

VIEWPOINT

Temperature does matter—an additional dimension in kinase inhibitor development

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Kinase inhibitors are a major focus in drug development. Recent work shows that subtle temperature changes in the physiologically relevant temperature range can dramatically alter kinase activity and specificity. We argue that temperature is an essential factor that should be considered in inhibitor screening campaigns. In many cases, high-throughput screening is performed at room temperature or 30 °C, which may lead to many false positives and false negatives when evaluating potential inhibitors in the physiological temperature range. As one example, we discuss a new anti-malaria compound that inhibits the highly temperature-sensitive kinase CLK3 (CDC2-like kinase 3) from *Plasmodium falciparum*.

Introduction

Pharmacological modulation of kinase activity using small molecules has been a central endeavor in drug development, in particular in the treatment of cancer [1]. Countless targeted studies and high-throughput screens in academia and industry have led to the identification of numerous inhibitors, some of which have made their way into the clinic [2]. In many cases, three-dimensional structures of target kinases in complex with small molecule inhibitors have been solved

and used to chemically modify and improve inhibitors [3]. However, recent work suggests an additional dimension that has been neglected in previous screening campaigns and validations: the impact of temperature, more specifically body temperature. In fact, common high-throughput screening platforms perform a kinase reaction at room temperature or 30 °C (e.g., [4–6] also see <http://www.kinase-screen.mrc.ac.uk/>), which is obviously below human body temperature,

Abbreviations

°C, degree Celsius; ³²P-γ-ATP, adenosine triphosphate, labeled on the gamma phosphate with nonbreaking space 32P; *Anopheles spec.*, *Anopheles* species; CLK, CDC2-like kinases; CLK1, CDC2-like kinase 1; CLK3, CDC2-like kinase 3; Kd, kinase domain; MBP, myelin basic protein; *P. falciparum*, *Plasmodium nonbreaking space falciparum*; *Pf*, *Plasmodium nonbreaking space falciparum*; *Plasmodium spp.*, *Plasmodium* species; SD, standard deviation; SR protein, serine/arginine-rich protein; SRPK, SR protein kinase; SRSF1, serine/arginine-rich splicing factor 1.

that is, the environment where the inhibitor should eventually be used. Here we suggest that this discrepancy in temperature may contribute to false positives and false negatives in high-throughput inhibitor screens and argue that temperature is an important variable that should be considered when screening for and validating kinase inhibitors.

The molecular basis for kinase temperature sensitivity

Recent work shows that the activity and/or specificity of at least two kinase families, p38alpha and CDC2-like kinases (CLKs), is highly responsive to temperature changes in the physiological temperature range [7,8]. P38alpha belongs to the family of mitogen-activated kinases [9], whereas CLKs phosphorylate SR proteins, a family of proteins that control pre-mRNA processing, for example, alternative splicing [10]. Recent work has shown that circadian oscillations in core body temperature of only 1–2 °C are sufficient to control CLK activity, which then alters the phosphorylation state of SR proteins and globally controls alternative splicing and gene expression [11,12]. This temperature-dependent change in CLK activity is mediated through subtle and reversible structural changes in the kinase activation segment, which in addition to controlling kinase activity is also involved in substrate binding and could thus alter substrate specificity and inhibitor binding [8]. Available CLK

inhibitors have been identified in screens at 30 °C or room temperature [13–16], whereas a screen at 37 °C or 38 °C, which induces the alternative conformation, has not been performed.

