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DHTP is an allosteric inhibitor of the kinesin-13 family of microtubule depolymerases

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ABSTRACT

The kinesin-13 family of microtubule depolymerases is a major regulator of microtubule dynamics. RNA interference-induced knockdown studies have highlighted their importance in many cell division processes including spindle assembly and chromosome segregation. Since microtubule turnovers and most mitotic events are relatively rapid (in minutes or seconds), developing tools that offer faster control over protein functions is therefore essential to more effectively interrogate kinesin-13 activities in living cells. Here, we report the identification and characterization of a selective allosteric kinesin-13 inhibitor, DHTP. Using high resolution microscopy, we show that DHTP is cell permeable and can modulate microtubule dynamics in cells.

1. Introduction

Microtubules form the cytoskeletal network that supports the shapes of different cell types. They are also the core component of the mitotic apparatus that is assembled to segregate chromosomes during cell division. Microtubules are intrinsically dynamic, stochastically switching between growth and shrinkage, a phenomenon called dynamic instability [1]. It is known that the dynamic properties of microtubules are essential for their functions [2]. In cells, microtubule polymerization dynamics are precisely regulated spatially and temporally by stabilizing and destabilizing factors. The kinesin-13 family of microtubule depolymerases is a major regulator of microtubule dynamics [3,4]. Loss of function studies have shown that kinesin-13 proteins are essential for many mitotic processes including spindle assembly, chromosome segregation and kinetochore-microtubule attachment error correction in multiple species [5–9]. Therefore, understanding how kinesin-13s regulate microtubule dynamics in mitosis is fundamental to understand the process of cell division. Although RNA interference (RNAi)-induced knockdown studies of three human kinesin-13s, Kif2a, Kif2b and Kif2c (or MCAK, Mitotic Centromere Associated Kinesin) and of kinesin-13s from other species have highlighted their functional importance and non-redundancy [7,8,10,11], the temporal resolution of this perturbation method is rather limited because of the time it takes to deplete the target proteins (in days). Since microtubule turnover, as well as many cellular processes such as spindle assembly and chromosome segregation are relatively rapid (in the time scale of seconds or minutes), developing tools that allow faster temporal control over protein function is an important step towards a better understanding of kinesin-13 functions in living cells. Small molecules are a convenient tool that fits this criterion, but selective kinesin-13 inhibitors are still lacking.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemicals were purchased from Bioshop except the following: ATP and GTP (Jena Bioscience), taxol/paclitaxel

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(LC laboratories), malachite green and ammonium molybdate (MP biomedicals), perchloric acid (Fisher) and casein (Sigma). DHTP *((Z)-2-(4-((5-(4-chlorophenyl)-6-(isopropoxycarbonyl)-7-methyl-3-oxo-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl)phenoxy) acetic acid*) was purchased from Specs.

2.1.2. Protein reagents

Tubulins were purified from bovine brain and modified using standard protocols [12]. Kinesin constructs were generated by amplifying the desired coding regions from plasmids containing the corresponding cDNAs via Polymerase Chain Reactions (PCR). All kinesin constructs were expressed in *Escherichia coli* and purified using standard affinity chromatography (Ni–NTA or glutathione). More detailed information is provided in the Supplementary material.

2.2. ATPase assay and high-throughput screening

A malachite green-based phosphate detection assay was used to screen for small molecule inhibitors of kinesin-13 ATPase activity and subsequent characterization of DHTP. The general composition of the ATPase reaction is as follows: BRB40 (40 mM PIPES pH 6.8, 0.5 mM EGTA, 0.5 mM $MgCl_2$), 10 μM taxol, X μM microtubules, X nM kinesin, X mM KCl, 0.25 mg/ml casein, 1 mM DTT, 1 mM $MgCl_2$, 0.02% Tween-20 and X mM ATP. Full description of the exact conditions used in different experiments is detailed in the Supplementary material.

