

channel currents reveal saturation of flux only at higher concentrations (Alkhalil et al., 2004).

When used, inhibitors were present at identical concentrations in both pipette and bath

compartments. Pipettes were fabricated from quartz capillaries by pulling to tip diameters < 0.5

µm with resistances of 1-3 MΩ in the recording solution. Seal resistances were > 100 GΩ.

Membrane holding potential was 0 mV; pulses to voltages between -100 mV and +100 mV were

applied using Clampex 9.0 software and an Axopatch 200B amplifier in voltage clamp mode

(Molecular Devices, Sunnyvale, CA). Acquired data was filtered at 5 kHz with an 8 pole Bessel

filter, digitized at 100 kHz (Digidata 1322A, Molecular Devices), and recorded. The single

channel traces in Figure 3E were subjected to postfiltering at 3 kHz and a 10-fold decimation to

maintain manageable size of the graphic; detection of events was not affected.

Analyses of single channel recordings were carried out using locally developed code, as

described previously (Desai, 2005). Compound **9**'s affinity was determined by measuring open

probability from up to 8 independent single channel patches at each concentration (total of 160 -

760 seconds of recording at each concentration). The code estimates open probability based on a

threshold midway between the closed and open states. Dwell time distributions were determined

from up to 360 seconds of recordings from single channel patches using a script that detects mid-

threshold crossings, uses linear interpolation of adjacent sample times, and corrects for a

Gaussian filter risetime of 66.4 µs.

Growth inhibition studies. *In vitro* parasite killing by identified transport inhibitors was

performed using a SYBR Green I-based fluorescence assay for parasite nucleic acid in 96-well

format. Parasite cultures were synchronized by incubation in 5% sorbitol before seeding at

0.5% parasitemia and 2% hematocrit in RPMI 1640 without phenol red, but supplemented with

25 mM HEPES, 5% serum, 50 mg/L hypoxanthine, and PSAC inhibitors at indicated

concentrations. Cultures were maintained for 3 days at 37 ^{o}C in 5% O_2, 5% CO_2. The plates

were then subjected to freeze-thaw before addition of SYBR Green I at twice the manufacturer's

recommended final concentration, incubation in the dark for 30 min, and measurement of

fluorescence (excitation/emission at 485/528 nm). Standard curves using serial dilutions of

parasite cultures revealed a linear relationship between fluorescence and parasitemia over the

range used in our study (not shown).

For each PSAC inhibitor, growth inhibitory IC_{50} values were estimated from measurements at a minimum of eight concentrations after subtraction of background fluorescence in matched cultures killed by 20 μM chloroquine.

Results

HTS assay development and optimization. Sorbitol, a poorly metabolized sugar alcohol, has high permeability into trophozoite-stage infected erythrocytes and negligible uptake via uninfected cells. Basic research labs have capitalized on this difference with routinely used protocols for synchronization of *in vitro* parasite cultures and for enrichment of mature infected cells on density gradients. It has also been extensively used to detect and characterize the parasite-induced permeability with a transmittance-based assay that tracks decreasing turbidity of infected cells as they undergo osmotic lysis in sorbitol (Figure 1A; Wagner et al., 2003). This kinetic assay yields estimates of sorbitol permeability and affinity of various inhibitors that are concordant with those from single channel and whole-cell patch-clamp under conditions that isolate PSAC activity (Alkhalil et al., 2004; Desai et al., 2005; Lisk et al., 2006).

To facilitate identification of improved inhibitors, we miniaturized this transmittance assay into 384-well format.   Although erythrocytes settle rapidly in this small geometry format, we determined that vertical transmittance of 700 nm light nevertheless increases upon lysis. This presumably reflects the greater scattering of light by intact cells, which contain locally high concentrations of hemoglobin.   Our studies determined that the magnitude of the lysis-associated transmittance change has a strong and complex dependence on hematocrit. We therefore empirically optimized hematocrit by comparing transmittances through infected cells lysed in sorbitol to those of intact cells in physiological saline. Under the optimized conditions

(see Materials and Methods), Z' factors calculated from positive and negative control wells were typically > 0.7, indicating suitability for high-throughput screening with a low rate of false-positives and false-negatives (Zhang et al., 1999).