Similarly, a substrate-specific change in activity between 33 °C and 39.5 °C has been reported for p38alpha, which is caused by a temperature-dependent conformational change, in this case in the substrate binding domain, which alters the affinity for different substrates [7]. Such structural rearrangements in close proximity to the substrate and nucleotide binding site switch these kinases on and off or change substrate specificity in a temperature range between 34 °C and 38 °C and will obviously affect the binding of potential inhibitors. Interestingly, altered conformational dynamics of the activation segment has been suggested to contribute to the emergence of tumors that display resistance to the kinase inhibitor imatinib through altered inhibitor binding [17]. This provides strong evidence that conformational dynamics of the activation segment, which is temperature-dependent, can indeed influence inhibitor binding. Screening at room temperature will thus result in a different set of hits than screening at 30 °C or 37 °C. This ambiguity will on the one hand contribute to the low percentage of initial hits that also perform at physiological temperatures at 37 °C. On the other hand, screening at room temperature may result in false negatives, as there may be promising candidates that only bind to the kinase in the 37 °C conformation and that are thus scored

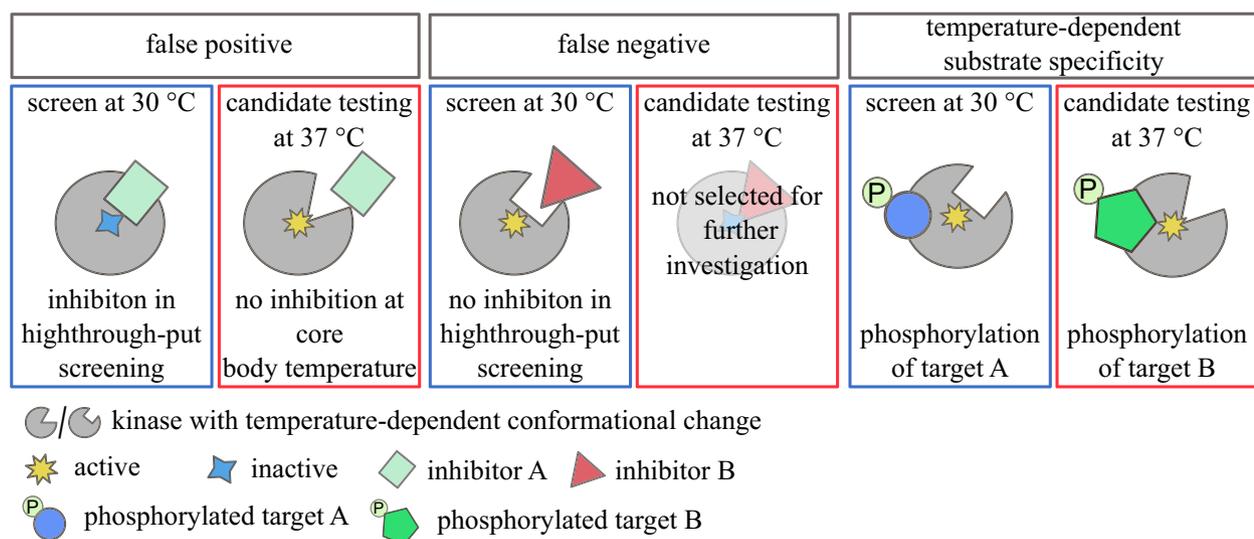


Fig. 1. Temperature-dependent conformational changes can influence the results of high-throughput screens. Model how temperature-dependent conformational changes in kinases can lead to false positives (left) and false negatives (middle) when performing high-throughput screening at room temperature or 30 °C; the right panel shows a temperature-dependent change in substrate specificity.

negative at lower temperatures (Fig. 1). Notably, comparing inhibitor activity *in vitro* (between room temperature and 30 °C) and in cell culture (at 37 °C) revealed in several cases strong differences [18,19], which is in line with the hypothesis discussed above. It should also be noted that the recombinant kinases used for inhibitor screens are in most cases expressed in bacteria and thus may lack functionally important post-translational modification. While this is a general concern, the CLK and p38 proteins in the examples discussed above were bacterially expressed and maintained temperature sensitivity, suggesting this trait to be independent of additional modifications.