2.3. Microtubule depolymerization assays

2.3.1. Microtubule sedimentation assay

Microtubule sedimentation assay was done as previously described [13]. Briefly, kinesin-13-induced reactions comprised of 50–75 nM enzyme, 1 mM ATP and 2 μM microtubules were set up similarly as those for the ATPase assay. After a 15-min incubation, reaction mixes were spun at 60–80 K RPM in a Sorvall S120AT3 rotor for 5 min. Supernatant and pellet fractions were then resolved by SDS–PAGE and processed as described [13].

2.3.2. Microscopy-based microtubule depolymerization assay

Microscopy-based microtubule depolymerization assay was done as follows: A flow chamber assay was set up similarly as described for kinesin-5-driven surface gliding assay using GST-Eg5(1-437) and X-Rhodamine-labeled GMP-CPP microtubules [14,15]. To simultaneously initiate Eg5-driven motility and kinesin-13 induced microtubule depolymerization, reaction mix containing kinesin-13 (50 nM MCAK-MD or Kif2a-MD, 1X BRB40, 50 mM KCl, 0.5 mg/ml casein, 1 mM MgATP, and 1X oxidation mix: 22.5 mM glucose, 0.22 mg/ml glucose oxidase (Sigma G-2133), 0.036 mg/ml catalase (Sigma C-40) and 4 mM DTT, was introduced into the chamber in the presence of either DHTP or DMSO. The movement and shortening of microtubules were captured by a time-lapse recording every 15 s using a Zeiss Axio-Imager Z1 microscope equipped with a 63 × 1.4 Plan-APOCHROMAT objective and a Zeiss AxioCam HRm camera. Recorded images were visualized and analyzed by kymography using MetaMorph (Molecular Devices).

2.4. Live imaging of microtubule dynamics

Drosophila S2 cells stably expressing tubulin-GFP were cultured in optical glass-bottom plates (Greiner bio-one) coated with concanavalin A (Sigma). Cells were treated with DMSO (vehicle) or with 10 μM DHTP. Images were acquired 15–20 min after treatment. Live imaging was performed using Deltavision elite microscope (Applied Precision) in a temperature-controlled environment (27 °C). For each condition at least 6 independent wells were treated. Images were acquired using a CoolSNAP HQ2 camera (Photometrics) every 5 s for 5–8 min. Images were processed using Softworx (Applied Precision), Image J (NIH) and Photoshop (adobe) software. To determine the microtubule dynamics the tips of individual microtubules were tracked manually using Image J package. A minimum of 56 microtubules and 6 individual cells were tracked and measured for each condition. For the tracking analysis, a displacement of less than or equal to 1 pixel (107 nm lateral) between 2 consecutive time frames (5s) was considered stationary (=displacement of 0) and therefore a "pause" event. A displacement of more than 1 pixel between 2 consecutive frames was considered either a shrinkage or a growth depending on its previous position. Digital traces of microtubule ends were then obtained by plotting the displacements from the origin (position at $t = 0$ s) against time.

3. Results and discussion

To identify small molecule inhibitors of the kinesin-13 family of microtubule depolymerases, we screened a chemical library of ~110000 compounds using an ATPase activity-based assay. The rationale is based on the fact that kinesin-13s are ATPases and ATP hydrolysis is required for catalytic depolymerization of microtubules [16,17]. To achieve this, we used a construct of the human kinesin-13 protein MCAK (MCAK-MD, minimal domain residues 187–589) that is competent in both ATP hydrolysis and

