Chemical Biology

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Relocation of Aurora B and Survivin from Centromeres to the Central Spindle Impaired by a Kinesin-Specific MKLP-2 Inhibitor**

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Mitotic kinesin-like protein 2 (MKLP-2), a member of the kinesin-6 family, is essential for cytokinesis. We screened 8900 small molecules for inhibition of the ATPase activity of MKLP-2 and identified the first inhibitor, (*Z*)-2-(1*H*-indol-3-yl)-3-(pyridin-3-yl)acrylonitrile, which we named paprotrain (PAssenger PROteins TRAnsport INhibitor; Figure 1b).[1] Paprotrain is a reversible inhibitor uncompetitive with ATP (inhibitor constant $K_i = (3.4 \pm 0.1)$ μM) and noncompetitive with microtubules (MTs; $K_i = (1.6 \pm 0.1)$ μM). It is specific for MKLP-2 as it does not inhibit other members of the kinesin superfamily. Paprotrain is cell permeable and incubation with 10 to 50 μM inhibitor results in binucleated cells, a characteristic phenotype similar to that observed following RNA interference (RNAi)-mediated depletion of MKLP-2. Additional mitotic spindle defects were also observed. Notably, paprotrain perturbs MKLP-2-mediated relocation of the chromosome passenger proteins Aurora B and survivin from the centromeres to the central spindle. Lack of relocation of passenger proteins to the central spindle is associated with failure of cytokinesis and generation of binucleated cells. In contrast, paprotrain does not impair kinesin family member 4 (Kif4)-mediated translocation of protein regulating cytokinesis 1 (PRC1), which emphasizes its specificity for MKLP-2. We conclude that paprotrain is a new lead compound targeting MKLP-2 by a novel mechanism of action.

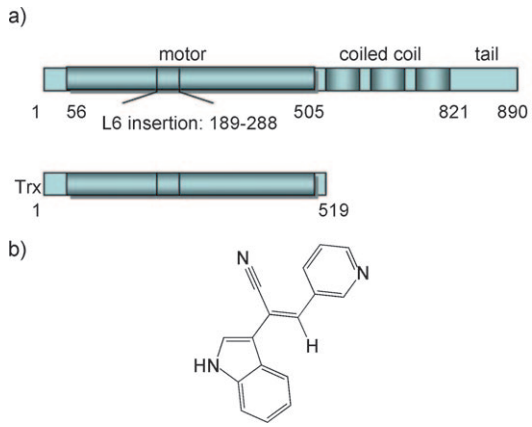

Figure 1. a) Bar diagram of human MKLP-2 and the protein construct used for inhibitor screening. MKLP-2 has an N-terminal motor domain (amino acids (aa) 56 to 505), an α-helical region (aa 519 to 821), and a C-terminal tail domain (aa 821 to 890). MKLP-2 contains an approximately 100-residue insertion (aa 189 to 288) in the motor domain that is unique for members of the kinesin-6 family. b) Chemical formula of paprotrain. Trx = thioredoxin.

The motor domain of human MKLP-2 (residues 1–519) was cloned, expressed, and purified (Figure 1a). Gel filtration and analytical ultracentrifugation showed that the protein is monomeric (data not shown) with an estimated molecular mass of (77 ± 5) kDa (see Table S1 in the Supporting Information).

The basal ATPase activity was (0.034 ± 0.012) s^{-1}, with a $K_{M,ATP}$ of 59.4 μM. The activity decreased with increasing NaCl concentration and inhibitor screening was therefore performed in the absence of salt. The MT-stimulated ATPase activity was 1.8 s^{-1}, which corresponded to an estimated 53-fold stimulation compared to the basal activity. The $K_{0.5,MT}$ was 1.6 μM (see Figure S1 and Table S2 in the Supporting Information).

We measured the inhibition of the basal and MT-stimulated ATPase activity in the presence of paprotrain (Figure 2). Calculated IC_{50} values were (1.35 ± 0.2) μM in basal (Figure 2a) and (0.83 ± 0.1) μM in MT-stimulated ATPase assays (Figure 2b). The measured rates of ATP hydrolysis in the presence of varying concentrations of paprotrain and MTs demonstrate that the inhibitor acts through a mixed noncompetitive mechanism with respect to MTs with an inhibitor constant $K_i = (1.6 \pm 0.07)$ μM (Figure 2c). We also analyzed the ATP hydrolysis rate at different ATP and inhibitor concentrations. Paprotrain is an ATP uncompetitive inhibitor with $K_i = (3.36 \pm 0.09)$ μM (Figure 2d).