Because lysis of individual cells is irreversible and results from accumulation of a threshold amount of sorbitol as previously quantified (Wagner et al., 2003), it should be possible to estimate relative inhibitor efficacy using a single defined concentration of each compound in a plate read at multiple timepoints. We tested this prediction with furosemide, a well characterized PSAC inhibitor with a $K_{0.5}$ of 2.7 µM (Desai, 2005; Desai et al., 2005). These studies revealed that increasing furosemide concentrations monotonically retarded the kinetics of osmotic lysis without affecting the plateau transmittance that corresponds to completed lysis of infected cells (Fig. 1B). Using this kinetic dose response, transmittance measurements at selected timepoints can be used to estimate furosemide concentration in a blinded solution. Conversely, furosemide's known $K_{0.5}$ and a standard curve from its dose response in this format can also be used to estimate relative inhibitory activities of each screening hit with multiple timed measurements at a single screening concentration. This approach successfully maximized the information content of our screen while minimizing the effort- and reagent-intensive culturing of parasites.

These microplate-based osmotic lysis experiments also determined that infected cells continue to accumulate sorbitol and accrue transmittance changes associated with osmotic lysis for prolonged durations despite use of nonphysiological conditions that do not support parasite growth (8 h in Fig. 1B). The transmittance changes reflect channel-mediated uptake rather than nonspecific cell lysis because they can be inhibited by PSAC inhibitors including furosemide and because uninfected cells are osmotically stable in sorbitol (red symbols, Fig. 1B). This

observation is consistent with the biochemical stability of PSAC activity in killed parasite

cultures (Alkhalil et al., 2004). We have also observed unchanged single channel properties in

cell-attached patch-clamp of infected cells killed by storage at 4 ^{o}C for 24 h (not shown).

Identification of a diverse collection of inhibitors. We then used the optimized assay to

execute a high-throughput screen of approximately 70,000 compounds from commercial,

synthetic, and natural product libraries under an investigator-initiated open access facility at the

Broad Institute (Tolliday et al., 2006). Each compound was tested at a 10 µM final concentration

along with DMSO alone control wells in each screening plate. Osmotic lysis was initiated by

addition of sorbitol and subsequently tracked with readings at t = 30 min, 2 h, and 18 h. With

each experiment, we also included one or more plates where sorbitol was replaced with buffered

NaCl solution containing furosemide as a zero lysis control. For each reading timepoint, we

defined a normalized statistic, % block(t), to quantify the relative level of inhibition by each test

compound (see Materials and Methods). Figure 2A shows the distribution of this statistic for

NaCl and DMSO controls superimposed upon those of all compounds tested for the 30 min and 2

h reads. Figure 2B shows the results for the 18 h read. These distributions reveal several

important points about the screen. First, the two types of control wells were tightly clustered

and well separated at each timepoint, indicating good reproducibility and a visual correlate of the

assay's high Z' statistic. Second, there was a spectrum of compound activities observed in our

screen. Although difficult to see in these distributions, the majority of compounds were without

measurable effect. There were also a large number of relatively weak inhibitors: a conservative

threshold of ≥ 50% block at the first timepoint yielded 6512 hits or a high hit rate of ~ 9 %. The

same threshold at the 2 h and 18 h timepoints yielded 1936 and 27 hits, corresponding to hits

rates of 2.7% and 0.03%, respectively. Third, we achieved increasing assay stringency with each

timed reading as indicated by the sigmoidal profile of the distributions in Fig. 2A and 2B. For

example, the subset of compounds producing > 90% inhibition at the first read averaged 75 ±

18% and 25 ± 20% inhibition at the second and third timepoints, respectively. By comparison to

standard curves generated from experiments with furosemide, we calculated that compounds

producing 50% inhibition at 30 min, 2 h, and 18 h have $K_{0.5}$ values of ~ 10 µM, 0.5-1.0 µM, and

< 200 nM, respectively. As confirmed below with single channel patch-clamp studies, our

screen permitted estimation of inhibitor affinity for hits spanning a broad range of activities in a

high-throughput format.

In keeping with the guidelines for screening at the Broad Institute, the structures and

activities of all compounds we screened are publicly available at

http://chembank.broadinstitute.org/.