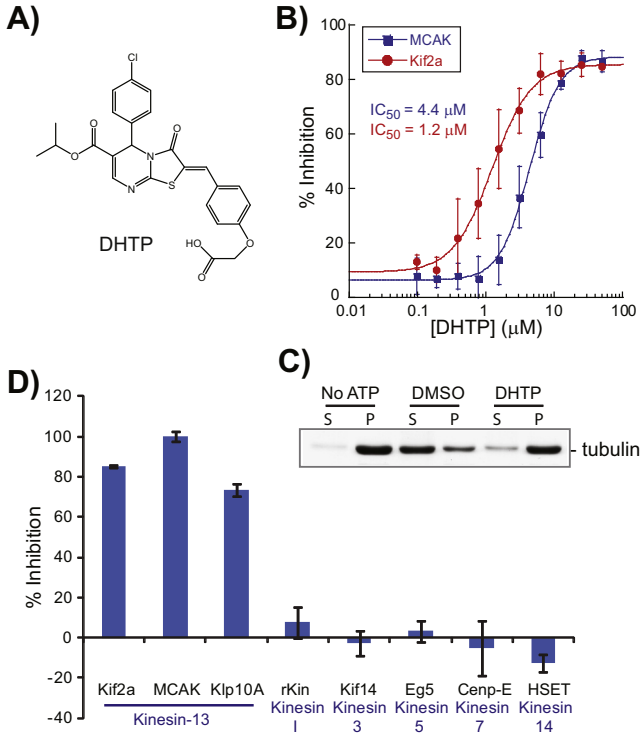

Fig. 1. (A) Chemical structure of DHTP. (B) Dose response curves of kinesin-13-induced ATPase activity versus increasing concentrations of DHTP used to determine the IC_{50} values of MCAK and Kif2a are shown. (C) Inhibition of 20 μM DHTP on MCAK-induced microtubule depolymerization by sedimentation assay. DMSO equivalent to the volume of the dissolved DHTP (1% of the reaction volume) was used as control. A representative coomassie blue-stained gel of the supernatant (S) and pellet (P) fractions is shown ($N > 3$). (D) The selectivity of DHTP for kinesin-13 proteins were tested against a panel of motile kinesins at 50 μM in the ATPase assay. For B and D, each data point represents average value from at least three independent experiments. Error bars = S.E.M. Values are expressed in percentage inhibition relative to the corresponding DMSO controls. Corresponding dose response plots of ATPase rates versus DHTP concentrations and the measured rates for all kinesins are provided in the Supplementary data (Fig. S2).

microtubule depolymerization. We verified that this MCAK construct was robust with kinetic parameters comparable to those of the published literature ([17–19] and Fig. S1). We established a robust malachite-green based phosphate detection ATPase assay in a 384-well format (with a *Z* score above 0.7) for the high-throughput screen (see Supplementary experimental methods). From the primary screen, we identified compounds that gave more than 25% inhibition as candidate hits, which were subsequently cherry-picked and reconfirmed in quadruplicates. We then tested the confirmed hits with another kinesin-13 construct, Kif2a-MD(126-527) and against other kinesin motors including kinesin-1 and kinesin-5, in order to identify potential kinesin-13 selective inhibitors. Among the candidate hits, DHTP emerged to be the most potent compound that inhibited both the ATPase activity and kinein-13 induced microtubule depolymerization (Fig. 1A–C). We determined the IC_{50} of DHTP in inhibiting kinesin-13-induced microtubule stimulated ATPase activity to be about 4.6 μM for MCAK and 1.2 μM for Kif2a (Figs. 1B, S2A). In addition, DHTP showed a drastic inhibition of MCAK-driven microtubule depolymerization in a centrifugation-based sedimentation assay which monitors depolymerization via the separation of the pelleted polymers from the dissociated tubulin dimers in the supernatant (Fig. 1C). These results prompted us to characterize this compound further. To assess the selectivity of DHTP against kinesin-13s, we tested microtubule-stimulated ATPase activity with three kinesin-13 proteins, MCAK, Kif2a and the *Drosophila* kinesin-13 ortholog KLP10A, and against a panel of kinesins from other families. We found that DHTP has no significant inhibitory effect on other families of kinesin motors even at 50 μM, a concentration at which DHTP almost completely blocked kinesin-13 induced ATPase activity (for both MCAK and Kif2a and to a lesser extent KLP10A, Figs. 1D, S2B). DHTP differs from other compounds that have been reported to target kinesin-13s [20,21], in its selectivity against other kinesins. For example, the thiazole compounds reported by Rickert et al. also inhibit other kinesins including kinesin-5 [20]. The effect of SQAGs (sulfoquinovosylacylglycerols), another type of compounds reported to target MCAK, on other kinesins has not been tested [21]. And with their low binding affinity to MCAK (K_d of 60–300 μM), SQAG's mode of inhibition is in doubt [21]. In comparison, our data show that DHTP is a potent and selective inhibitor of kinesin-13 proteins.