***Plasmodium falciparum* CLK3 and an antimalaria compound as an example**

Interestingly, CLKs from different organisms have adapted to the physiologically relevant temperature range of the respective species, from mammals to flies, reptiles, and parasites ([8] and see below). We will discuss one example in detail, the CLK3 homolog from *Plasmodium falciparum*, to highlight the importance of considering temperature when screening for kinase inhibitors. Malaria is one of the most frequent infectious diseases, and, with more than 200 million new infections and 405 000 deaths per year (WHO malaria report 2019), represents a global health threat. There is an urgent need to develop new antimalaria therapeutics, especially due to developing resistance against conventional treatments [20]. The disease is transmitted via the mosquito *Anopheles* from which the protozoan parasite *Plasmodium* spp. is transferred into the human host (reviewed in Ref. [21]). The parasite has a complex life cycle and needs to undergo dramatic morphological changes to adapt to the different hosts, which in turn requires massive changes in gene expression. Some of these changes appear to be mediated through alternative splicing, as, for example, the splicing regulatory protein *PfSR1*, a homolog of human SR protein SRSF1, is required for *Plasmodium* spp. proliferation in human red blood cells [22–25]. Thus, alternative splicing is increasingly recognized as potential point for therapeutic intervention. Alternative splicing is globally controlled through the phosphorylation status of SR proteins, which are phosphorylated by two classes of kinases, CLKs and SRPKs. In *P. falciparum*, the importance of CLK3 (*PfCLK3*) has been highlighted by the finding that it plays an essential role in maintaining the asexual blood stage of the parasite [26]. In recent work, Alam *et al.* [22] identified and validated a potential new antimalaria drug that specifically inhibits *PfCLK3*. A high-throughput screen (kinase reaction

performed at room temperature) revealed the compound TCMDS-135051 as a highly selective and specific *PfCLK3* kinase inhibitor. This was confirmed *in vivo* by the finding that parasite strains that developed resistance against TCMDS-135051 acquired mutations in *PfCLK3*. Gene expression analysis showed that upon inhibitor treatment transcription was impaired and intron-containing genes were overrepresented in the downregulated genes, pointing to a potential connection of *PfCLK3* and splicing, which, however, was not further analyzed [22].

As mentioned above, the best-characterized substrates of CLKs are SR proteins. To further elucidate the role of *PfCLKs* in controlling SR protein phosphorylation, we used our established *in vitro* kinase assay with an SR repeat as substrate. We have previously shown that the *in vitro* activity faithfully mimics the activity of the respective kinase in living cells [8]. Our assays demonstrate that the activity of both, *PfCLK1* and *PfCLK3*, strongly responds to temperature changes in the physiologically relevant temperature range (Fig. 2). The temperature optima of both kinases are around 22 °C and remarkably, both kinases are basically inactive toward phosphorylating an SR repeat at 37 °C. Interestingly the optimal temperature for *Anopheles* populations is 20–26 °C [27], which is also the optimal temperature for *P. falciparum* development and transmission to humans [28]. This temperature range will induce maximal CLK activity, whereas the activity is switched off upon transmission into the human host simply by the altered temperature (Fig. 2). We hypothesize that *P. falciparum* uses the different host temperature leading to altered CLK activity and SR protein phosphorylation to induce the massive changes in gene expression required in different life cycle stages, but this idea awaits experimental validation.

The *PfCLK* temperature-response curve raises the question, how TCMDS-135051 can be active as an antimalaria compound, if its target, *PfCLK3*, is not active when the parasite is in the human host. The answer may lie in changed substrate specificity conferred by higher temperature. Alam *et al.* [22] used myelin basic protein (MBP) as *in vitro* substrate, which is not an SR protein and may thus not be the physiologically relevant substrate, but they clearly observed phosphorylation by *PfCLK3* at 37 °C. Furthermore, autophosphorylation of *PfCLK3* was still weakly active at 37 °C (Fig. 2, also see Ref. [22]), suggesting that higher temperature primarily affects the phosphorylation of SR proteins but that other targets may still be phosphorylated. This could point to a temperature-dependent change in substrate selectivity.