A novel furoquinoline scaffold is highly effective against organic solute uptake and

confirms a primary role for PSAC. Although some hits were from natural product libraries

and may contain mixtures of compounds whose structures remain to be decoded, many hits were

from commercial libraries with high purity and known structures. Additional hits came from the

Broad Institute's in-house diversity-oriented syntheses (Tolliday et al., 2006). Starting with

compounds having known structures, we selected 16 compounds available for secondary

evaluation and obtained a 100% retest rate using cuvette-based osmotic lysis experiments in our

NIAID laboratory. Representative compounds along with estimates of $K_{0.5}$ values for inhibition

of sorbitol uptake are shown in Table 1. Each of these compounds also inhibited *in vitro* parasite

growth (IC_{50} values in Table 1).

13

To examine the structural basis of inhibition, we selected a tricyclic furoquinoline scaffold that was consistently active in the high throughput-screen (Fig. 2, blue symbols; compound **1**, Table 1) and obtained an additional 87 derivatives. Although these derivatives were distinct from compounds in the primary screen, all 87 compounds produced significant inhibition of sorbitol uptake at a 1 μM concentration (Fig. 3A), confirming that the furoquinoline moiety is responsible for inhibition by these compounds. Several of these derivatives were significantly more effective than the parent compound, presumably because they carry substituents that enhance interactions with critical sites on the channel. One compound, **9**, had a $K_{0.5}$ of 6 nM for inhibition of sorbitol uptake, making it the most potent inhibitor of parasite-induced permeability known to date (Fig. 3B and C).

We next evaluated the effect of these furoquinolines on parasite-induced Cl⁻ currents with single channel patch-clamp and found high level inhibition of PSAC activity at concentrations consistent with their effects on sorbitol uptake (Fig. 3D and 3E). With both compounds **1** and **9**, inhibition was evident as long periods of inactivity in single PSAC recordings. As illustrated with a condensed timescale in Fig. 3E, these block durations were especially long with compound **9**, suggesting that its improved efficacy is due to longer residence in a shared site on the channel. Figure 3F tallies the durations of these long block events and demonstrates that they are absent from recordings without inhibitor.

We used open probability determinations to estimate an inhibitory $K_{0.5}$ of 18 ± 9 nM for compound **9** from single channel recordings at 0, 20, and 100 nM (n = 8, 3, and 4 patches, respectively). Precise quantification of single channel inhibition is complicated for compounds that produce block of very long durations. One error arises because of the relatively small number of closing events that can be captured in achievable recording durations; another results

14

from underestimation of inhibition because events from two channel molecules in a patch may be incorrectly attributed to a single channel in the setting of an inhibitor that imposes long block durations. Taking into consideration these limitations, this estimate is in good agreement with the value of 6 nM obtained for this inhibitor in macroscopic sorbitol uptake measurements (Fig. 3C). Thus, patch-clamp studies confirm that the sorbitol-induced osmotic lysis assay can quantify inhibitor potency in a high-throughput format.

Because the furoquinoline scaffold was not nonspecifically identified in other screens (see below) and because of quantitatively concordant inhibition of sorbitol uptake and patch-clamp currents by compound **9**, these studies support PSAC as the primary parasite-induced channel responsible for both Cl^- and sorbitol transport across the infected erythrocyte membrane.

Recent functional studies suggest that existing inhibitors of this channel have reduced effectiveness against a subset of permeating solutes (Lisk et al., 2007). Solutes exhibiting this effect appear to be recognized and transported through PSAC by a distinct mechanism (Bokhari et al., 2008). To explore whether these improved inhibitors are susceptible to similar interactions with permeating solutes, we examined the efficacy of compound **9** against uptake of 3 solutes that exhibit these interactions—phenytrimethylammonium ($PhTMA^+$), proline, and arabinose (Fig. 4). **9** was significantly less effective at inhibiting the uptake of these solutes than of sorbitol, alanine, or hydroxyproline, solutes that do not exhibit these interactions. We also found that reducing the temperature in these experiments from 37 ^{o}C to 15 ^{o}C largely ameliorated the reduced efficacy of **9**. Of note, both the list of solutes exhibiting this effect and its relatively steep temperature dependence parallel previous findings with furosemide and other weak inhibitors. Thus, inhibitors with diverse chemical structures and a broad range of PSAC affinities are susceptible to these interactions.