To assess the mode of inhibition, we first determined whether DHTP is an ATP competitive inhibitor. We performed ATPase assays with a 10-fold change in ATP concentration in the presence of DHTP. The rationale is that for competitive inhibitors, the IC_{50} will change with varying ATP concentrations. As an example of competitive inhibition, we used ADP which binds to the same nucleotide-binding pocket as ATP does. We found that while a 10-fold increase in ATP concentration from 0.1 mM to 1 mM resulted in about a 20-fold increase in the IC_{50} value for ADP, it did not considerably affect the IC_{50} value for DHTP (Fig. 2A). When we measured the MCAK-induced ATPase rates with increasing ATP

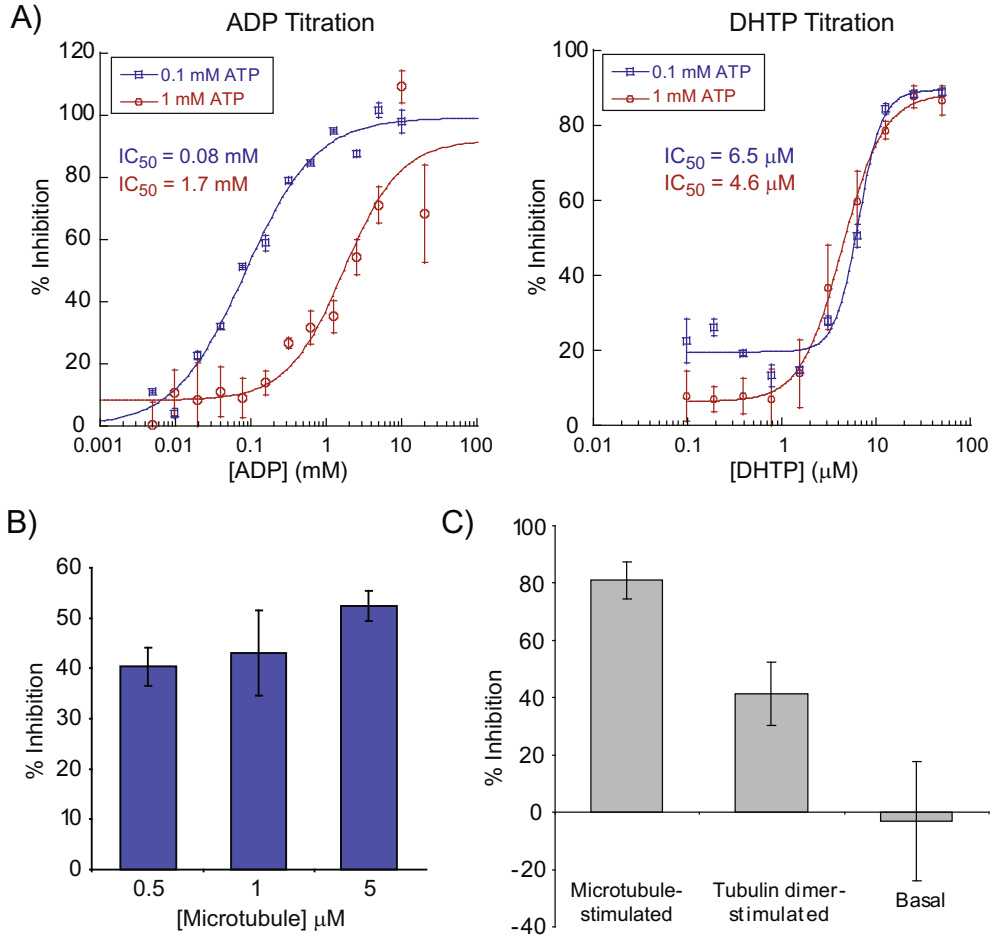

Fig. 2. (A) Competition experiment with ATP: 10-fold changes in ATP concentrations were used in the ATPase assay with MCAK. Dose response curves of ADP (as an example of competitive inhibition, left) and DHTP (right) are shown. Error bars = S.E.M. (B) Competition experiment with microtubules in MCAK-induced ATPase assay: percentage inhibition was obtained with 2 or 10-fold changes in microtubule concentration. Error bars = S.E.M. (C) Effects of DHTP on the basal (enzyme alone), tubulin dimer-stimulated and microtubule-stimulated ATPase activity of MCAK. Error bars = Standard deviations. DHTP was used at 5 μM and 50 μM in B and C, respectively. All data sets were obtained from at least 3 experiments.

concentrations, we also found that DHTP progressively decreased the maximal turnover rates (Fig. S3). These results indicate that DHTP is not an ATP competitive inhibitor. Next we considered the possibility that DHTP could target microtubules instead of binding to the enzymes directly. If this is true, increasing the microtubule concentration should decrease the potency of the compound. To test this, we varied the microtubule concentrations from 0.5 μM to 5 μM in the ATPase assay. We found that even a 10-fold increase in microtubule concentration did not decrease the percentage of inhibition of DHTP at around the IC_{50} concentration (5 μM, Fig. 