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Discovery and biochemical characterization of selective ATP competitive inhibitors of the human mitotic kinesin KSP

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Abstract

The kinesin spindle protein (KSP, also known as Eg5) is essential for the proper separation of spindle poles during mitosis, and inhibition results in mitotic arrest and the formation of characteristic monoaster spindles. Several distinct classes of KSP inhibitors have been described previously in the public and patent literature. However, most appear to share a common induced-fit allosteric binding site, suggesting a common mechanism of inhibition. In a high-throughput screen for inhibitors of KSP, a novel class of thiazole-containing inhibitors was identified. Unlike the previously described allosteric KSP inhibitors, the thiazoles described here show ATP competitive kinetic behavior, consistent with binding within the nucleotide binding pocket. Although they bind to a pocket that is highly conserved across kinesins, these molecules exhibit significant selectivity for KSP over other kinesins and other ATP-utilizing enzymes. Several of these compounds are active in cells and produce a phenotype similar to that observed with previously published allosteric inhibitors of KSP.

Keywords: Kinesin; Inhibitor; KSP; Nucleotide; Motor

The mitotic spindle is a complex and elegant apparatus that carries out the essential task of segregating replicated chromosomes to daughter cells during the process of cellular replication. As such, it has become a well validated target for cancer chemotherapeutic drugs, such as the taxanes, the vinca alkaloids, and colchicine. All of these drugs have as their ultimate target the microtubules which form the principal structure of the mitotic spindle [1]. However, because microtubules play important roles in a number of other cellular processes, the microtubule-binding chemotherapeutics also have side effects related to non-mitotic microtubule functions, e.g., peripheral neuropathy [1].

Over the past decade, the field has shifted towards identification of novel targets that have essential roles in the assembly and function of the mitotic spindle and that are often over-expressed and/or amplified in a variety of tumor backgrounds. A mitosis-specific compound screen identified one of these targets denoted KSP (Hs_Eg5, Kifl1), a member of the kinesin-5 family, which is essential for formation of a normal bipolar spindle [2,3]. KSP is thought to crosslink and slide microtubules relative to each other, pushing apart interpolar microtubules, and may also play a role in bundling microtubules together [4,5].

The discovery of monastrol [3], a selective and cell-active inhibitor of KSP has triggered a dramatic increase in research on inhibition of this motor protein, which has been shown in cell culture to lead to monoastral arrays

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of chromosomes on microtubules, mitotic arrest, and cell death. Inhibition of KSP *in vivo* causes similar effects on proliferating cells, leading to efficacy in xenograft tumor models [6–8]. Unlike tubulin and microtubules, KSP's biological function appears to be much more specific for mitosis, and so KSP inhibitors might provide efficacy in cancer without the neurotoxic side effects seen with anti-microtubule agents. Accordingly, there has been considerable interest in the development of KSP inhibitors for the treatment of cancer, and numerous groups have reported progress towards potent, selective, and cell permeable inhibitors of KSP [8–21]. Many of these molecules, including monastrol, share a common allosteric binding site, formed by an induced-fit pocket between helix α3 and the L5 insertion loop [10,22]. As the L5 insertion loop is not highly conserved between KSP and other kinesins, inhibition by binding to this site seems to be highly specific to KSP. Kinetic studies of L5 binding allosteric inhibitors clearly show that these molecules do not interfere with KSP binding to nucleotides in a competitive fashion [8,23–25].

Herein we introduce a series of small molecule KSP inhibitors with a novel mechanism of action. These thiazole molecules are competitive with ATP and uncompetitive with microtubules in steady-state ATPase assays. Surprisingly, these molecules also show selectivity for KSP over other kinesins and other ATP-utilizing enzymes.

Methods

KSP ATPase

The primary assays for KSP ATPase activity are based on a colorimetric determination of inorganic phosphate released from ATP after hydrolysis by the enzyme. The human KSP motor domain (Uniprot P52732) (1–367) was expressed in bacteria with a His_6 tag for purification [22]. Microtubules were polymerized from tubulin at 37 °C, in the presence of 1 mM GTP and 10 μM paclitaxel, and were then separated from unpolymerized tubulin by centrifugation. Assays were performed in a 96 well plate, with each well containing 1 μL of a DMSO solution of the compound being tested. Compounds were tested as an 11-point titration series, with 3-fold dilutions between each step, ranging from a concentration of 50 μM (in assay) to 800 pM. The reaction was started by adding 39 μL of the reaction buffer to each well, and final reaction conditions were 80 mM K-HEPES (pH 6.8), 40 mM KCl, 1 mM EGTA,[1] 0.01% BSA, 1 mM DTT, 2 mM $MgCl_2$, 1 mM ATP, 0.5 μM tubulin (as polymerized microtubules), 2.5% DMSO, and 5 nM KSP. After 50 min of incubation at room temperature, reactions were quenched by the addition of 40 μL of a solution containing 50 mM EDTA and 1.8 M KCl. Reactions were visualized by the addition of 120 μL of a solution containing 65 μg/ml quinaldine red, 0.093% polyvinyl alcohol, and 4.1 mM ammonium molybdate in 0.38 M sulfuric acid. After 5 min for color development, plates were read for optical absorbance at 540 nM. ATPase activity in the absence of microtubules was measured similarly, but with a higher concentration of KSP (100–200 nM final concentration). Synergy between inhibitors was tested similarly following the guidelines outlined in Chou and Talalay [26], using individual inhibitor concentrations ranging from 0.1 to 10 IC_{50} over an 11 point range. The mixture of both inhibitors was tested over the same concentration range, with each inhibitor present at an equivalent ratio to its IC_{50} as determined individually.