Validation of PSAC as a drug target with a family of derivatives exhibiting a spectrum of efficacies. To examine whether inhibition of PSAC interferes with parasite growth, we searched for and identified a family of compounds exhibiting a spectrum of channel affinities. In contrast to those carrying the furoquinoline scaffold, compounds carrying a dibenzothiazepinone scaffold did not uniformly produce high level inhibition in our high-throughput screen (compound **2**, Table 1). We then obtained 72 derivatives that share this scaffold, but carry various substitutions at positions 8 and 10 on the scaffold. For each compound, we performed dose response experiments using the osmotic lysis assay to estimate $K_{0.5}$ for inhibition of PSAC. Because adsorption of some compounds by serum may adversely affect their PSAC affinities under *in vitro* culture conditions, these transport studies were performed in the presence of serum. $pK_{0.5}$ were calculated from these values and are graphically presented with each compound's *in vitro* growth inhibitory pIC_{50} in Figures 5. With serum present at identical concentrations in transport and growth studies, there was a significant correlation between PSAC inhibition and *in vitro* parasite growth inhibition (Spearman's rank correlation coefficient, r_s, of 0.424; $P < 0.0005$ for the null hypothesis of statistical independence). Importantly, compounds producing high level channel inhibition ($K_{0.5} < 200$ nM) were invariably effective against parasite growth with IC_{50} values uniformly below 3 µM. Although the relationship between channel activity and parasite growth is complex and non-linear, the observed correlation provides chemical validation of PSAC as an antimalarial target.

Specificity of these scaffolds for PSAC. We then examined whether these inhibitors are specific for PSAC and whether they are suitable starting points for antimalarial drug discovery.

16

To this end, we searched for activities of compounds **1** and **2** against other targets screened at the Broad Institute using the public ChemBank database (Seiler et al., 2008). ChemBank stores raw values associated with each screen as well as a number of statistical parameters that are designed to evaluate each screen's signal-to-noise ratio, the reproducibility of measurements, and whether individual compounds are considered hits. According to this database, compounds **1** and **2** have been used for screening in 18 and 20 separate projects, respectively. Statistical criteria used by ChemBank reproducibly identified both as "standard hits" in our PSAC inhibitor screens. Compound **1** was judged to be a standard hit in one other project, but this was not clearly reproducible (positive in 6/24 trials against that target). Compound **2** was judged to be a standard hit in two other projects, but again neither was clearly reproducible (positive in 2/6 and 2/18 trials each). Thus, these compounds do not appear to be nonspecific inhibitors of diverse targets.

Compounds **1** and **2** also meet all of Lipinski's rule of five criteria (Lipinski et al., 2001), suggesting that they are sufficiently drug-like for exploration as starting points for antimalarial drug discovery targeting PSAC.

Discussion

We describe a high-throughput screen that successfully identified potent inhibitors of malaria parasite-induced transport across the erythrocyte membrane. The screen was based on selective osmotic lysis of infected erythrocytes in the sugar alcohol sorbitol. Compounds that inhibit parasite-induced sorbitol uptake abrogate osmotic lysis and were detected in our screen as wells with higher absorbance. Statistical measures indicate that our screen had high reproducibility with clear separation of positive and negative controls. Because osmotic lysis of individual cells

is an irreversible process resulting from accumulation of a threshold amount of sorbitol (Wagner et al., 2003), sequential readings of each plate permitted estimation of inhibitory $K_{0.5}$ values over almost 4 orders of magnitude in a high-throughput format.

Our assay and screening algorithm has a number of important conceptual and technical advantages. Most importantly, our approach combines the advantages of target-based and cell-based screens. Target-based screens, typically carried out with recombinant purified protein, generally have high confidence that identified hits will directly interact with the target in question. Their main disadvantage is that the compound may fail to be active against the target in the context of living cells; important examples of this include inability of the compound to access its intracellular target because of low membrane permeability and reduced stability of the compound due to cellular metabolism (Payne et al., 2007). Cell-based screens overcome this limitation by identifying activity in intact cells. However, most cell-based screens are plagued by the identification of hits that act against multiple unrelated targets. A major limitation of most cell-based screens is, therefore, that downstream studies to definitively identify the target of key hits may be both arduous and unsuccessful (An and Tolliday, 2009). Our screen overcomes these various limitations by using a cell-based assay and implementing a readout that is specific for a single parasite activity.