2B), suggesting that DHTP's inhibition of kinesin-13 ATPase activity is not through binding to microtubules. This assessment is consistent with the observation that DHTP is kinesin-13 selective as evident by its lack of effect on other kinesins (Fig. 1D). Next we determined whether DHTP can inhibit kinesin-13 activity in the absence of microtubules. We examined both the basal activity and tubulin dimer-stimulated activity of MCAK. We found that DHTP had a much lesser effect on dimer-stimulated activity, and had no inhibitory effect on the basal activity of MCAK at a concentration as high as 50 μM (Fig. 2C). Together, these data show that DHTP inhibits kinesin-13 most potently in the microtubule-bound state in a manner that is neither ATP- nor microtubule-competitive.

Although the microtubule sedimentation assay can detect the effect of DHTP inhibition on kinesin-13 induced microtubule depolymerization and can be quantitative, it only measures microtubule depolymerization in bulk. To visualize how DHTP affects MCAK-driven microtubule depolymerization at the single filament level, we developed a microscopy-based flow chamber assay to monitor the progression of depolymerization on individual microtubules. In this assay, fluorescently-labeled microtubules are anchored on a glass coverslip via surface immobilized GST-Eg5 (a kinesin-5) in a flow chamber. To initiate microtubule depolymerization, a reaction mix containing kinesin-13 proteins (MCAK-MD or Kif2a-MD) and ATP was flowed into the chamber. Time lapse imaging of X-Rhodamine-labeled microtubules showed that both kinesin-13-induced microtubule shortening and kinesin-5 driven motility could be readily detected. In the presence of DHTP, we found that while the rate of MCAK-induced microtubule depolymerization was greatly reduced (by 74% at 5 μM and by 89% at 10 μM), Eg5-driven microtubule gliding was unaffected (Fig. 3). Similar results were obtained for Kif2a (not shown). This shows the selectivity of DHTP towards kinesin-13 proteins over kinesin-5, which is consistent with what we observed in the ATPase assay (Fig. 1D).