The ATPase activity of a panel of 12 other kinesins[2,3] was not inhibited by paprotrain, thus demonstrating high specificity within the kinesin superfamily (see Figure S2a in the Supporting Information). Notably, paprotrain does not inhibit

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MKLP-2 = mitotic kinesin-like protein 2.

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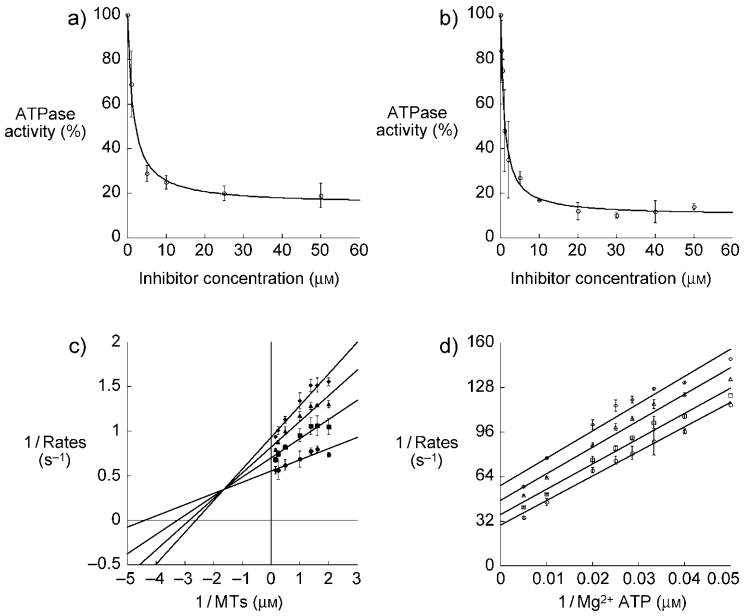


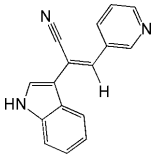
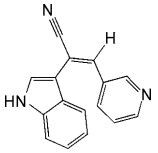
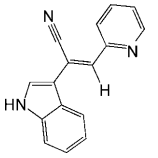
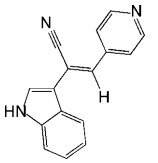
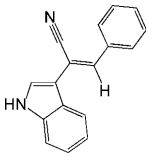
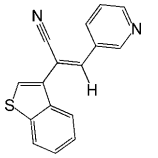
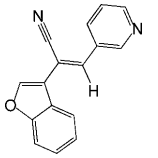
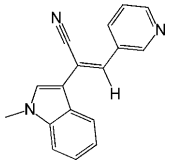
Figure 2. a) Inhibition of the basal MKLP-2 ATPase activity in the presence of paprotrain. b) Inhibition of the MT-activated MKLP-2 ATPase activity by the inhibitor. c) Paprotrain is noncompetitive when mixed with MTs: ● 0; ■ 0.35; ▲ 0.7; ◆ 1.1 μM MKLP-2 inhibitor. d) The inhibitor is uncompetitive with ATP: ○ 0; □ 0.7; △ 1.5; ◇ 2.5 μM inhibitor.

the two closest related kinesins involved in cytokinesis, MKLP-1 and M-phase phosphoprotein 1 (MPP1).

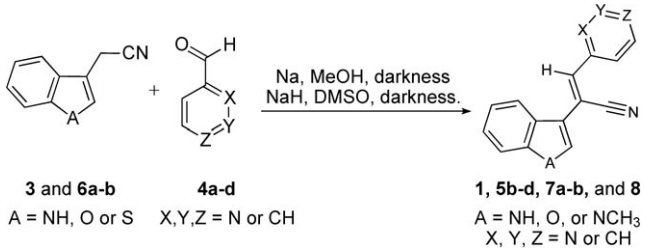
MKLP-2–MT co-sedimentation assays showed that the inhibitor did not influence the binding of MKLP-2 to MTs (Figure S2b). Compared to nocodazole and taxol, paprotrain has no significant effect on MT polymerization (Figure S2c).