Another common problem with cell-based screens arises from the observation that some compounds in nearly all screening collections have detergent-like activity. Weak detergent activity is often sufficient to produce a false positive in many screens. Our screen is not subject to this concern because the osmotic lysis assay identifies hits based on their abilities to retard or abolish infected cell lysis. Thus, compounds having detergent activity may produce cell lysis

18

and be incorrectly classified as negatives, but they cannot yield false positive results in the screen presented here.

We also used a simple and inexpensive absorbance readout, avoiding false positive detection of autofluorescent compounds as commonly seen in fluorescence-based assays. Because compounds that significantly absorb 700 nm light might be detected as false positives in our screen, we screened several collections of compounds in the osmotic lysis buffer without addition of infected cells and did not find a single compound that altered absorbance significantly. This may reflect the generally poor absorbance of small molecules at near-infrared wavelengths, physico-chemical limitations on absorption by compounds present at relatively low screening concentrations, or the much larger absorbance change associated with lysis of erythrocytes. Indeed, all of the screening hits shown here as well as a number of weaker hits examined in secondary studies appear to specifically target PSAC activity.

The results of our high-throughput screen provide several important insights into parasite biology. One important unanswered question is the number of distinct ion channels required to account for the diverse collection of organic and inorganic solutes with increased permeability after infection. Some studies have suggested that there may be separate ion channels for various subsets of the solutes (Ginsburg and Stein, 2005; Bouyer et al., 2006; Akkaya et al., 2009); in this scenario, the combined activities of these channels would then be responsible for the overall profile of parasite-induced host cell permeability. Although quantitative examination of the effects of inhibitors on solute transport should be able to determine whether there are multiple separate routes or a single shared route, previous transport inhibitors were unable to address this debate because they had relatively low affinities and uncertain specificities (Staines et al., 2007). Our study provides evidence for a single, shared ion channel because high affinity inhibitors

identified through a sorbitol permeability screen are also potent inhibitors of amino acid, sugar, and organic cation uptake (Fig. 4). Our single channel studies add Cl^- to this list of solutes and implicate PSAC as the common mechanistic basis of the parasite-induced permeabilities. Nevertheless, definitive resolution of the relative contributions of various proposed channels will be possible only when the responsible genes are identified.

We present two novel scaffolds that interact directly with one or more sites on the channel. Although medicinal chemistry to systematically evaluate derivatives of these scaffolds is pending, the furoquinoline scaffold was almost invariably associated with high-level channel inhibition and one derivative, compound **9**, is the most potent reversible inhibitor of PSAC identified to date. In contrast, the dibenzothiazepinone scaffold was also highly active, but was more susceptible to adverse effects of substitutions on the scaffold. A more rigorous examination of structure-activity relationships around this scaffold may provide a better understanding of how these molecules achieve block. Our analyses of single channel recordings with both scaffolds suggest that the most potent inhibitors induce very long block durations (≥ 1 s) without reducing open channel amplitudes, suggesting an allosteric mechanism of action.

Previous studies have surveyed derivatives of furosemide and dantrolene and achieved substantial improvements in PSAC affinity (Staines et al., 2004; Kang et al., 2005). While those scaffolds also deserve consideration, our study makes several fundamental advances that should facilitate drug discovery against this target. First, we have identified a large number of diverse scaffolds by high-throughput screening, all available online at http://chembank.broadinstitute.org/. Multiple, unrelated scaffolds are generally considered necessary because of attrition in hit-to-lead chemistry due to considerations of chemical tractability for lead optimization, pharmacokinetic liabilities, specificity for the desired target,

20

cost, and other issues (Zhao, 2007). Second, drug discovery will be assisted by our single

channel studies, which provide mechanistic insights into how derivatives may achieve greater

potency. Finally, another advantage of our screen is that each of the hits described here is

immediately available for basic research studies (ChemDiv, San Diego).