It is known that depletion of individual kinesin13s by RNAi-induced knockdown results in a variety of mitotic defects such as monopolar spindle formation (for Kif2a or Kif2b) and misaligned chromosomes (for MCAK) [7,8]. However, there was also a report of the lack of monopolar spindle phenotype upon Kif2a or Kif2b knockdown [22]. Being cognizant of this, we treated cultured cells with DHTP to determine if we could observe any mitotic defects. Despite our numerous attempts, we did not observe any obvious phenotypes similar to those observed in individual kinesin-13 knockdowns in HeLa or U2OS cells [7,8] (data not shown). To account for the lack of mitotic phenotypes, we considered two possibilities. First, DHTP might not be cell permeable. Second, small molecule inhibition of kinesin-13 proteins could differ from RNAi-based knockdown. To distinguish these two possibilities and because of the controversy of knockdown phenotypes in mammalian cells, we decided to monitor microtubule dynamics directly upon DHTP treatment in *Drosophila* S2 cells. We chose S2 cells for the following reasons. First, DHTP also inhibits the *Drosophila* kinesin-13 protein KLP10A, in addition to human kinesin-13s (Fig. 1D). Second, it is feasible to monitor polymerization dynamics of individual microtubule filaments in S2 cells, as we and others have

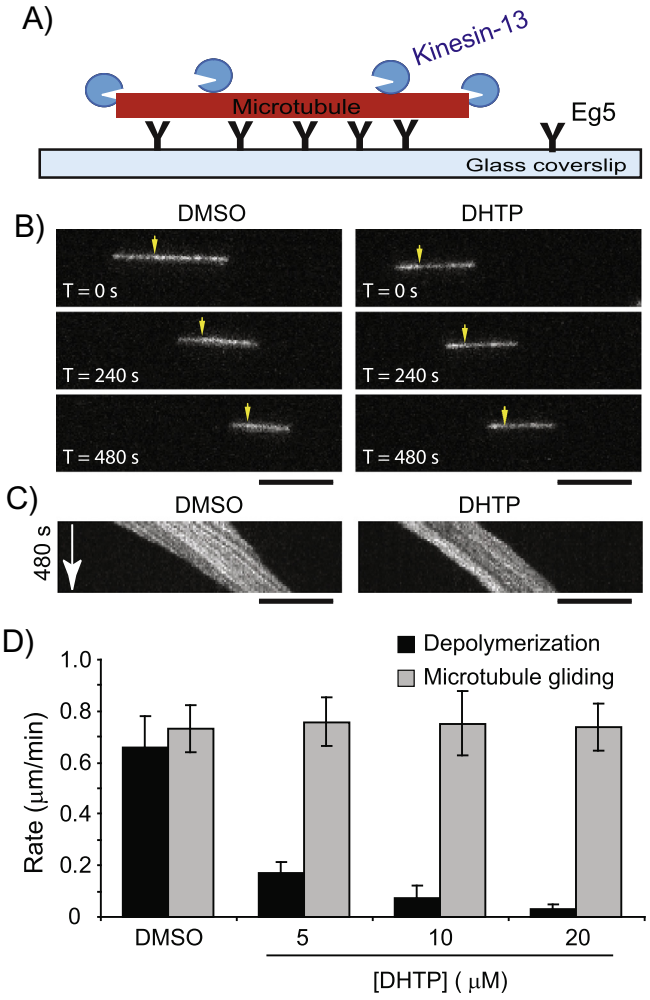

Fig. 3. (A) An illustration depicting a microscopy-based kinesin-13-driven microtubule depolymerization assay. (B) Three frames of a time-lapsed recording of microtubule gliding (by Eg5) and depolymerization (by MCAK-MD) in DMSO control and DHTP are shown. A reference point in the middle of shown microtubule (arrowhead) is indicated to show the movement and shortening of microtubules. (C) Corresponding kymographs of the microtubules shown in B. Bar, 5 μm. (D) Quantification on the rates of GST-Eg5(1-437)-driven microtubule gliding and MCAK-MD-driven depolymerization (from 3 independent experiments, $n = 60$ microtubules for each condition, error bars = standard deviations).