We synthesized and characterized seven additional inhibitor analogues (see Schemes S1–S4 in the Supporting Information) and assessed the inhibition of MKLP-2 (Scheme 1 and Table 1). The two heterocycles in **1** are in the *trans* position to each other. To evaluate the biological effect of the double bond's stereochemistry, the *trans* double bond of compound **1** was isomerized by photochemical irradiation to afford compound **2** with a *cis* double bond. This *cis* stereochemistry was confirmed by a coupling constant of 9 Hz between the H3 proton and the C1 carbon atom in the heteronuclear multiple bond correlation (HMBC) spectrum. Compound **2** inhibits MKLP-2 with considerably higher IC_{50} values. In **1**, the pyridinyl group is connected in the *meta* position to the double bond and it is in the *trans* position with the indole scaffold. Two additional compounds that have the pyridinyl group connected in either the *ortho* (**5b**) or *para* position (**5c**) do not inhibit MKLP-2. Compound **5d**, in which the pyridine is replaced by a phenyl group, is a less potent inhibitor of MKLP-2. Equally striking are the results obtained with two other compounds, in which the nitrogen atom of the indole ring is substituted in the same position by either sulfur (**7a**) or oxygen (**7b**). Neither compound inhibits MKLP-2. Finally, the N-methylated indole analogue (**8**) is also inactive.

Table 1: Inhibitor analogues investigated to study the structure–activity relationship of paprotrain-related compounds.

Compound		Basal ATPase activity IC_{50} [μM]	MT-stimulated ATPase activity IC_{50} [μM]
1		1.35 ± 0.2	0.83 ± 0.1
2		20.1 ± 3.7 (35%)[a]	9.1 ± 2.5 (25%)[a]
5b		61.4 ± 9.0 (55%)[a]	n.i.[b]
5c		n.i.[b]	n.i.[b]
5d		25.0 ± 6.8 (55%)[a]	52.0 ± 5.2 (50%)[a]
7a		n.i.[b]	n.i.[b]
7b		n.i.[b]	n.i.[b]
8		n.i.[b]	n.i.[b]

[a] Percentage of maximal inhibition. [b] n.i.: no inhibition.



Scheme 1. Synthesis of inhibitor analogues.

Paprotrain was cell permeable and effective in HeLa cells. Previous studies have shown that RNAi-mediated depletion of MKLP-2 led to increased levels of binucleated cells.[4,5] After 8 h of incubation, the number of binucleated cells (Figure 3a and b, left panels) increased to more than three times that of untreated cells (11 ± 1 vs. 3.5 ± 0.5%, respectively). Exposure of cells to dihydrocytochalasin B[6] (DCB; 10 μM) resulted in 32 ± 12% binucleated cells (Figure S3a in the Supporting Information). After 24 h of treatment with paprotrain (10–50 μM), a significant number of cells were in apoptosis, confirmed by immunodetection of cleaved poly-(ADP-ribose) polymerase (PARP) and activated caspase 3 (Figure S3b).

MKLP-2 function is essential for the relocation of the chromosome passenger protein complex (CPC) from centromeres to the spindle midzone during the metaphase-to-anaphase transition[7] (Figure 3a). The inhibitor did not affect the inner centromere recruitment of Aurora B in metaphase, but it inhibited the relocation of Aurora B and survivin to the spindle midzone in anaphase (Figure 3b). A similar phenotype was observed in cells following RNAi depletion of MKLP-2 (see Figure S3c).[7] In the presence of the inhibitor, Aurora B and survivin were diffusely associated with the chromosomes in anaphase, a phenotype, which resembles Aurora B localization following RNAi-mediated depletion of survivin.[8] The absence of passenger proteins at the spindle midzone is also linked to failure of cytokinesis and may explain the appearance of binucleated cells following paprotrain treatment. The motor proteins, Kif4, MKLP-1, and MPP1, are also thought to participate in cytokinesis by interacting with PRC1, a MT-bundling protein necessary for central spindle formation.[9,10] In the presence of paprotrain, the normal PRC1 localization pattern in anaphase was observed (Figure 3b, right images). Therefore, we conclude that paprotrain does not affect Kif4- or MKLP-1-mediated transport of PRC1 to the spindle midzone during cytokinesis.

In proliferation assays, paprotrain inhibits growth with EC_{50} values between 7.6 and 22 μM (see Table S3 in the Supporting Information). Paprotrain is not a substrate for the efflux pump P-glycoprotein (see Table S4 in the Supporting Information). The inhibitor induces additional spindle defects that are not observed in cells following MKLP-2 depletion by RNAi. Metaphase spindles appeared to have an incomplete chromosome complement aligned to the metaphase plate (metaphases in Figure 3b). In untreated cells 91.5 ± 5% of metaphase spindles contained a normal bipolar spindle (Figure 3a, upper panels). In contrast, after treatment with paprotrain the majority of metaphase spindles (70 ± 10% of total metaphase cells) had misaligned chromosomes (Figure S3d). Higher concentrations of paprotrain (50 μM) increased the percentage of multipolar spindles at the expense of normal bipolar spindles (14 ± 3% of total metaphase cells at 50 μM versus 6 ± 1.6% at 20 μM; Figure S3e). Phenotypic dissimilarities have also been reported for the Kif18A[11] inhibitor and might reflect differences originating from distinct experimental approaches (inhibition caused by RNAi versus small-molecule compounds) requiring further evaluation of the cellular phenotypes.