Although multiple studies have suggested that inhibition of PSAC may adversely affect the

intracellular parasite, evidence supporting this hypothesis has been based only on small numbers

of relatively weaker inhibitors that may also interfere with other parasite activities (Kutner et al.,

1987; Kang et al., 2005; Desai et al., 2005). These so-called "off-target effects" represent a

major hurdle for drug discovery projects and appear to be commonplace (Kell, 2006). Although

it is difficult to definitively exclude off-target effects (Dorato and Engelhardt, 2005), the

statistically significant correlation between PSAC inhibition and *in vitro* parasite growth

inhibition observed for a large family of dibenzothiazepinone inhibitors supports an essential role

of PSAC in intracellular parasite survival. Additional studies, possibly facilitated by these potent

inhibitors, will be necessary to determine whether this essential role is related to nutrient

acquisition (Desai et al., 2000) or other proposed functions (Brand et al., 2003; Lang et al., 2004;

Mauritz et al., 2009).

Although they are effective at inhibiting *in vitro* parasite growth, these PSAC inhibitors had

IC_{50} values for *in vitro* parasite growth inhibition generally higher than their $K_{0.5}$ values for

inhibition of sorbitol uptake. For example, the most active dibenzothiazepinone inhibited

sorbitol uptake in serum-containing solutions with a $K_{0.5}$ of 70 nM and was also the best in this

class at parasite growth inhibition (Fig. 5); however, its IC_{50} for parasite growth inhibition was

approximately 10-fold higher (670 nM). Similar or larger discrepancies have been seen with

other parasite targets (Freundlich et al., 2007; Baldwin et al., 2005; Rosenthal et al., 1991); they

21

have frequently been attributed to either poor membrane permeability of inhibitors or expression of the target enzyme at levels higher than minimally required for parasite growth. Two factors specific to PSAC may also contribute to this discrepancy in our studies. First, if PSAC-mediated uptake of organic solutes does function in nutrient acquisition, use of RPMI formulations, which contain supra-physiological concentrations of essential nutrients, may lead to reduced efficacy of these compounds in culture-based growth inhibition studies. Second, the reduced efficacy of these and other PSAC inhibitors against a subset of permeating solutes (Fig. 4; Lisk et al., 2007; Bokhari et al., 2008) may also contribute to the observed discrepancy if these solute-inhibitor interactions occur in parasite cultures. Inhibitors not susceptible to these interactions are being sought and may be better starting points for drug development against PSAC.

A major hurdle in both parasite cell biology and drug discovery targeting PSAC is the identification of PSAC's genetic basis. Definitive identification has in part been hindered by uncertainties about whether the activity reflects proteins encoded by the parasite or human proteins selectively modified by the parasite (Staines et al., 2007). The availability of potent small molecule ligands for PSAC now renders biochemical purification of the protein on an affinity column followed by peptide sequencing a viable approach to gene identification. Such an approach would not require assumptions about the origin or number of proteins involved in this important activity.

Acknowledgments

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25

Footnotes

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Competing Interests: S.A.D. and A.D.P. are named inventors on a provisional patent application describing compounds presented in this manuscript. The other authors do not have competing interests to declare.

Figure Legends

Fig. 1. A miniaturized osmotic lysis assay for PSAC inhibitors suitable for high-throughput screening. A, Continuous transmittance measurements through suspensions of enriched infected cells in buffered sorbitol using a 1 mL cuvette format. Curves show osmotic lysis kinetics in sorbitol with indicated concentrations of furosemide (in µM), normalized to 100% lysis without inhibitor at 1 h. Infected cells do not undergo lysis in PBS (red trace) because of their very low Na^+ permeability (Cohn et al., 2003). B, Assay adapted to 384-well format with a vertical optical path. Symbols represent mean ± S.E.M. absorbance values for infected cells in sorbitol with indicated concentrations of furosemide (in µM). Uninfected cells without furosemide are shown as a control (red circles). A single 384-well plate having 48 wells for each condition was read at 15 min intervals without cell resuspension. Note that increasing furosemide concentrations slow the kinetics of osmotic lysis without affecting the endpoint absorbance plateau, corresponding to 100% lysis. Weak inhibition (*e.g.*, by 2 µM furosemide) is easily detected with readings in the first hour, while conditions producing potent block can be distinguished from each other at later timepoints.