shown [13,23]. Third, KLP10A knockdown phenotypes have been reported in S2 cells, including the effect on microtubule dynamics [11]. Based on live cell imaging in S2 cells expressing tubulin-GFP, we observed that microtubules were in general less dynamic in DHTP-treated cells, as evident by the increase in time they spent in pause (neither growing nor shrinking) (Fig. 4), consistent with what was observed upon KLP10A depletion [11]. In addition, these microtubules showed more persistent growth towards the cell edge and had fewer catastrophes as evident by microtubule end tracking and dynamic parameter measurements (Fig. 4B and D). Together, these data show that DHTP is a cell permeable kinesin-13 inhibitor and can modulate microtubule dynamics in cultured cells.

In summary, we report here the identification of a cell permeable kinesin-13 selective inhibitor. By treating cells with DHTP, we observed an outcome consistent with kinesin-13 inhibition – change in microtubule dynamics. In particular, we showed that DHTP treatment resulted in more persistent growth of microtubules with decreased frequency of catastrophe in cultured cells as evident by our analysis of individual microtubules. Currently, one of the limitations of DHTP is that it inhibits multiple kinesin-13 proteins (different isoforms and orthologs from other species

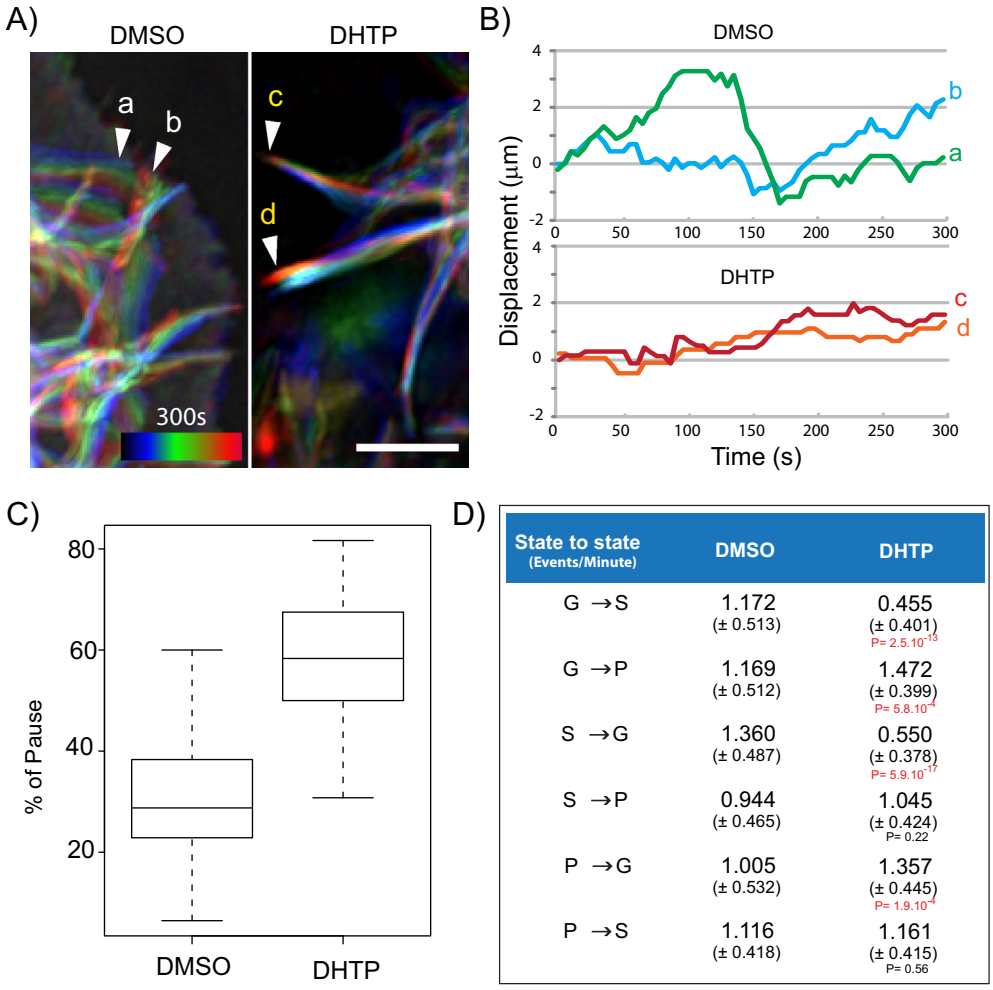

Fig. 4. (A) Time-lapse projection of microtubules in live cell imaging of GFP-tubulin expressing *Drosophila* S2 cells. Time frames of the GFP channel are overlaid and shown in heat map (blue to red, 60 frames every 5 s). Bar = 5 µm. The corresponding movies are included in the Supplementary material. (B) Representative traces of displacement of the microtubules indicated by arrowheads in (a). (C) Quantification of percentage of time microtubules spent on pause (neither growing nor shrinking), as a measure of dynamicity. The plot shows the percentage of time spent by individual microtubules on pause. Boxes show top and bottom quartiles, horizontal bold lines show median values, and vertical lines show minimal and maximal values. Each dot represents an individual microtubule. *P*-value = 7.25×10^{-23}. (D) Measurements of dynamic parameters by tracking individual microtubule ends in cells treated with DMSO control and DHTP. State transitions of microtubules are quantified and shown (G: growth, S: shrinkage, and P: pause). *P*-values of <0.05 are shown in red.