Kinesin counterscreens

MCAK and *A. nidulans* BimC motor domain were purchased from Cytoskeleton (MK01, BM01). All other kinesins were assayed as isolated motor domains, cloned from the corresponding human sequences (Kifl1, the mouse ortholog of KSP, was cloned from the mouse sequences), and expressed and purified similarly to the procedure used for KSP [Kif11: 1–366, CENP-E: 1–340, uKHC: 1–337, nKHC: 1–340, Kif1B: 1–350, Kif3A: 1–350, MKLP-1: 1–435, CMKrp: 342–720; also see Table 3]. Enzyme concentrations were adjusted to optimize sensitivity and linearity of the initial reaction rates for each enzyme tested: Kif11: 10 nM, CENP-E: 5 nM, uKHC: 1.5 nM, nKHC: 10 nM, Kif1B:5 nM, Kif3A: 2 nM, MKLP-1: 50 nM, Kif14: 50 nM, MCAK: 50 nM, and BimC: 200 nM.

Kinetic competition

Assays followed methods similar to that described above for the primary KSP ATPase assay. The reaction buffer was as described above, but both ATP and microtubule concentrations were varied. For either study, compounds were tested at eight different concentrations, from 0 to either 0.8 or 0.02 μM as appropriate for the potency of the compound. Either ATP or microtubules were varied independently from the compound over 12 different concentrations—ATP was varied from 15 μM to 1 mM, while microtubules were varied from 50 to 1000 nM. When ATP was varied, microtubule concentrations were fixed at 1 μM. When microtubules were varied, ATP was fixed at 1 mM. The enzyme concentration tested was 5 nM. Reactions for each condition were initiated in a total volume of 120 μL. Ten microlitres aliquots were quenched by addition of 90 μL of a solution containing 25 mM EDTA and 0.9 M KCl at 1, 2, 3, 4, 6, 8, 10, and 12 min. Phosphate was detected by addition of 150 μL of the quinaldine red reagent described above, and incubated for 5 min before reading the absorbance at 540 nM. The data were first analyzed as a function of time, to derive linear rates for each reaction condition. The velocity data were then analyzed with respect to Eq. (1) below (described as a Michaelis–Menten) using a non-linear least-squares method within SigmaPlot. The equation is almost identical to equation IV-11 from [27], but αK_I is replaced with K_{IA} to improve the quality of the curve fit. In this equation, v represents the observed enzymatic rate of reaction, [I] represents the concentration of the inhibitor, and [S] represents the concentration of either ATP or microtubules. V_{max}, K_M, K_I, and K_{IA} are all derived from the modeled data; V_{max} and K_M are the normal Michaelis–Menten coefficients for the substrate, K_I reflects the affinity of the inhibitor for the enzyme in the absence of the substrate, and K_{IA} reflects the affinity of the inhibitor for the enzyme in the presence of bound substrate. With a strictly competitive inhibitor, where $K_{IA} >> K_I$, the fourth term in the denominator drops out, and the equation simplifies to the classical model of Eq. (2). With a strictly uncompetitive inhibitor, where $K_I >> K_{IA}$, the third term in the denominator drops out, and the equation simplifies to Eq. (3), the classical model for uncompetitive inhibition but using the term K_{IA} where K_I is used in equation III-31 from [27]. With a pure noncompetitive inhibitor, $K_{IA} = K_I$ but the equation still follows the form of Eq. (1).

$$v = \frac{V_{max} \cdot [S]}{K_M \cdot \left(1 + \frac{[S]}{K_M} + \frac{[I]}{K_I} + \frac{[S]\cdot[I]}{K_M \cdot K_{IA}}\right)} \quad (1)$$

$$v = \frac{V_{max} \cdot [S]}{K_M \cdot \left(1 + \frac{[S]}{K_M} + \frac{[I]}{K_I}\right)} \quad (2)$$

$$v = \frac{V_{max} \cdot [S]}{K_M \cdot \left(1 + \frac{[S]}{K_M} + \frac{[S]\cdot[I]}{K_M \cdot K_{IA}}\right)} \quad (3)$$

[1] *Abbreviations used:* BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate; FACS, fluorescence automated cell sorting.