Fig. 2. Distribution of small molecule inhibitory activities at each timepoint in the high-throughput screen.   % block calculated according to Equation 1 for individual wells in 384-well plates containing each of ~70,000 compounds (yellow or blue dots), DMSO alone (red dots), or PBS + 2 mM furosemide (green dots). Panel A shows values for each well at 30 min and 2 h, while panel B shows the values from the 18 h read against the 30 min read.   49 compounds containing a furoquinoline scaffold are shown as blue dots. Note the marked

separation of positive and negative controls and the sigmoidal profile in each panel, reflecting the higher stringency of late readings.

Fig. 3. Potent inhibition by furoquinolines results from direct action on PSAC. A, Histogram showing distribution of % block at 20 min for 87 furoquinoline derivatives, calculated according to Equation 1. B, Structure of compound **9**, a more potent furoquinoline derivative. C, Dose responses for inhibition of sorbitol-induced osmotic lysis by compounds **1** and **9** (triangles and filled circles, respectively) measured as in Fig. 1A and normalized to 1.0 without inhibitor. Each symbol represents the mean ± S.E.M. of up to 9 measurements. Solid curves represent best fits to $y = K_{0.5}/(K_{0.5} + x)$. Panels D and E, Single PSAC recordings in the presence of indicated concentrations of compounds **1** or **9** (D and E, respectively). While each trace in D is 800 ms long, those in E are 9 seconds long. In both panels, membrane potential is -100 mV for all traces; horizontal dashes represent the closed channel level. Vertical scale bar represents 3 pA for both panels; horizontal scale bar represents 150 ms for panel D and 1500 ms for panel E. F, Closed channel dwell time distributions from recordings as in panel E without or with 100 nM compound **9** (black and red histograms, respectively). The distributions are presented on a log-log scale and are normalized to the total number of closed channel events to facilitate visual estimation of blocking rate constants, as described previously (Sigworth and Sine, 1987). The broad peak at 100-1000 ms in the distribution with **9** corresponds to inhibitor-induced block events because they are absent from control recordings. Events shorter than ~ 20 ms correspond to PSAC's intrinsic closings, which are not affected by addition of **9**.

Fig. 4. Compound 9 exhibits previously identified solute-inhibitor interactions. Osmotic lysis kinetics in indicated solutions without (black trace) or with 500 nM compound **9** (green and red traces). Lysis kinetics were tracked at 37 $^{\circ}$C (black and green traces) or at 15 $^{\circ}$C (red traces). Notice that **9** is less effective at inhibiting uptake of PhTMA^{+}, proline, and arabinose than of sorbitol, alanine, and hydroxyproline at 37 $^{\circ}$C, but that this difference is largely abolished at 15 $^{\circ}$C. In the absence of inhibitors, this temperature reduction has only a modest effect on lysis kinetics without inhibitors (Bokhari et al., 2008).

Fig. 5. Chemical validation of PSAC as an antimalarial target with a family of dibenzothiazepinone derivatives. *In vitro* parasite growth inhibition pIC_{50} values for each of 72 dibenzothiazepinone derivatives is plotted against p$K_{0.5}$ values determined from dose responses for inhibition of osmotic lysis in the sorbitol lysis solution supplemented with 5% human serum. pIC_{50} and p$K_{0.5}$ were calculated as -log(IC_{50}) and -log($K_{0.5}$) in molar units, respectively; the most effective compounds are at the top right of the plot. Compound **2**, the parent compound for this family, is shown as a star. Although some derivatives appear to have additional "off-target" effects (*e.g.*, compounds to the upper left of the profile), there is a significant correlation between channel inhibition and parasite killing.