such as *Drosophila* KLP10A). In order to examine the roles of individual kinesin-13 proteins, it would be essential to develop selective DHTP analogs targeting each of the three kinesin-13s in human in the near future. Given the fact that DHTP inhibits both MCAK and Kif2a, as well as the *Drosophila* kinesin-13 KLP10A and that it is not ATP competitive, we postulate that DHTP must target a common allosteric site among kinesin-13 proteins. One interesting possibility is the site analogous to the monastrol binding site on kinesin-5 [24]. This pocket is formed by the loop L5, which varies considerably among kinesins. Like kinesin-5, kinesin-13 has one of the longest L5 loops. Therefore, it is logical to think that this allosteric pocket is also present in kinesin-13s, as evident by the crystal structures of MCAK and Kif2a ([25] and PDB code of KIF2a: 2GRY). Since allosteric sites are in general more divergent and often under lesser selective pressure than the catalytic pocket, obtaining sub-family selectivity is an achievable goal. However, as suggested by our data, DHTP may only bind to a microtubule-bound conformation of kinesin-13 or to a lesser extent a tubulin dimer-bound conformation. Therefore, it is still technically challenging to determine exactly how DHTP binds to the proposed allosteric pocket solely based on the crystal structures because of the fact that DHTP does not inhibit the basal activity of both Kif2a and MCAK, and the loop L5 is not resolved in the aforementioned kinesin-13 crystal structures.

Our discovery of DHTP as a pan-kinesin-13 selective inhibitor provides the first step towards developing sub-family specific inhibitors and using them as chemical biology tools to examine the regulation of microtubule dynamics mediated by kinesin-13 proteins. In addition, since overexpression of kinesin-13 proteins (MCAK and Kif2a) has been reported in multiple cancer cells and has been suggested to be a possible cause for resistance to microtubule-targeting drugs such as taxol [26], kinesin-13 inhibitors will therefore open new possibilities to develop alternative chemotherapeutics and to overcome some of the drug resistance issues associated with tubulin poisons.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.05.024.

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