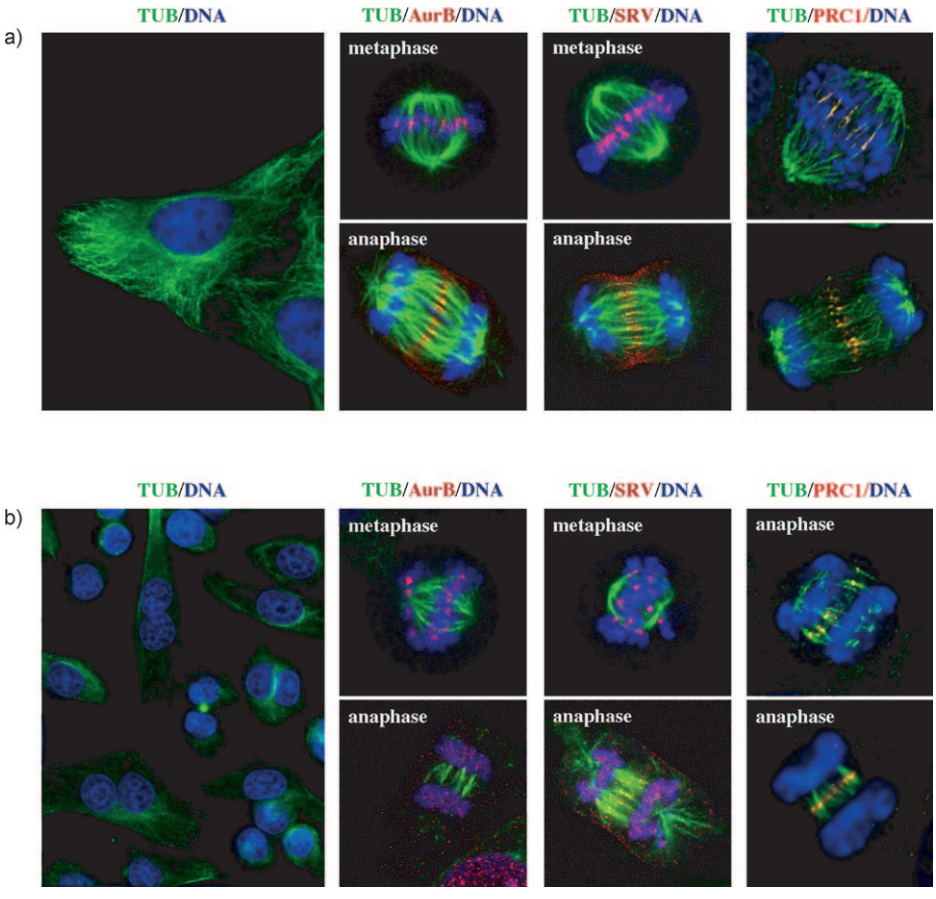


Figure 3. Paprotrain induces binucleated cells and impedes the relocation of the CPC from the inner centromeres to the central spindle during anaphase. Immunostaining of a) control untreated cells and b) cells treated with paprotrain (50 μM) after 8 h. MTs (TUB) were stained with fluorescein isothiocyanate (green), Aurora B (AurB), survivin (SRV), and PRC1 with cyanine dye Cy3 (red), and DNA with 4′,6-diamidino-2-phenylindole (blue).

To date, kinesin inhibitors have only been reported for Eg5,[12] CENP-E,[13,14] and Kif18A.[11] Herein, we have identified and characterized the first inhibitor of the mitotic kinesin MKLP-2. Paprotrain will be a useful tool for chemical genetics to study the function of MKLP-2 in cytokinesis and to gain mechanistic insights, for example, to exploit mechanistic differences between the various kinesins. In the case of MKLP-2, it might shed

light on the question of whether MKLP-2 is functionally involved in intracellular transport involving the Golgi.[15] Finally, inhibitors that target cytokinesis-specific kinesins may help to validate these proteins as potential targets for drug development in cancer chemotherapy.[16]

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