29

Table 1. High affinity chemical entities identified by the high-throughput screen along with their $K_{0.5}$ values for inhibition of sorbitol uptake and IC_{50} values for *in vitro* parasite growth inhibition.
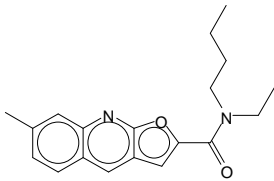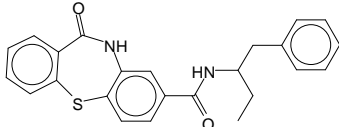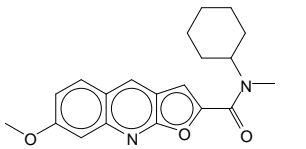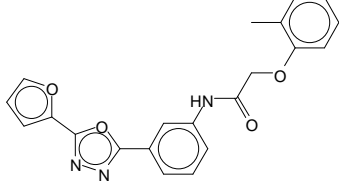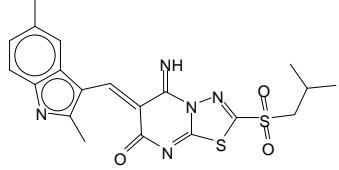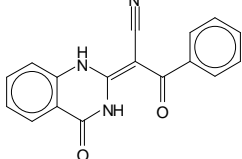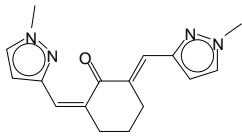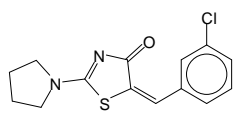
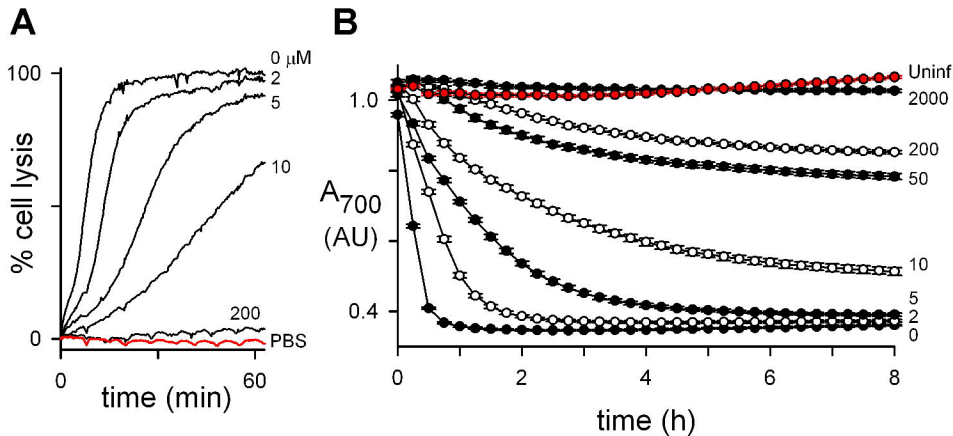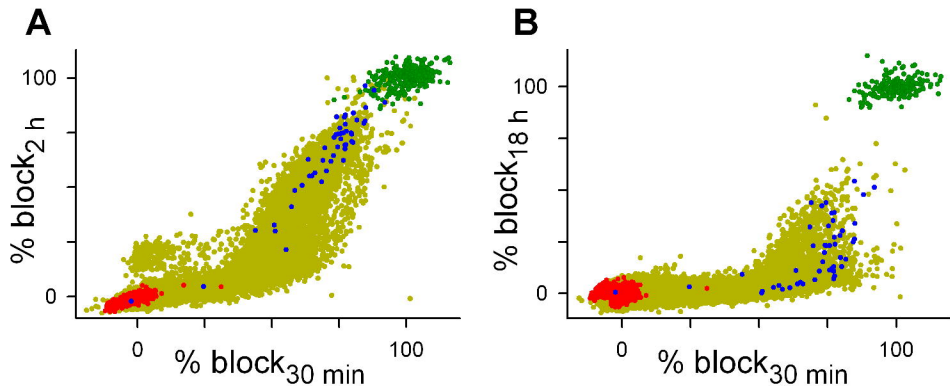|	Compound	mean $K_{0.5}$ (nM) ± S.E.M.	mean IC_{50} (µM) ± S.E.M.
1		87 ± 9	6.0 ± 1.0
2		84 ± 6	2.2 ± 0.4
3		33 ± 3	4.7 ± 2.4
4		124 ± 8	15.2 ± 1.5
5		81 ± 13	13.9 ± 1.0
6		83 ± 6	6.4 ± 0.4
7		98 ± 7	3.4 ± 0.9
8		44 ± 9	6.6 ± 1.3

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

growth inhibition pIC_{50} versus transport p$K_{0.5}$