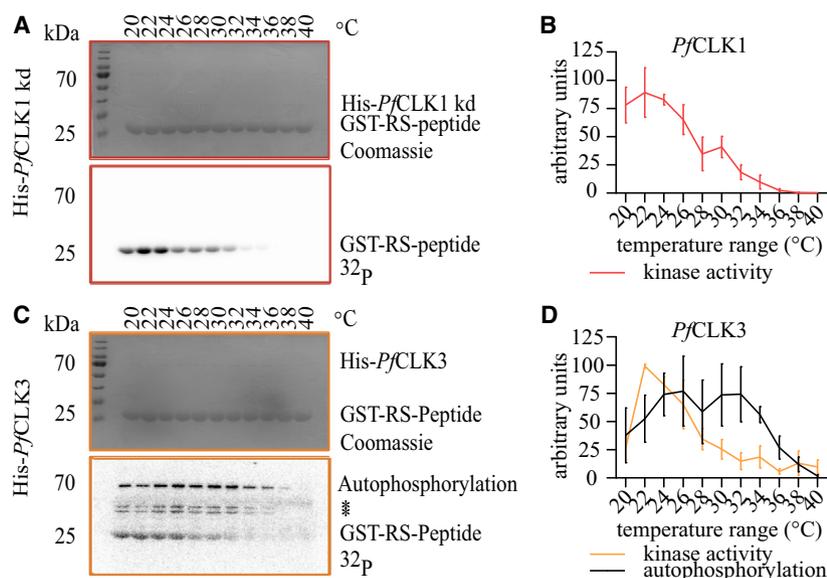


Fig. 2. Kinase activity of *PfCLK1* and *PfCLK3* is temperature-dependent in the physiological relevant temperature range of the vector *Anopheles* spec. (A) An *in vitro* kinase assay with bacterially expressed His-tagged *PfCLK1* kinase domain (kd) and a GST-RS-peptide as substrate. Kinase and substrate were incubated at the indicated temperatures with ³²P-γ-ATP as described in Ref. [6]. A representative gel is shown. The top gel is the Coomassie stain after SDS/PAGE to confirm equal loading and the bottom gel the autoradiograph (³²P) of the same gel. (B) Quantification of substrate phosphorylation (red) relative to the highest activity ($n = 3$, mean \pm SD); autophosphorylation was not detectable. (C) *In vitro* kinase assays with bacterially expressed His-tagged *PfCLK3* full length performed as in (A). *PfCLK3* degradation products are marked with asterisks. (D) Quantification of substrate (orange) and autophosphorylation (black) relative to the highest activity ($n = 3$, mean \pm SD).

Interestingly, *PfCLK3* autophosphorylation happens at tyrosine 526 [26] and MBP has two predicted tyrosine phosphorylation sites and these substrates were both phosphorylated at 37 °C. In contrast, an SR repeat was not phosphorylated at 37 °C, pointing to a temperature-dependent change in target amino acid specificity. This could be controlled through conformational changes of the activation segment, which makes direct contact to the substrate and could thus inhibit or favor binding of specific substrates. A similar phenomenon has been described for p38alpha, where changes in the physiological temperature range alter substrate affinity and specificity by subtle conformational changes [7], suggesting this to be a general phenomenon. Thus, while TCMDS-135051 is clearly acting as an antimalaria agent, it may do so by inhibiting the phosphorylation of noncanonical and as of yet unknown targets with equally unknown functionality.

Conclusion

Together these data emphasize that temperature should be considered as an important variable in kinase inhibitor screening campaigns, as even subtle

changes within the physiologically relevant temperature range can have a dramatic impact on kinase activity and substrate specificity. Furthermore, we hypothesize that repeating screens at 37 °C will yield new promising lead structures that were false negatives in screening campaigns at lower temperature.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MS performed experiments. MS and FH analyzed and interpreted data and wrote the manuscript. FH conceived and supervised the work.

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